Safety Assessment of Malaria Vaccines in African Populations: Exemplified by the Assessment of PfSPZ based Vaccines in Tanzania and Equatorial Guinea

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…….. to my beloved wife, children, parents, brothers and sisters.

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List of abbreviations

AE	Adverse Event
AVAREF	African Vaccine Regulatory Forum
BCTF / U	Bagamoyo Clinical Trials Facility / Unit
BDH	Bagamoyo District Hospital
BIMEP	Bioko Island Malaria Elimination Program
BRTC	Bagamoyo Research and Training Centre
BSPZV1 / 2 / 3	Bagamoyo Sporozoite Vaccine Trial Number 1 / 2 / 3
CHMI	Controlled Human Malaria Infection
CONSORT	Consolidated Standards of Reporting Trials
CPK	Creatine Phosphokinase
CRC	Clinical Research Center
CVac	Chemo-attenuated Vaccine
DNA	Deoxyribonucleic Acid
EC	Ethical Committee
ECG	Electrocardiogram
EDTA	Ethyl Diamine Tetra acetic Acid
EG	Equatorial Guinea
EGSPZV1 / 2 /3	Equatorial Guinea Sporozoite Vaccine Trial Number 1 / 2 / 3
EKNZ	Ethics Committee of Northwestern and Central Switzerland (Ethikkommission Nordwest- und Zentralschweiz)
EMEA	European Agency for the Evaluation of Medicinal Products
EPI	Expanded Program on Immunization
FBC	Full Blood Count
FDA	Food and Drugs Authority
GCLP	Good Clinical and Laboratory Practice
GCP	Good Clinical Practice
GIVS	Global Immunization Vision and Strategy
GLP	Good Laboratory Practices
GMP	Good Manufacturing Practice

List of abbreviations

GSK	GlaxoSmithKline
HIV	Human Immunodeficiency Virus
ICH	International Conference on Harmonization
ID	Intradermal
IHI	Ifakara Health Institute
IP / IMP	Investigational Product / Investigational Medicinal Product
IPTi	Intermittent Preventive Treatment in infants
IPTp	Intermittent Preventive Treatment in pregnant women
IRB	Institutional Review Board
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
IV	Intravenous
LCL	Lower confidence limit
MCDI	Medical Care Development International
MoHSW	Ministri of Health and Social Welfare
MRC	United Kingdom Medical Research Council
NIAID	National Institutes of Allergy and Infectious Diseases
NIMR	National Institute of Medical Research
NS	Normal saline recipients
OR	Odds ratio
P27A	Synthetic peptide from Plasmodium falciparum trophozoite exported protein 1
Pf	*Plasmodium falciparum*
PfSPZ	*Plasmodium falciparum* Sporozoite
PhD	Doctor of Philosophy
QA	Quality Assurance
R21	A virus-like particle vaccine
RA	Regulatory Authority
RTS,S	Repeated Tandem region of the circumsporozoite protein (CSP) fused in hepatitis B antigen
SC	Subcutaneous
SOP	Standard Operating Procedure

List of abbreviations

Swiss TPH	Swiss Tropical and Public Health Institute
TFDA / TMDA	Tanzanian Regulatory Authority
UCL	Upper confidence limit
UNICEF	United Nations Children's Emergency Fund
US	United States of America
V	Vaccine recipients
VE	Vaccine Efficacy
VRC	Vaccine Research Center
WBC	White Blood Cell
WHO	World Health Organization

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First and foremost, all praise and thanks are for Allah by whose favor righteous deeds are fulfilled.

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Summary

Introduction

Malaria is a major global health problem, with persistent public health challenges especially in sub-Saharan Africa. An upswing in malaria clinical cases is reported by the WHO from 214,200,000 in 2015 to 219,000,000 in 2017. The latest WHO's World malaria report, has estimated 627,000 malaria deaths worldwide in 2020 representing about 69,000 more deaths in 2020 compared to 2019 (WHO, 2021). Although, disruptions in the provision of malaria prevention, diagnosis and treatment during the Covic-19 pandemic were linked with approximately two thirds of these additional deaths, evidence exists which suggest an existing real threat to the effectiveness of insecticide-based vector control and antimalarial drugs due to developing resistance towards these tools (Namias et al., 2021; Vaulin et al., 2019). Apparently, if the goal of malaria elimination is to be achieved interventions that can prevent infection and thereby block transmission are needed. Integration of malaria interventions with immunization, complements Global Immunization Vision and Strategy (GIVS), an agenda created by the WHO and UNICEF (Lindstrand et al., 2021; WHO, 2020). The WHO rainbow tables outline several malaria vaccine candidates at different clinical development stages including; Pre-erythrocytic stage projects (eg. RTS,S-A01, PfSPZ Vaccine etc), blood stage projects (eg. P27A Vaccine etc), other projects (eg. PfSPZ-CVac (PfSPZ Challenge + chloroquine)) most of which have been or are being tested across different countries including malaria endemic regions in Africa. Complexities of conducting field trials have been the center of discussion since the early periods of searching for malaria vaccine on proper safety assessment involving areas where the prevalence, importance and epidemiology of malaria is known (Liheluka et al., 2013; McGregor, 1979; Powell, 1979). Over years, research institutions in Africa have developed capacity and are now able to conduct good clinical (GCP) and laboratory practices (GLP) compliant clinical trials and such interest is growing. This important milestone will help to accelerate clinical development programs of several vaccine

candidates and other investigational products of public health importance in endemic countries. As a matter to assess, build and strengthen the capacity of different sites to implement these trials, early trials were designed to reproduce the results obtained in Europe and America with minor modification of protocols and standard operating procedures (SOPs) to fit the situation. The need to achieve optimal safety assessment, proper interpretation and reporting, for local investigators, cannot be overstated. The broader concept of safety assessment must be understood as, the process beyond mere assessment of findings from the individual parameters following exposure to IMP, but rather as, the process of safety assessment that is adequate for; relevant participant selection, safe administration of IMP, proper safety follow-up, compliance to safety reporting to local regulatory authorities and relevant final clinical safety report. Interpretation of safety findings in reference to the socio-cultural and baseline epidemiological and clinical characteristics of the local population being assessed has potential to provide fundamental bridge between the intervention being assessed and the local healthcare system, enabling proper use of resources as well as the relevant and timely key policy decisions to be made. Known epidemiological characterization among different African populations may provide an indication that, these communities are similar and with further exploration, procedures may be optimized and synchronized at a level that allows operational shift from the so called African sites to African research centers.

Methods and findings

In the first part of this thesis (Chapter 4), the aim was to generate the safety and tolerability profiles of PfSPZ based malaria vaccine candidates namely PfSPZ Vaccine and PfSPZ-cVac in malaria endemic African population, targeting to develop and implement a vaccine that can be used as additional tool for malaria intervention and possibly elimination, that is well tolerated and safe. As a toolkit for malaria control in Africa, the PfSPZ vaccines are designed to prevent infection. We worked on assessing safety of PfSPZ vaccine candidates by conducting a series of randomized, placebo-controlled,

double-blind clinical trials in two African countries (Tanzania and Equatorial Guinea) through the program built on strong South-South / North-South collaboration platform. We designed and conducted the clinical trial protocols covering spectrum of age groups ranging from adults (with and without HIV infection) to the target pediatric population of healthy adolescents, children and infants. The clinical development plan that we pursued focused on addressing safety concerns related to PfSPZ vaccines, including; (i) feasibility of administration via direct venous inoculation (DVI) across age groups; (ii) possibility of breakthrough malaria infections due to inadequate attenuation; (iii) possibility of significant local side effects at the injection site that sporozoites may contribute; (iv) possibility of significant systemic reactogenicity caused by PfSPZ Vaccine; (v) possibility of effects on the rates of AEs related to geographical locations among African population, age or dose; (vi) possibility of significant increase in AEs with repeated dosing; (vii) possibility that the accelerated vaccination schedule regimens of PfSPZ Vaccine are significantly intolerable. Through this work, we performed; The first demonstration of PfSPZ Vaccine efficacy against PfSPZ Challenge in healthy adults population (PAPER I); the first demonstration of safety and tolerability of PfSPZ Vaccine in infants and children along with demonstration of the safety and exploration of efficacy of Sanaria's PfSPZ vaccine using PfSPZ-CHMI in African adults (Paper II and Paper III); the demonstration of safety and tolerability of Sanaria's chemo-attenuated vaccine (PfSPZ-CVac) in Equatorial Guinea (Paper IV); the first demonstration of safety and tolerability of PfSPZ Vaccine and exploration of efficacy of Sanaria's PfSPZ vaccine using PfSPZ-CHMI in individuals living with HIV (Paper V); the demonstration of safety and tolerability of accelerated vaccination regimens of PfSPZ Vaccine and down-selection of optimal regimen for pivotal trials in Africa (Paper VI). A meta-analysis of AE data for adults and children using forest plots of total solicited AEs in vaccinees and placebos in the randomized, double-blind, placebo-controlled trials analyzed to date has shown in all cases, 95% confidence intervals cross a ratio of 1, indicating no differences between vaccinees and controls.

In the second part of this thesis (Chapter 5), the aim was to optimize recruitment and enrolment process through better understanding of the social-cultural, epidemiological and clinical characteristics of the local population. Due to factors ranging from social-cultural to the local clinical research implementation and regulation systems, synchronization of recruitment and enrolment strategies with the local population characteristics, will potentially contribute to the successful clinical development plan for interventions. Fundamental to such success are the components such as, proper use of resources, better alignment; to the daily social-cultural activities and the local healthcare systems, timely recruitment, preparation of the risk mitigation plans relevant to the communities being assessed, reliable interpretation of safety results and precision in estimating risk benefit ratio of the intervention to the local community. In these regards, we designed and conducted pilot epidemiological assessments within study areas located in eastern and western parts of Africa, (Bagamoyo, Tanzania) and (Bioko Island, Equatorial Guinea) respectively. The pilot assessments were implemented through separate protocols linked to the processes in main trial protocols on community engagement and sensitization to ensure that, all members of the target population including infants and children, plus HIV positive individuals are able to participate in GCP compliant clinical research in Africa. Through these activities, we formulated a simple and yet very effective categorization of recruitment and screening criteria across the protocols being; (1) criteria assessing GCP compliance; (2) criteria assessing protocol compliance; (3) clinical criteria assessing health status and (4) laboratory criteria assessing health status. By applying this seemingly simple categorization of criteria we piloted relevant allocation of limited resources between community-based and clinic-based recruitment and screening processes. Over 6,000 people were screened with subsequent registration of potential participants for future clinical trials (Paper VII).

In the third part of this thesis (Chapter 6), the aim was to optimize the processes for safety assessment, interpretation and reporting of results through the application of locally relevant toxicity grading tools in reference to the standard toxicity grading systems adopted to the local clinical and

laboratory population parameters. Reporting requirements to the local regulatory authorities' entail expedited reporting for certain AE grades following IP administration. It is critical that, sponsors and investigators adhere to such requirements for compliance and that the local use of standard toxicity grading systems recommended by the US-FDA, CTCAE and other relevant systems must be properly adopted and hence, optimized to enable proper clinical care for participants and to avoid over- or under-reporting of important safety observations. US-FDA recommends for the local reference values to be considered when the parameter limit values are defined in the guidance or some cases may even require the exercise of clinical judgment. The main reason for this is, such reference systems were developed primarily using data collected from certain population, and therefore are only practically relevant if directly used to the population with similar characteristics. Even for population from the same geographical locations, ethnic differences may require proper adjustments to be made. To achieve this optimization, we applied the methodology stipulated by the CLSI for analysis of reference intervals based on local clinical and laboratory data to and developed a listing of clinical and laboratory reference intervals applicable for local populations which were then integrated to the known standard toxicity grading systems (USFDA, CTCAE ect) to develop locally relevant toxicity grading scales. Site based, QA-controlled manual of reference intervals and toxicity grading was developed for the local population and has since been used for standardized interpretation and reporting of results during safety assessments at screening, enrolment and follow-up.

Conclusion

Evaluation of potential relevant health interventions tailored to collaborations involving South-South / North-South partnerships linking multiple sites in Africa, is the peculiar platform to demonstrate and promote the potential reproducibility of processes and results, with reasonable justification for their application across sites. Through such a platform, standardizations that we made on the optimization-approaches for enrolment practices and for interpretation of results and assessment of abnormal

clinical and laboratory parameters, played essential role enabling reproducibility of procedures across sites. Fundamental to the benefit of such standardizations, is the potentially reasonable justification for application of results from evaluations across sites. In the scope of this thesis, the assessments that we performed, provided results indicating that, the safety profile of malaria vaccine candidates is determined by the product itself rather than the type of African population in which it is tested. Strategic partnerships created an enabling platform for interactions with multiple regulatory authorities, ethical committees and IRBs, a critical component reflecting independent reviews for the protocols and reporting of results. With the spectrum of available malaria vaccine candidates including the PfSPZ based, these findings will enable accelerations of clinical development plans not only for clinical trials accessing malaria vaccines but all other related interventions. Our perspective is to maintain the portfolio of activities and skill base that will streamline implementation for subsequent programs of work. In addition, this work has spearheaded the establishment of the clinical research capacity for conduct of regulatory trials in Equatorial Guinea, with primary support from the Bagamoyo Clinical Trials Facility investigators from Tanzania, and used the clinical trials platform to support several masters and PhD training of colleagues in Tanzania, Equatorial Guinea and from partner institution's outside Africa. This work is built on the strong South-South and North-South collaborations enabling and strengthening synchronization of procedures among clinical trials conducted in different African communities at a level that will allow operational shift from the so called African research sites to African research centers.

CHAPTER 1: Introduction

1.1 Rationale for the development of a malaria vaccine

Malaria transmission is reported at the end of 2015 to be ongoing within 95 countries at an estimated 214 million malaria cases and at an estimated 438,000 malaria deaths globally. Though still high, these numbers reflect remarkable improvement from the reported figures in 2000. Impact of malaria interventions since 2000 accounted for 76% decline in parasite prevalence among children aged 2–10 years, and for 70% of the reduced number of cases (WHO, 2021). Evidence exists to suggest an existing real threat to the effectiveness of insecticide-based vector control and antimalarial drugs due to developing resistance towards these tools (Namias et al., 2021; Vaulin et al., 2019). To address existing and emerging challenges, more efforts are needed to supplement success reached by the global malaria community (WHO, 2021). Integration of other critical health interventions with immunization has been outlined among the four primary aims of Global Immunization Vision and Strategy (GIVS), an agenda created by the WHO and UNICEF (Lindstrand et al., 2021; O'Brien, 2009; WHO, 2020). This approach is in-line with the constantly emerging challenges facing the available tools used in the war against malaria (2013; Chattopadhyay et al., 2007; Dhanawat et al., 2009; Horii, 2008; Wells and Poll, 2010; Wkly, 1995). In response to the fact that malaria vaccines are considered amongst the most important modalities, great effort integrating many groups is ongoing for potential prevention of malaria disease and reduction of malaria transmission.

1.2 Status of malaria vaccines

The WHO rainbow tables outline several malaria vaccine candidates at different clinical development stages including; Pre-erythrocytic stage projects (eg. RTS,S-A01, PfSPZ Vaccine etc), blood stage projects (eg. P27A Vaccine etc), other projects (eg. PfSPZ-CVac (PfSPZ Challenge + chloroquine)). Despite all these efforts, no licensed malaria vaccine is currently available (Graves and Gelband, 2006a; b; Hoffman et al., 1997; Hoffman et al., 2015a; b; Horii, 2008; WHO). Most malaria vaccine candidates have been or are being tested across different countries including malaria endemic regions

in Africa. RTS,S has received a positive scientific opinion from the European Medicines Agency (EMA) and is so far the only malaria vaccine that is recommended for use by WHO, calling for second generation malaria vaccines development efforts to increase supply and potentially meet the anticipated high demand.

1.2.1 RTS,S

This recombinant protein-based malaria vaccine RTS,S was created back in 1987 in GSK laboratories and developed for infants and young children living in malaria endemic regions in sub-Saharan Africa. RTS,S is a pre-erythrocytic malaria vaccine acting against the first stages of malaria in human host. During the 30 years of research and development, its efficacy was established in a Phase 3 trial that concluded in 2014 involving a network of African research centres and was approved for use by European regulators in July 2015 (2010; Agnandji et al., 2015; Agnandji et al., 2012; Agnandji et al., 2011; Aide et al., 2010; Alonso et al., 2004). It is the first malaria vaccine recommended for broad use by WHO In October 2021, as the world's first malaria vaccine, for use in children at risk in sub-Saharan Africa and in other regions with moderate to high transmission of malaria caused by Plasmodium falciparum. Following the Gavi Board approval for an investment to support malaria vaccine introduction, procurement and delivery for in sub-Saharan Africa in 2022-2025, the use of the malaria vaccine will include country decision-making on whether to adopt the vaccine as part of national malaria control strategies (WHO, 2022)

1.2.2 R21

The R21 vaccine is a virus-like particle based on the C-terminal portion of the circumsporozoite protein from P falciparum strain NF54 fused to the N-terminus of HBsAg. R21 is being manufactured using the Matrix-M (MM) adjuvant produced by Novavax vaccine that might have an important role in preventing malaria (Datoo et al., 2022). With higher efficacy than many of the published RTS,S trials reported, additional work is needed at a larger scale focusing on identifying unforeseen safety issues,

synchronization that will allow direct comparison between vaccinations timed to occur with the beginning of each malaria season and case detection methods (Moorthy and Binka, 2021).

1.2.3 PfSPZ vaccine candidate

Sanaria® PfSPZ Vaccine is comprised of aseptic, purified, cryopreserved, Pf sporozoites (PfSPZ) that are manufactured according to GMPs for parenteral injection. They are diluted in phosphate buffered saline (PBS) and human serum albumen (HSA) to obtain the desired dose and concentration, and are administered by DVI without an adjuvant. The PfSPZ are attenuated by exposure to radiation during manufacture and are unable to cause parasitemia in humans or undergo transmission to mosquitoes that might bite an individual following immunization (Bastiaens et al., 2015; Druilhe and Barnwell, 2007; Epstein et al., 2011b; Hoffman et al., 2010; Kanoi and Egwang, 2007; Mikolajczak et al., 2014; Richie et al., 2015b; Vaughan et al., 2010).

1.2.3.1 Nonclinical Investigations of PfSPZ Vaccine

The safety and immunogenicity of PfSPZ Vaccine administered by subcutaneous (SC), intradermal (ID), and IV routes were demonstrated in New Zealand White rabbits and mice. Three preclinical toxicology studies in rabbits, giving 5-7 doses of $1.35x10^5$ PfSPZ by the ID, SC and IV routes were performed. In all 3 studies, PfSPZ Vaccine was safe and well tolerated. Formal biodistribution studies were performed, which examined the distribution and persistence in mouse tissues of Pf DNA following SC, ID, or IV inoculation of a single $1.35x10^5$ PfSPZ dose of PfSPZ Vaccine. The studies showed that PfSPZ Vaccine was safe and well tolerated in mice and that there was no persistence of PfSPZ in the host tissue beyond 144 hours. The dose of sporozoites in these studies was equivalent to the highest dose administered in the first two Phase 1 clinical trials of PfSPZ Vaccine, a dose that has now been shown to be safe and well tolerated in adult and pediatric participants administered PfSPZ Vaccine by SC, ID, and/or IV routes (Nussenzweig and Nussenzweig, 1986; Rieckmann et al., 1974).

1.2.3.2 Clinical Experience with PfSPZ Vaccine

There are 18 clinical trials of PfSPZ Vaccine that have been conducted or are ongoing at 4 sites in the USA, 1 site in Germany and 6 countries in Africa. In these trials, as of December, 2019, 5603 doses of PfSPZ Vaccine have been administered to 1727 subjects aged 5 months to 65 years; 873 doses have been administered by DVI to 330 infants, and the PfSPZ have been safe and well tolerated. Highlights of these studies are presented here.

- *First clinical trial of PfSPZ Vaccine:* PfSPZ Vaccine was administered ID or SC in a first-in-humans Phase 1 clinical trial in 80 healthy, malaria-naïve adults at the Naval Medical Research Center and the University of Maryland (Epstein et al., 2011b). PfSPZ Vaccine was safe, fully attenuated by irradiation (no breakthrough blood stage infections) and well tolerated in human subjects. Furthermore, the vaccine induced antibody and T-cell immune responses, and protected several volunteers against controlled human malaria infection (CHMI). However, when administered into or under the skin, PfSPZ Vaccine did not confer adequate protection. Studies of PfSPZ Vaccine in non-human primates demonstrated that ID and SC routes of administration were suboptimal, whereas intravenous (IV) administration greatly increased PfSPZ-specific immune responses, especially the frequency of liver resident CD8+ T cells recognizing PfSPZ (Epstein et al., 2011b). This finding justified clinical testing of PfSPZ Vaccine using the IV route.
- *100% protection achieved after IV administration:* In October 2011, a study of the safety, tolerability, immunogenicity and VE of PfSPZ Vaccine administered IV was initiated at the Vaccine Research Center (VRC), National Institutes of Allergy and Infectious Diseases (NIAID), NIH, Bethesda (Seder et al., 2013b). A catheter was placed in the peripheral vein of the test subjects, and the vaccine administered as an IV push through the catheter, resulting in much improved immunogenicity. This trial, called VRC 312, showed that PfSPZ Vaccine was safe and well tolerated as the doses were escalated from $2x10^3$ to $7.5x10^3$ to $3x10^4$ to $1.35x10^5$ PfSPZ/dose, with concurrence of the Safety Monitoring Committee (SMC) and FDA at each

dose escalation. There were no SAEs considered related to vaccination and no allergic reactions. A vaccine dose response with regard to antibody and T cell responses was observed. CHMI in the higher dose groups (4 or 5 doses of $1.35x10^5$ PfSPZ, total dosage of $5.4\ x10^5$ to $6.75x10^5$ PfSPZ) was performed 3 weeks after the last immunization using homologous parasites. VE was 67% (6/9) in subjects who received 4 doses and 100% (6/6) in subjects who received 5 doses (Seder et al., 2013b).

- *VE extends to heterologous parasite strains, lasts >1 year, can be achieved after 3 doses, and can be achieved using condensed regimens:* The publication of the landmark VRC312 study in *Science* in 2013 led to an enthusiastic international response, and multiple research groups joined the International PfSPZ Consortium (I-PfSPZ-C), partnering with Sanaria to conduct 16 more Phase 1 or Phase 2 trials of PfSPZ Vaccine. The first of these new trials (VRC 314) also used the IV catheter route, but all subsequent studies have used the simpler, more rapid, DVI route, where the vein is punctured directly and nearly painlessly with a 25-gauge needle and the vaccine in 0.5 mL of diluent is injected over a few seconds, a procedure that in most recipients is well tolerated and causes no AEs (Ishizuka et al., 2016c). DVI is now the standard for administration.

1.3 Role of partially effective vaccines as part of integrated approach in malaria control

Malaria remains a primary cause of childhood illness and death in sub-Saharan Africa – and the situation is worsening (WHO, 2021). Despite considerable progress with the scale up of insecticide treated nets (ITNs), deployment of chemoprevention approaches, and the adoption of highly effective treatment since 2000, WHO and partners have called for new tools, including malaria vaccines, to help get malaria control efforts back on track. WHO advises countries to move away from a “one-size fits all” approach and apply a mix of tools, tailored to local contexts, for maximum benefit. A malaria vaccine is

a breakthrough addition to the malaria toolkit and can help get malaria control back on track (WHO, 2022).

1.4 Rationale for Performing Clinical Evaluation of Vaccines choosing a focus on safety in Africa

The regulatory process of vaccines at the developmental stage consists of two main parts, pre-clinical research and development, and clinical research and development. At the clinical development stage, clinical trials in humans are classified into: phase I, phase II, phase III and post marketing period as demonstrated in Figure *1* showing the timescale variation and number of participants as indicated by the figure sizes.

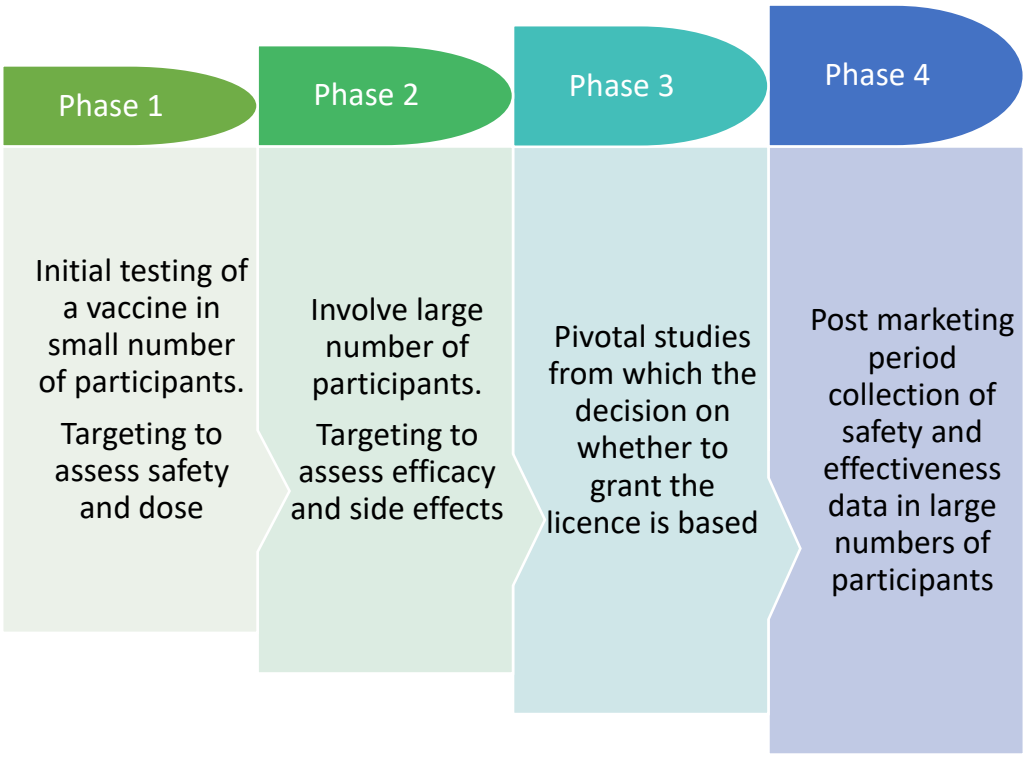


Figure 1: Phases of clinical development stages. Figure sizes indicate increase in both number of participants and length of individual studies from phase 1 to phase 4. [USFDA]

During early clinical research and developmental phases, vaccines are typically given to healthy individuals and the question on safety is primary to the ethics and regulatory authorities as well as to the investigators, other scientists and general community. (Griffiths and Knezevic, 2003; Moxon and Siegrist, 2011). For many years, phase 1 clinical trials have been conducted in developed countries. Complexities of conducting field trials have been the center of discussion since the early periods of searching for malaria vaccine on matters such as proper collection of relevant clinical information, proper assessment and interpretation of the results as well as the need to involve areas where the prevalence, importance and epidemiology of malaria is known (Liheluka et al., 2013; McGregor, 1979; Powell, 1979). Over years, research institutions in Africa have developed capacity and are now able to conduct good clinical (GCP) and laboratory practices (GLP) compliant Phase I clinical trials and such interest is growing. This is considered to be important in a way that it will help to accelerate clinical development program of several vaccine candidates and other investigational products of public health importance in endemic countries. As a matter to assess the capacity of different sites to implement these trials, early trials were designed to reproduce the results obtained in Europe and America, hence same protocols and standard operating procedures (SOPs) were used with minor modification to fit the situation. The key question is, whether such modifications are relevant for proper assessment and interpretation of safety data collected from the local population. In the development of the toxicity grading scales for healthy volunteers, we chose parameter limit values based on published information, when such values were available (CPI, 2009; FDA 2005D-0155, 2007; NCI, 2017; NIH-DAIDS, 2017; NIH, 2005; Sibille et al., 2010). For example, the Brighton Collaboration has developed case definitions and guidelines to evaluate some adverse events associated with administering vaccines (CPI, 2009). In some cases, parameter limit values were based on clinical experience and experience reviewing vaccine clinical trials that enroll normal healthy subjects. Toxicity grading scales for laboratory abnormalities should consider the local laboratory reference values when the parameter limit values are defined. The characterization of laboratory parameters among some populations of healthy adults and adolescents may require the exercise of clinical judgment, for example, consideration of the potential

for ethnic differences in white blood cell (WBC) counts or gender differences in creatine phosphokinase (CPK) values.

In the present work, we developed clinical trial protocols designs specifically aligned to address key questions on the safety assessment of PfSPZ based malaria vaccine candidates in human subjects from endemic population. To accomplish this, we designed and implemented additional pilot, epidemiological protocols tailored to help characterize key baseline local epidemiological parameters related to safety assessment and interpretation among local populations.

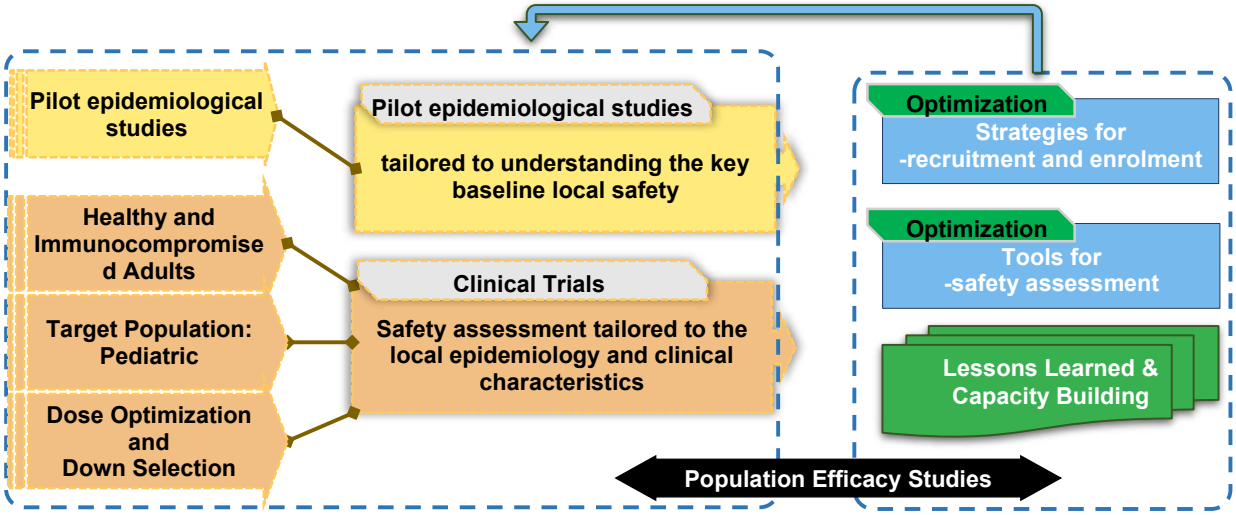


Figure 2: Overview: Key thesis components relevant for safety assessment

CHAPTER 2: Aims of the thesis, research questions and rationale

Investigational products of malaria vaccine candidates have been and are being tested across several research sites, primarily for safety and tolerability in Africans population. With the worldwide increase in availability of vaccines, and the rise of vaccine research conducted in developing countries, there seems to be a room for improvement with respect to vaccine clinical trials conducted in malaria endemic populations (Muehlhans et al., 2012). Guidance is available on various aspects of clinical trials from several bodies such as the WHO guidelines (WHO; WHO; 2014), the International Conference on Harmonization (ICH), the Tanzanian Regulatory Authority (TFDA / TMDA), the African Vaccine Regulatory Forum (AVAREF), the European Agency for the Evaluation of Medicinal Products (EMEA), the United States Food and Drug Administration (FDA), the United Kingdom Medical Research Council (MRC) and the Brighton collaboration (CPMP, 1998; OECD; US-FDA, 1997; WMA). Fundamental to the core of the guidance from the bodies above, is to ensure that, human subjects participating in clinical trials are adequately protected and the implementation of clinical trials, follow the process and yield data that is adequate for safe administration of IP, proper monitoring of participants safety and proper safety reporting to relevant regulatory authorities. The aspect of benefits for the aims we pursued in this thesis are in the public interest of African communities, namely; the establishment of standardized safety profiles of reference with potential applications across African populations, the process to develop and apply the recruitment and enrolment criteria relevant to local African population and the process to develop the population-based toxicity grading systems. Potential benefit to these interests is the population-based process that will have impact to potentially reduce harm to the participants, optimize the use of human and financial resources and reduce the lead time from regulatory approval of malaria vaccine to recommendation for its use in African communities beyond the two African communities that this work has been conducted. This chapter provides details and rationale for each of the three thesis aims in the respective sections below.

2.1 Aim 1

In the first part of this thesis (chapter 4), we aimed to evaluate the safety and tolerability of PfSPZ malaria vaccine candidates namely PfSPZ Vaccine and PfSPZ-CVac amongst populations residing in malaria endemic regions, targeting to support the clinical development plan for a well-tolerated and safe vaccine that can be used as additional tool for malaria intervention and possibly elimination. Our overall hypothesis was that, “the safety profile of malaria vaccine candidates is determined by the product itself rather than the type of African population in which it is tested”. A firm understanding of the study subjects and patterns / characteristics of safety results is needed for investigators to implement clinical trials appropriately and achieving successful clinical development pathways. To address aim 1, we designed a clinical development pathway for proper characterization of safety procedures prior and following the administration of PfSPZ based candidate vaccines across age groups ranging from infants to adults (Including adults living with HIV). We implemented a series of randomized, placebo controlled, double-blind trials amongst populations residing in malaria endemic regions from; the Eastern Coast of Africa (Bagamoyo and Dar es salaam - Tanzania) and Western Coast of Africa (Bioko Island – Equatorial Guinea) (Papers I to VI). The primary objective of these trials was:

- To assess tolerability and safety; following administration of PfSPZ candidate vaccines with CHMI, among adults with or without HIV and following administration of PfSPZ candidate vaccines among adolescents, children and infants from malaria endemic African populations.

With respect to aim 1, results that we published on the general and specific safety concerns with PfSPZ malaria vaccine candidates are discussed in details for each publication in Chapter 4. This thesis aimed to answer the following questions:

1. What will be the feasibility of administering PfSPZ Vaccine intravenously across different age groups and sex?
2. Are there significant differences in safety and tolerability due to dose escalation?

3. Are there significant differences in safety and tolerability associated with age de-escalation?
4. Are there significant differences in safety profiles associated with the type of African population?
5. Are there significant differences in safety and tolerability due to HIV infection status?
6. Are there significant differences in safety and tolerability following CHMI administration?
7. Are the accelerated vaccination schedule regimens of PfSPZ Vaccine safe and tolerable and what regimen will be down-selected for further pivotal trials?

Safety profiles generated among the respective populations from Tanzania and Equatorial Guinea, will provide potential indication on the applicability of safety results among trials investigating the same product in different African populations. Since safety is the primary end-point in this objective, results that we published on the general and specific safety concerns with PfSPZ malaria vaccine candidates are discussed in details for each publication in Chapter 4.

2.2 Aim 2

In the second part of this thesis (chapter 5), we aimed to optimize the recruitment and enrolment process by referencing criteria application to the locally applicable regulatory, epidemiological, sociao-economical, sociocultural and clinical elements for the implementation of the GCP compliant clinical trials among the local African populations. Based on several factors such as dietary, risk exposures to diseases, genetics and hereditary disorders, some of the characteristics of population living in Europe and America may differ from those living in malaria endemic areas of Africa. Hence, the direct extrapolation of criteria set originally designed to determine eligibility of participants for clinical trial protocols implemented for malaria naïve population from elsewhere may not be appropriate. The thesis objectives for aim 2 were as follows:

1. To describe the common practices used to develop and apply criteria for recruitment and enrolment of participants among clinical trials conducted in different populations

2. To describe the process for developing criteria that is relevant for recruitment and enrolment of participant for clinical trials in African populations
3. To assess the impact of integrating local components during the development and application of criteria for recruitment and enrolment of participants in reference to the clinical trials conducted within the scope of this thesis work

With respect to aim 2, this thesis aimed to answer the following questions:

1. Which practices are potentially relevant for development and application of criteria for recruitment and enrolment of participants to local regulations and populations?
2. What were the challenges associated with development and application of criteria for recruitment and enrolment of participants in African settings?
3. What optimizations were essential in enabling the criteria to be relevant for local population?
4. What was the average rate of lost to follow-up among enrolled participants in clinical trials conducted within the scope of this thesis work, in Tanzania and Equatorial Guinea?
5. What proportion of trials were completed within the planned time frame, among those clinical trials conducted within the scope of this thesis work, in Tanzania and Equatorial Guinea?

To address aim 2, we conducted pilot studies and clinical trials in two sites located at the eastern (Bagamoyo, Tanzania) and western (Bioko Island, Equatorial Guinea) parts of Africa. This optimization is expected to potentially extend the non-redundant and dynamic knowledge base, in serving other populations with comparable characteristics in or outside African continent. Further details on aim 2 of this thesis are provided in chapter 5.

2.3 Aim 3

In the third part of this thesis (chapter 6), we aimed to optimize safety assessment process with the intention to achieve the ultimate goal in clinical trials that, safety assessment procedures are adequate

for proper screening and enrolment, safe administration of IP, safety follow-up of participants and proper regulatory reporting. Fundamental to such optimization is the basis and the process in place, for interpretation of safety results. Mostly, the safety assessments required at screening and follow-up of participants, such as those defining triggers for stopping rules are based on the requirements of sponsors (usually based on pre-clinical and clinical data not necessarily from the same population). Furthermore, regulatory reporting requirements are defined by the reporting mechanisms stipulated by the relevant regulatory authorities (TMDA, 2017). The process of severity grading systems that are based on the investigator's clinical judgement, are generally, subjective and may potentially be less reliable in accomplishing such tasks. Recommended toxicity grading systems (CPI, 2009; FDA 2005D-0155, 2007; NCI, 2017; NIH-DAIDS, 2017; NIH, 2005; Sibille et al., 2010) provide guidance on assessing the severity of clinical and laboratory abnormalities in volunteers enrolled in clinical trials. In this regard, the thesis objectives for aim 3 were as follows:

1. To assess the common practices used for the assessment of abnormal clinical and laboratory findings in clinical trials
2. To develop the population-based toxicity grading system relevant for clinical trials in African populations in reference to the available toxicity grading guidance
3. To describe the potential benefits based on the experience of using population-based toxicity grading systems in the clinical trials conducted within the scope of this thesis work

With respect to aim 3, this thesis aimed to answer the following questions:

1. What is the rationale for choosing the recommended practice over existing practices for the assessment of abnormal clinical and laboratory findings in clinical trials?
2. What were the challenges experienced when adopting toxicity grading systems of reference that are originally generated from the populations other than African population?
3. What criteria were used at the sites to select the relevant reference toxicity grading system for different protocol specific populations being assessed?

4. What steps and quality assurance processes were followed in adopting toxicity grading systems of reference, in clinical trials conducted African settings?
5. What components were essential for adopting and optimizing the recommended toxicity grading systems in African settings?
6. How the population-based toxicity grading systems were used to improve clinical care of participants?
7. How the population-based toxicity grading systems were used to improve clarity; on implementation of protocol-based and on meeting the regulatory safety reporting?

To address aim 3, we utilized clinical and laboratory data collected from the local population to develop population-based reference intervals which were used to adopt the relevant toxicity grading systems. The resulting, population-based toxicity grading system and further details related to this aim are provided in chapter 6.

CHAPTER 3: Methodology

3.1 Study Sites

3.1.1 Tanzania - IHI-Bagamoyo Clinical Trials Facility (BCTF)

The Ifakara Health Institute (IHI) is a not-for-profit, human health research and resource institute operating in the United Republic of Tanzania founded in 1957 (Tanner et al., 1994). Over the past decades, IHI has been involved in the evaluation of human health interventions against major causes of morbidity and mortality, and assessments of local and national public health systems in Tanzania. IHI uses the Bagamoyo Clinical Trial Facility to conduct high quality clinical trials in close collaboration with national and international institutions and organizations including: Muhimbili National Hospital, National Institute for Medical Research (NIMR), Swiss Tropical and Public Health Institute (Swiss TPH), University of Lausanne, Medicines for Malaria Venture, Malaria Vaccine Initiative, European Vaccine Initiative, Novartis, GSK, and Sanaria Inc (USA). The team has several years of experience in the conduct of clinical trials from phase I to IV. The Investigators team have been trained on Protecting Human Research Participants. In addition, the site team has been trained on the principles of ICH-GCP. Regular advanced life support trainings (including certificates) are conducted on site by the emergency unit from Muhimbili National Hospital. The unit has access to the institutional research clinical laboratory with adequate facilities to perform screening assessment, analyses on participant safety throughout the study. The laboratories are GCLP certified. The site is supported by the institutional quality assurance team.

3.1.2 Equatorial Guinea; BIMEP-CRC, Bioko Island

The clinical trials within the scope of this thesis were performed at the Bioko Island Malaria Elimination Program (BIMEP), Clinical Research Center (CRC) in Malabo, Baney Clinical Research Facility, Bioko Island, Equatorial Guinea. The facility is equipped with ample office space, clinical examination rooms, large inpatient wards, a nursing area, a pharmacy with a biosafety cabinet for vaccine preparation, a full laboratory, internet access, a back-up generator and back-up water supply.

There is oxygen, a cardiac defibrillator, 12-lead ECG machine and other resuscitation supplies and medications on site. BIME-CRC implement high quality clinical trials in close collaboration with Equatorial Guinea-Ministry of Health and Social Welfare, MCDI, IHI, Swiss TPH and Sanaria Inc. The team has several years of experience in the conduct of clinical trials. The Investigators team have been trained on Protecting Human Research Participants. In addition, the site team has been trained on the principles of ICH-GCP. Regular advanced life support trainings (including certificates). The unit has access to the Reference Baney research clinical laboratory where Clinical laboratory, molecular biology and other research assays were performed with adequate facilities to perform screening assessment, analyses on participant safety throughout the study (Jongo et al., 2022a; Olotu et al., 2018a).

3.2 Collaborations

3.2.1 Internal

3.2.1.1 IHI

Interventions and Clinical Trials Department

- Salim Abdulla - for advice and discussion on general conduct of the studies

Biostatistics unit:

- Ali Ali and Ummi Abdul: for advice and discussion on Analysis of reference intervals

3.2.1.2 Equatorial Guinea

- Hon. Mitoha Ondo'o Ayekaba: Vice Minister of Health and Social Welfare of Equatorial Guinea at Government of Equatorial Guinea. For support from local health-care system.

3.2.1.3 Swiss TPH

Department of Medical Parasitology and Infection Biology

- Claudia Daubenberger, Clinical Immunology Unit – for advise on clinical protocol design and implementation

Department of Medicines Research:

- Andrea Kümmerle - for advice and discussion on safety reporting
- Christian Burri – for information on trial methodology

Department of Biostatistics:

- Amanda Ross - for advice and discussion on quantitative statistical analysis

3.2.2 External Collaboration

- Thomas Richie - for advice and discussion on general conduct of the studies

3.3 RESEARCH AND ETHICAL CLEARANCE

All studies received relevant EC and RA approvals as stipulated in each manuscript.

Table 1:Summary of IRBs, ECs and RAs

Tanzania	Ifakara Health Institute – Institutional Review Board (IHI IRB)
	Tanzania Medicines & Medical Devices Authority (TMDA)
Equatorial Guinea	Equatorial Guinea National Ethics Committee (CENGE)
	Director General of Pharmacy and Traditional Medicine, Ministry of Health and Social Welfare, Equatorial Guinea
Switzerland	Ethics Committee of Northwestern and Central Switzerland (Ethikkommission Nordwest- und Zentralschweiz, EKNZ) (administrative review)
USA	Prime IRB, USA (administrative review)
	United States Food and Drug Administration (FDA)

CHAPTER 4: Safety of PfSPZ based malaria vaccine candidates in different populations in Africa

4.1 General overview of safety assessments

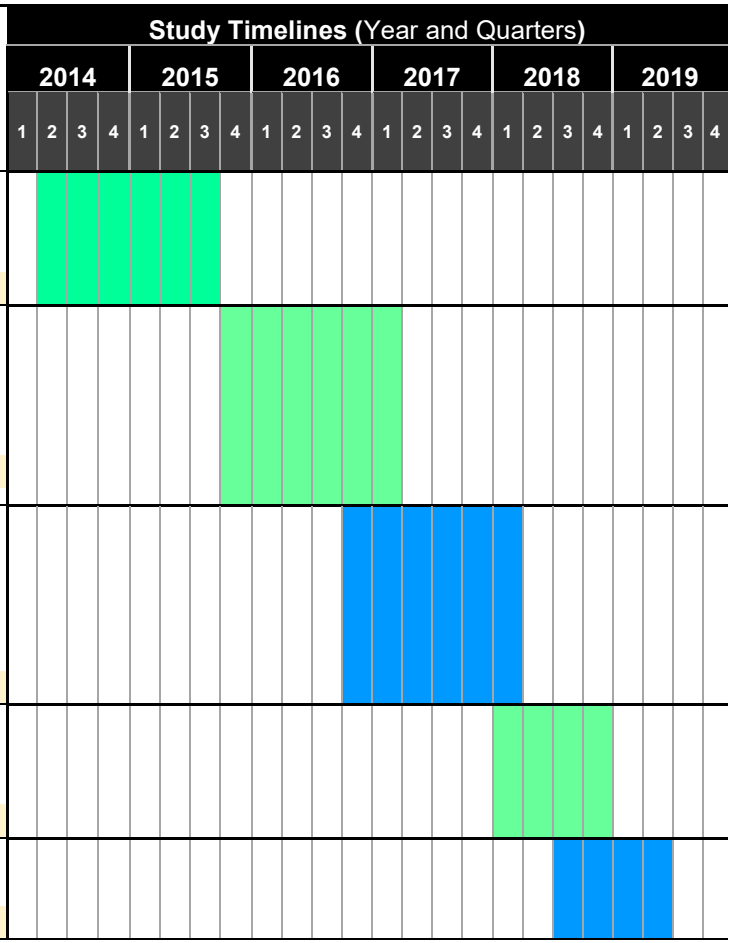
This section provides details substantiating the description provided for thesis aim 1 in section 2.1 for each publication on the general and specific safety concerns with PfSPZ malaria vaccine candidates. The work that we performed within the scope of this thesis, was primarily focused on generating early tolerability and safety data adequate to support further assessment of PfSPZ-based malaria vaccine candidates among other African communities. An overview of studies is provided in Table 2. Summaries for each work is given below and individual referenced papers are provided in sections 0 to 4.7.

4.1.1 Paper-I: Safety following immunization with PfSPZ Vaccine and infectivity assessment using PfSPZ-CHMI

The work that we presented in section 0, published the results from the first trial in Africa to assess the safety in African population challenged with PfSPZ-CHMI after immunization with PfSPZ Vaccine (BSPZV1, thesis Paper I, Section 0) (Jongo et al., 2018b). In this study, we demonstrated that, the PfSPZ-CHMI model which was previously assessed for the first time in Tanzania for Africans adult population, could be safely used to assess VE following immunization with PfSPZ Vaccine. The same PfSPZ Vaccine dosage regimen was less immunogenic and protective in Tanzanians than in Americans and VE against homologous CHMI in Tanzania was lower (or similar) to VE against intense field exposure to heterogeneous Pf parasites in Mali (Sissoko et al., 2017b). Due to the risk level perceived at the time, only young adult male Tanzanians from higher level institutions were enrolled. The safety results from paper I, were used as the proof of concept in using the CHMI model as a reliable approach to assess protective efficacy in African population, not only for vaccines, but also for other malaria interventions and all types of adult populations. Further details substantiating paper I are published in an article presented in section 4.3.

Table 2: Chronological Listing of Trials conducted within the scope of this thesis

Study Identifier (Clinicaltrials.gov)	Study population (Malaria Exposed) Age & Sample size Vaccinees / NS Controls	Study Design Summary (Papers / Manuscripts)	Study Timelines (Year and Quarters)
BSPZV1 **Tanzania** **(NCT02132299)**	**Adults total: 67** 49 / 8 CHMI Controls: 10	Phase 1, randomized, double- blind placebo-controlled* with CHMI (by needle and syringe) in Tanzania **(Paper I)**	
BSPZV2 **Tanzania** **(NCT02613520)**	**Adults total: 18** 12 / 6 **Children total: 54** 36 /18 **Infants total: 21** 15 / 6	Phase 1 dose escalation, double-blind, randomized, placebo-controlled* with CHMI (by needle and syringe) **(Paper II and Paper III)**	
EGSPZV2 **Equatorial Guinea** **(NCT02859350)**	**Adults total: 52**** 40 / 12 **Children total: 48** 36/12 **Infants total: 19** 15/4	Phase 1 dose escalation, randomized double-blind, placebo-controlled* with head-to-head PfSPZ Vaccine and PfSPZ-CVac comparison **(Paper IV)**	
BSPZV3a **Tanzania** **(NCT03420053)**	**Adults total: 21** HIV-: 6 / 3 HIV+: 9 / 3	Phase 1 dose escalation, randomized double-blind, placebo-controlled* with CHMI **(Paper V: Manuscript)**	
EGSPZV3 **Equatorial Guinea** **(NCT03590340)**	**Adults total: 104** 84 / 20	Phase 1 double-blind, randomized, placebo-controlled* with CHMI **(Paper VI)**	



***The placebo control used in all trials is normal saline; ** 20/52 adult volunteers in EGSPZV2 received PfSPZ-CVac.**

4.1.2 Paper-II: Safety in age de-escalation and dose escalation among adults and pediatric populations

The work that we present in sections 0 and 0, published results from the first trial in the world to assess the tolerability and safety following PfSPZ Vaccine through age-de-escalation and dose-escalation among healthy adults, adolescents, children and pediatric populations in Africa (Jongo et al., 2019). The design included interim safety reviews going from healthy adults to younger population. We, therefore, evaluated PfSPZ Vaccine in 93 Tanzanians aged 45 years to 6 months in a randomized, double-blind, normal saline placebo-controlled trial. There were no significant differences in adverse events between vaccinees and controls or between dosage regimens. Because all age groups received three doses of 9.0×10^5 PfSPZ of PfSPZ Vaccine, immune responses were compared at this dosage. The safety data were used to support initiation of trials in > 300 infants in Kenya and Equatorial Guinea (Jongo et al., 2021a; Oneko et al., 2020; Oneko et al., 2021). Because PfSPZ Vaccine-induced protection is thought to be mediated by T cells, the T-cell data suggest PfSPZ Vaccine may be more protective in children than in adults, whereas infants may not be immunologically mature enough to respond to the PfSPZ Vaccine immunization regimen assessed. Further details substantiating paper II are published in an article presented in section 0.

4.1.3 Paper-III: Safety in dose-escalation among adults

The work that we present in sections 4.4, published results that partially supported previous PfSPZ Vaccine studies indicated increasing numbers of PfSPZ per dose would increase VE (Jongo et al., 2020b). Our results indicated for the first time that, increasing from 9.0×10^5 to 1.8×10^6 PfSPZ was associated with reduction of VE from 100% at 23 or 79 days after last vaccine dose to 33% at 52 days after last vaccine dose (P = .0224). There were no safety concerns related to higher doses. Prior to these findings, we have pursued an empiric development process in which we have altered

PfSPZ/dose, and dose numbers and intervals to identify an optimal immunization regimen. With similar phenomenon being described in mice, these results provided an indication to concentrate on optimizing 9.0 × 10^5 PfSPZ/dose. Further details substantiating paper III are published in an article presented in section 0.

4.1.4 Paper IV: Parallel assessment on the safety of PfSPZ Vaccine and PfSPZ-CVac

This is the first trial double-blind, randomized, placebo-controlled trial to directly compare the VE of PfSPZ-CVac and PfSPZ Vaccine and was conducted in Equatorial Guinea among adults 18- to 35-year-olds. Sanaria PfSPZ-CVac is identical to PfSPZ Vaccine, except it is chemo-attenuated by Chloroquine phosphate administered weekly beginning 2 days before the first dose through to 12 days after the final dose. With the PfSPZ Vaccine dose of 2.7x10^6 PfSPZ/dose, the resulting VE of 27% is consistent with the findings in Tanzania, that increasing the dose beyond 9.0x10^5 apparently reduces VE against CHMI. PfSPZ-CVac dose of 1.0x10^5 PfSPZ/dose, the resulting VE of 55% was not optimal (Paper IV) (Jongo et al., 2021a). Further details substantiating paper IV are published in an article presented in section 4.6.

4.1.5 Paper-V; Manuscript: Safety of condensed accelerated regimens among adults with and without HIV

The PfSPZ of PfSPZ Vaccine although metabolically active, do not expand in size and cannot undergo schizogony due to radiation-induced attenuation. Thus, a competent immune response by the immunized participant is not required to attenuate the vaccine organism, unlike other live, whole organism vaccines such as oral polio vaccine that undergo replication in the human host. In addition, there is no evidence that PfSPZ Vaccine causes liver damage. Thus, even in an individual with HIV infection, administration of PfSPZ Vaccine should be safe. The BSPZV3a trial enrolled HIV+ study subjects, and tested the safety of five doses of 9.0x105 PfSPZ of PfSPZ Vaccine. The study showed

that the vaccine was safe in this population, with no changes in CD4 cell counts or viremia associated with immunization. Based on these data, it is expected that three injections with $9.0x10^5$ PfSPZ as planned in this trial will be safe in other populations with chronic diseases or infections (Paper V). Further details substantiating paper V are under review for publication in the manuscript presented in section 5.2.

4.1.6 Paper-VI: Optimization and down-selection for condensed accelerated regimens

In a previous trial we reported that increasing the dose of PfSPZ in PfSPZ Vaccine from 9.0x10^5 to 1.8x10^6 (3 doses at 8-week intervals) in Tanzanian adults significantly reduced vaccine efficacy (VE) against controlled human malaria infection (CHMI) from 100% to 33% (Paper III). When 9.0x10^5 PfSPZ were administered on days 1, 3, 5, 7 (priming) with a 16-week boost, VE against heterologous CHMI in U.S. adults at 12 weeks was higher than with any previous regimens (Lyke et al., 2021b). We administered the same priming regimen (1,3,5,7) followed by a 4-week boost to healthy Tanzanian adults and showed 80% VE against CHMI (Paper V). A condensed administration schedule of PfSPZ Vaccine with high VE would facilitate its deployment. Following these results, we conducted a regimen optimization study of a radiation attenuated, aseptic, purified, cryopreserved PfSPZ Vaccine (Paper VI). In this double blind, placebo-controlled trial in 104 Equatorial Guinean adults, four multi dose priming regimens were tested. The regimens were:

- **Regimen 1:** Warfighter – USA: Best Regimen: Days 1, 3, 5, 7, boost at week 16, 40% VE against heterologous CHMI 3 months later
- Permutations
    a. **Regimen 2:** Days 1, 3, 5, 7, no boost (permutation of regimen 1)
    b. **Regimen 3:** Days 1, 3, 5, 7, boost at day 29 – (permutation of regimen 1: assessed for the first time in Africa in Bagamoyo BSPZV3a (Paper V))

- **Regimen 4:** Mavache-German: Best Regimen: Days 1, 8, boost at day 29, 83.3% VE against heterologous CHMI at 10 weeks

Vaccine was tolerable and safe when given in condensed multi-dose prime regimens. This study found that; (1) The 3-dose regimen (days 1, 8 and 29) is as good as or better than a 5-dose regimen (whether boosts at 29 days or 113 days) and (2) The boost dose, apparently improves vaccine efficacy. These observations, indicate that the Most Optimal multi-prime regimen in this dose optimization trial is 9x10^5 PfSPZ given on days 1, 8 and 29. (Paper VI). Further details substantiating paper VI are published in an article presented in section 4.7.

4.2 Overall safety profile of PfSPZ based vaccine-candidates

At baseline, clinical and laboratory safety parameters were assessed among African populations from the two geographical locations, and final eligibility for enrolment was performed after reviewing other criteria related to GCP and protocol compliance. Eligible participants were enrolled and administered with IP (PfSPZ malaria vaccine candidates) under close monitoring for the solicited and unsolicited local and systemic AEs and laboratory safety parameters. Specific details for each of the trials listed in Table 2 are provided in CHAPTER 4:. The side effect profile of PfSPZ Vaccine recorded in 18 Phase 1 and 2 clinical trials has been benign, and not been different from that of NS placebo controls as indicated in the forest plots below.

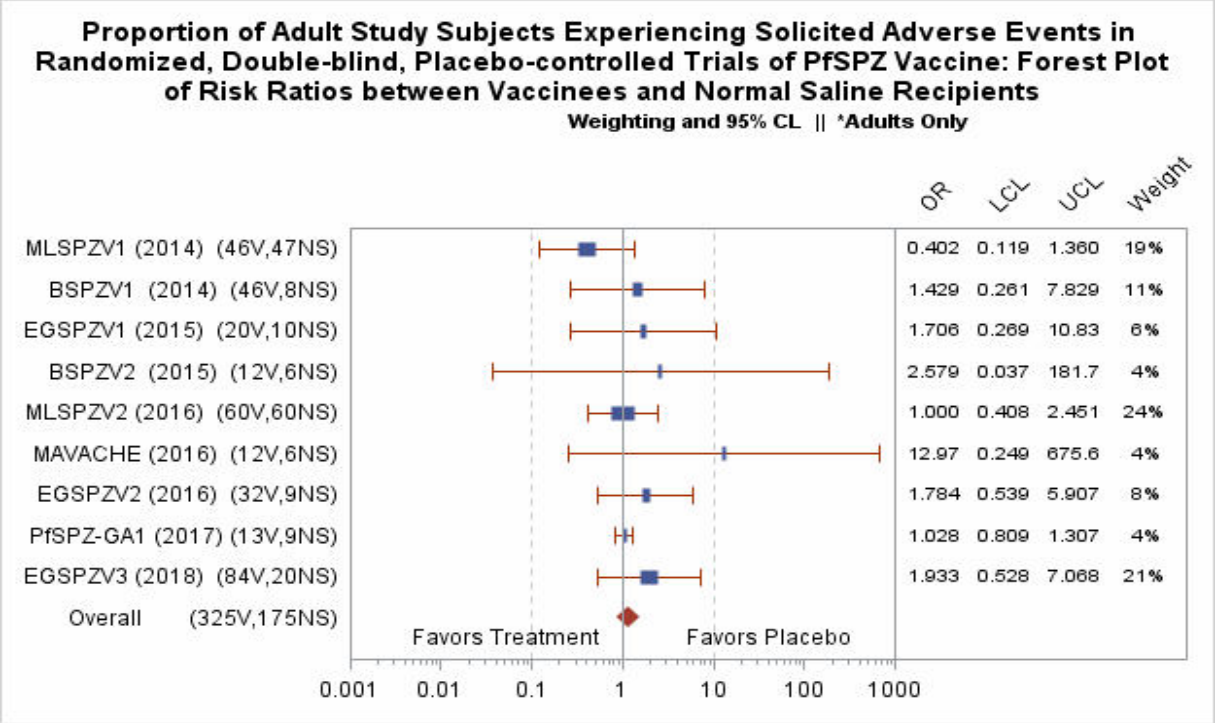


Figure 3: Forest plot showing, risk ratios between adults subjects (vaccinees vs NS recipients)

Trial sites included: Mali (MLSPZV1, MLSPZV2); Bagamoyo, Tanzania (BSZPV1, BSPZV2); Bioko Island, Equatorial Guinea (EGSPZV1, EGSPZV2, EGSPZV3); Tübingen, Germany (MAVACHE); and Leiden and Nijmegen, the Netherlands (PfSPZ-GA-1). Weight is based on sample size and is represented by the width of the blue bar

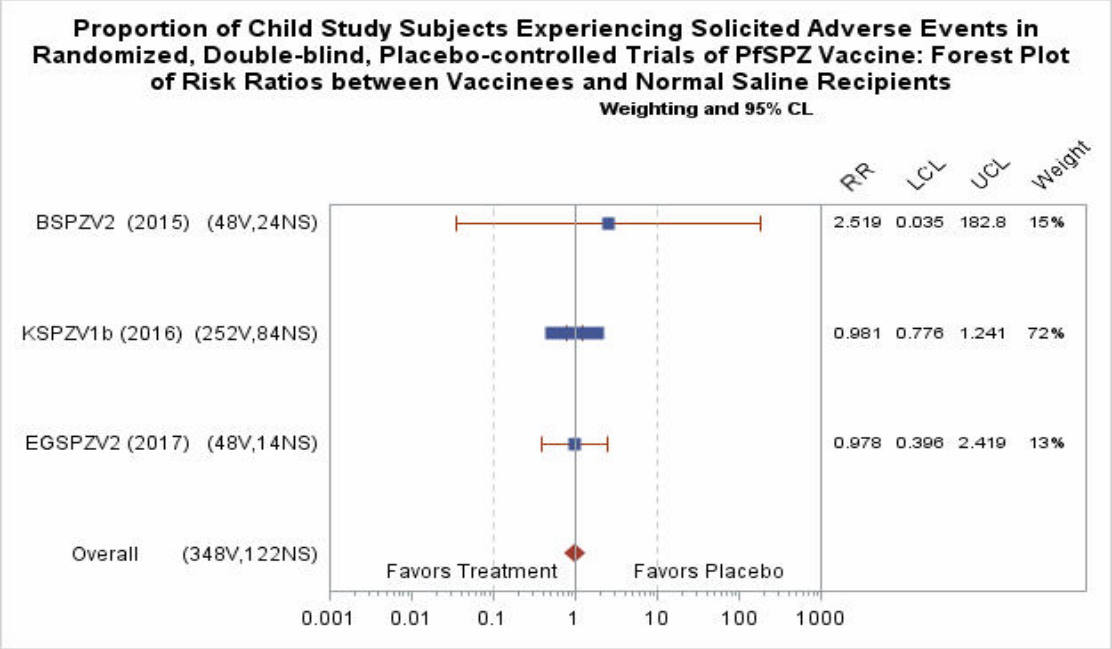


Figure 4: Forest plot showing, risk ratios between pediatric subjects (vaccinees vs NS recipients)

Trial sites included: Bagamoyo, Tanzania (BSPZV2); Siaya, Kenya (KSPZV1b); and Bioko Island, Equatorial Guinea (EGSPZV2. V = vaccine recipients, NS = normal saline recipients, OR = odds ratio, LCL = lower confidence limit, UCL = upper confidence limit. Weight is based on sample size and is represented by the width of the blue bar.

4.3 Paper I: Published Article

Safety, Immunogenicity, and Protective Efficacy against Controlled Human Malaria Infection of Plasmodium falciparum Sporozoite Vaccine in Tanzanian Adults: Randomized Controlled Trial

Am J Trop Med Hyg. 2018 Aug;99(2):338-349. doi: 10.4269/ajtmh.17-1014.Epub 2018 Jun 21.

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Safety, Immunogenicity, and Protective Efficacy against Controlled Human Malaria Infection of *Plasmodium falciparum* Sporozoite Vaccine in Tanzanian Adults

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Abstract. We are using controlled human malaria infection (CHMI) by direct venous inoculation (DVI) of cryopreserved, infectious *Plasmodium falciparum* (Pf) sporozoites (SPZ) (PfSPZ Challenge) to try to reduce time and costs of developing PfSPZ Vaccine to prevent malaria in Africa. Immunization with five doses at 0, 4, 8, 12, and 20 weeks of 2.7×10^5 PfSPZ of PfSPZ Vaccine gave 65% vaccine efficacy (VE) at 24 weeks against mosquito bite CHMI in U.S. adults and 52% (time to event) or 29% (proportional) VE over 24 weeks against naturally transmitted Pf in Malian adults. We assessed the identical regimen in Tanzanians for VE against PfSPZ Challenge. Twenty- to thirty-year-old men were randomized to receive five doses normal saline or PfSPZ Vaccine in a double-blind trial. Vaccine efficacy was assessed 3 and 24 weeks later. Adverse events were similar in vaccinees and controls. Antibody responses to Pf circumsporozoite protein were significantly lower than in malaria-naïve Americans, but significantly higher than in Malians. All 18 controls developed Pf parasitemia after CHMI. Four of 20 (20%) vaccinees remained uninfected after 3 week CHMI ($P = 0.015$ by time to event, $P = 0.543$ by proportional analysis) and all four (100%) were uninfected after repeat 24 week CHMI ($P = 0.005$ by proportional, $P = 0.004$ by time to event analysis). *Plasmodium falciparum* SPZ Vaccine was safe, well tolerated, and induced durable VE in four subjects. Controlled human malaria infection by DVI of PfSPZ Challenge appeared more stringent over 24 weeks than mosquito bite CHMI in United States or natural exposure in Malian adults, thereby providing a rigorous test of VE in Africa.

INTRODUCTION

In 2015 and in 2016, there were an estimated 429,000–730,500 deaths caused by malaria.[1–3] *Plasmodium falciparum* (Pf) is the cause of > 98% of malaria deaths and > 80% of malaria cases in sub-Saharan Africa. Our goal is to field a vaccine that will prevent infection with Pf and thereby prevent all manifestations of Pf malaria and parasite transmission from humans to mosquitoes.[4]

Plasmodium falciparum sporozoites (SPZ) are the only immunogens that have ever prevented Pf infection in > 90% of subjects.[5–7] Sanaria® PfSPZ Vaccine (Sanaria Inc., Rockville, MD) is composed of radiation-attenuated, aseptic, purified, cryopreserved PfSPZ.[8,9] When administered by rapid intravenous injection, PfSPZ Vaccine protected 100% (6/6) of malaria-naïve subjects in the United States against mosquito bite–controlled human malaria infection (CHMI) with Pf parasites similar to those in the vaccine (homologous) 3 weeks after the last immunization,[10] and 65% at 24 weeks.[11] Protection was durable against homologous mosquito bite CHMI for at least 59 weeks[12] and heterologous (parasites different than in vaccine) mosquito bite CHMI for at least 33 weeks.[13] PfSPZ Vaccine also prevented naturally transmitted heterogeneous Pf in adults in Mali for at least 24 weeks (vaccine efficacy [VE] 52% by time to event and 29% by proportional analysis).[14]

We used the same dosage regimen as in the United States and Mali to evaluate the tolerability, safety, immunogenicity, and VE of PfSPZ Vaccine in young adult male Tanzanians. Previously, we had conducted the first modern CHMI in Africa and showed that injection of aseptic, purified, cryopreserved PfSPZ, Sanaria® PfSPZ Challenge, consistently infected Tanzanian volunteers and subsequently repeated in multiple other countries.[15–21] In this study, we took advantage of this capability to assess VE of PfSPZ Vaccine by CHMI with PfSPZ Challenge (NF54). The same PfSPZ Vaccine dosage regimen was less immunogenic and protective in Tanzanians than in Americans,[11] and VE against homologous CHMI in Tanzania was lower (or similar) to VE against intense field exposure to heterogeneous Pf parasites in Mali.[14]

MATERIAL AND METHODS

Study design and population. This double-blind, randomized, controlled trial was conducted in Bagamoyo, Tanzania, between April 2014 and August 2015. Sixty-seven healthy male volunteers of 18–35 years of age were recruited from higher learning institutions in Dar es Salaam. After initial screening, prospective volunteers were invited to the Bagamoyo Clinical Trial Unit of the Ifakara Health Institute (IHI) to complete informed consent and screening.

All had to complete a 20-question assessment of trial understanding with a 100% correct response rate on the first or second attempt (Supplemental Table 1) to be eligible. Volunteers were screened using predetermined inclusion and exclusion criteria (Supplemental Tables 2 and 3). History of malaria in the previous 5 years or antibodies to Pf exported protein 1 (PfEXP1) by an enzyme-linked immunosorbent assay (ELISA) above a level associated with a single, recent Pf infection by CHMI[19] (see the Antibody assays section) were the exclusion criteria. Hematology, biochemistry, and parasitology testing, including malaria thick blood smear (TBS), stool,

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and urine by microscopy was carried out. Tests for human immunodeficiency virus and hepatitis B and C were performed after counseling; volunteers were excluded if positive and referred for evaluation and management by appropriate local physicians. Volunteers were excluded if they had significant abnormalities on electrocardiograms.

The trial was performed in accordance with Good Clinical Practices. The protocol was approved by institutional review boards (IRBs) of the IHI (Ref. No. IHI/IRB/No:02-2014), the National Institute for Medical Research Tanzania (NIMR/HQ/R.8a/Vol.IX/1691), the Ethikkommission Nordwest-und Zentralschweiz, Basel, Switzerland (reference number 261/13), and by the Tanzania Food and Drug Authority (Ref. No. TFDA 13/CTR/0003); registered at Clinical Trials.gov (NCT02132299); and conducted under U.S. FDA IND application.

Investigational products (IPs). The IPs were Sanaria® PfSPZ Vaccine[8–14] and Sanaria® PfSPZ Challenge.[15–20] PfSPZ Vaccine consists of aseptic, purified, vialed, metabolically active, nonreplicating (radiation attenuated), cryopreserved PfSPZ (NF54 strain). It was stored, thawed, diluted, and administered by direct venous inoculation (DVI) in 0.5 mL through a 25-gauge needle.[11,14,18,20] PfSPZ Challenge is identical to PfSPZ Vaccine except it is not radiation attenuated. It was handled and administered like PfSPZ Vaccine. Preparation of IPs was supervised by the study pharmacist. After labeling the syringe, the pharmacist handed it to the clinical team through a window.

Allocation and randomization. Volunteers were allocated to five groups (Table 1; Figure 1). Forty-nine received PfSPZ Vaccine and eight normal saline (NS). Ten were additional infectivity controls. The clinical team and volunteers were blinded to assignment to vaccine or NS until study end.

Group 1. Three volunteers received consecutive doses of 3×10^4, 1.35×10^5, and 2.7×10^5 PfSPZ of PfSPZ Vaccine at 4-week intervals to assess safety (Group 1).

Groups 2 and 3. Volunteers were randomized to receive 1.35×10^5 PfSPZ of PfSPZ Vaccine ($N = 20$) or NS ($N = 4$) (Group 2), or 2.7×10^5 PfSPZ of PfSPZ Vaccine ($N = 20$) or NS ($N = 4$) (Group 3) at 0, 4, 8, 12, and 20 weeks.

Group 4. Six volunteers were immunized with 2.7×10^5 PfSPZ of PfSPZ Vaccine on the same schedule as Group 3.

Group 5. Ten volunteers served as unblinded infectivity controls during CHMIs (see in the following paragraph): two with CHMI #1, two with CHMI #2, and six with CHMI #3.

Vaccine efficacy. *Controlled human malaria infection.* Vaccine efficacy was assessed by CHMI by DVI of 3.2×10^3 PfSPZ of PfSPZ Challenge. Controlled human malaria infection #1 was 3 weeks after the last immunization in Group 2. Controlled human malaria infection #2 was 3 weeks after the last immunization in Group 3. Controlled human malaria infection #3 was 24 weeks after the last immunization in Groups 3 and 4 and included the four volunteers in Group 3 who did not develop parasitemia after CHMI #2 and the six Group 4 volunteers. Volunteers were inpatients from day 9 after PfSPZ Challenge injection for observation until diagnosed and treated for malaria or until day 21; daily outpatient monitoring for TBS-negative volunteers continued until day 28. Thick blood smears were obtained every 12 hours on days 9–14 after CHMI and daily on days 15–21 until positive or until day 21. Thick blood smears could be performed more frequently, if volunteers had symptoms/signs consistent with malaria. After initiation of treatment, TBSs were assessed until two consecutive daily TBSs were negative and on day 28.

Detection of Pf parasites and parasite DNA. Slide preparation and reading for TBSs were performed as described.[19] Sensitivity was 2 parasites/μL blood unless the volunteer was symptomatic, in which case four times as many fields were read. Parasitemia was also determined by quantitative polymerase chain reaction (qPCR) with sensitivity of 0.1 parasites/μL blood based on a multiplex assay detecting *Plasmodium* spp. 18S genes and the human RNaseP gene as endogenous control.[22] A second, more sensitive qPCR assay with a sensitivity of 0.05 parasites/μL blood and targeting the Pf-specific telomere-associated repetitive element 2[23] was used to reanalyze all samples that were negative by 18S-based qPCR. After the start of CHMI, the time of first blood sample positivity by qPCR was used to determine infection status and for the calculation of prepatent period. Volunteers were continuously monitored by qPCR until malaria treatment based on TBS positivity. The World Health Organization International Standard for Pf DNA Nucleic Acid Amplification Techniques (NIBSC, Hertfordshire, United Kingdom) was used as standard for calculation of parasite densities. DNA was extracted from 100 μL whole blood and eluted with 50 μL Elution Buffer using Quick-gDNA Blood MicroPrep Kit (Zymo Research, Irvine, CA). Blood samples were analyzed retrospectively by qPCR after storing at –80°C after the conclusion of CHMIs. To exclude field strain infections, parasite genotyping was performed on samples randomly chosen as described.[24] In all cases in which TBS was negative and qPCR was considered positive, two consecutive samples were positive by qPCR.

Adverse events (AEs). Volunteers were observed as inpatients for 48 hours after administration of IP and discharged with diaries and thermometers for recording AEs and temperatures and followed with daily telephone calls. Symptoms and signs (solicited and unsolicited) were recorded and graded by physicians: mild (easily tolerated), moderate (interfere with normal activity), severe (prevents normal activity),

TABLE 1
Demographic characteristics of volunteers

	Vaccinees	Normal saline controls	Infectivity controls
Number of volunteers	49	8	10
Percentage males	100%	100%	100%
Mean age in years (range)	24 (20, 30)	23 (20, 28)	25 (21, 28)
Percentage Africans	100%	100%	100%
Mean body mass index (range)	22.33 (18.00, 29.70)	21.91 (19.00, 24.20)	21.68 (18.40, 24.30)
Number (%) heterozygous for alpha thalassemia	22 (44.9%)	4 (50%)	5 (50%)
Number (%) with LTBI* (QuantiFERON positive)	17 (34.7%)	3 (36.5%)	1 (10%)
Number (%) positive on screening of urine or stool for parasitic infection	0 (0%)	1 (12.5%)	0 (0%)
Number (%) students	49 (100%)	8 (100%)	10 (100%)

* Latent tuberculosis infection.

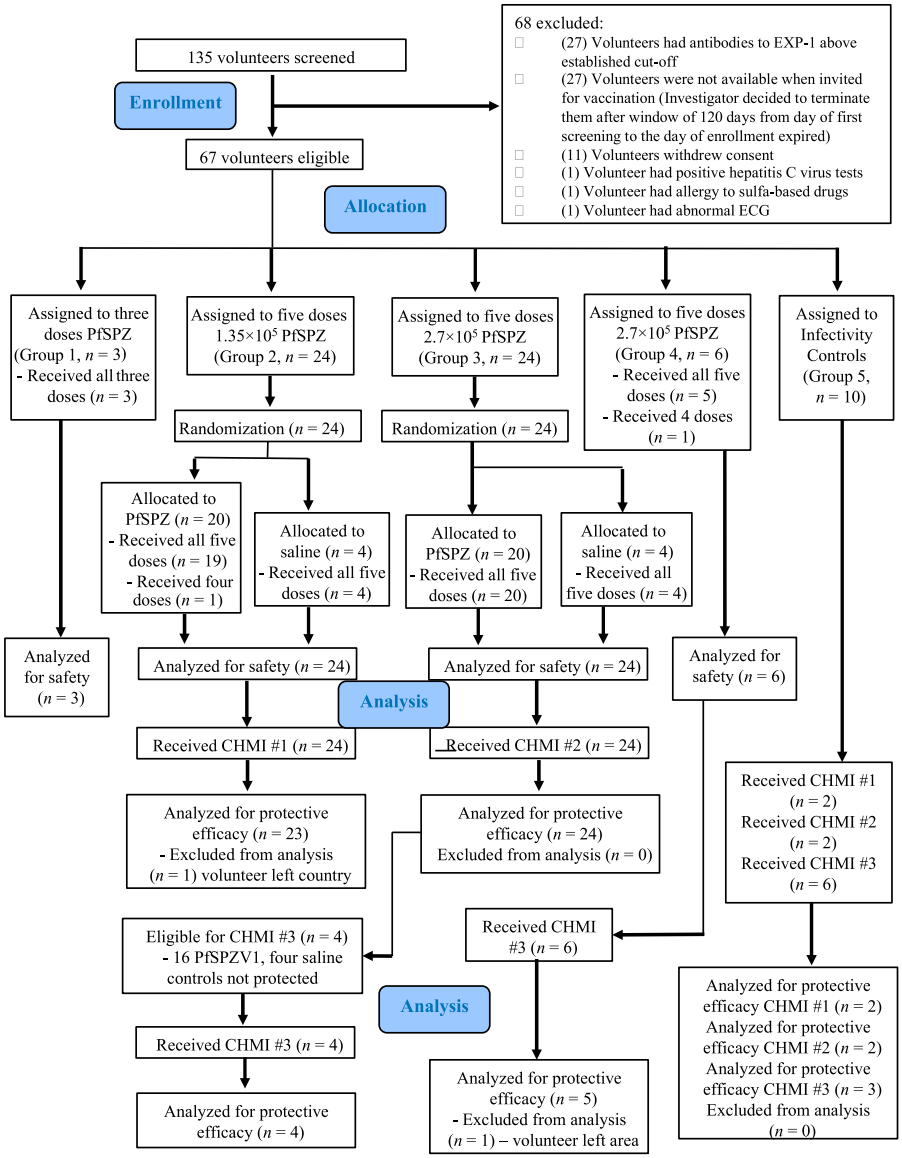


FIGURE 1. Volunteer participation (CONSORT 2010 diagram). This figure appears in color at www.ajtmh.org.

or life threatening. Axillary temperature was grade 1 (> 37.5–38.0°C), grade 2 (> 38.0–39.0°C), grade 3 (> 39.0–40.0°C), or grade 4 (> 40.0°C). Hematological and biochemical abnormalities were also assessed using standard clinical assays.

During the first 7 days after injection of IPs, prespecified local (site of injection) and systemic AEs were solicited. Open-ended questioning was used to identify unsolicited AEs through day 28 (Supplemental Table 4). All AEs were assessed for severity and relatedness to IP administration. Adverse events were classified as definitely related, probably related, possibly related, unlikely to be related, and not related. Definitely, probably, and possibly were considered to be related. Unlikely to be related and not related were considered to be unrelated. For CHMIs, volunteers returned on day 9 for admission to the ward for diagnosis and treatment of malaria. Events during the 8–28 day period were assessed for relationship to Pf infection and considered related if the event was within 3 days before and 7 days after TBS was first positive.

Antibody assays. Sera were assessed for antibodies by ELISA, immunofluorescence assay (aIFA), and inhibition of sporozoite invasion (ISI) assay as described (see Supplemental Table 5).[25] For ELISAs, the results are reported as the serum dilution at which the optical density (OD) was 1.0. Enzyme-linked immunosorbent assay for PfEXP1 was used to screen volunteers for possible malaria exposure (Supplemental Table 6). Any subject with an OD 1.0 of ≥ 600 was excluded. This was because we had previously determined in Tanzanians who underwent CHMI[19] that antibodies to PfEXP1 at this level were a sensitive indicator of recent Pf infection (unpublished).

T-cell assays. T-cell responses in cryopreserved peripheral blood mononuclear cells (PBMC) were measured by flow cytometry in a single batch after the study as described.[12] After stimulation, cells were stained as described.[26] The staining panels are in Supplemental Table 7 and antibody clones and manufacturers are in Supplemental Table 8. All antigen-specific frequencies are reported after background

subtraction of identical gates from the same sample incubated with control antigen. Data were analyzed with FlowJo v9.9.3 (TreeStar, Ashland, OR) and graphed in Prism v7.0a (GraphPad, San Diego, CA).

Statistical analysis. Comparisons of categorical variables between groups were analyzed using 2-tailed Fisher's exact test. Comparisons of continuous variables between groups were analyzed by 2-tailed nonparametric tests. For multiple group comparisons, the Kruskal–Wallis test was used. Time to event was assessed by the Kaplan–Meier curves and log-rank test. Vaccine efficacy by time to event was quantified using Cox proportional hazards ratios. Time to event data were analyzed from CHMI injection until positive TBS result or positive qPCR result. Controlled human malaria infection follow-up period lasted until day 28 after CHMI injection. Analyses of immunological data are described with the data.

Role of the funding source. The funders were involved in study design, study management, data collection, data analysis, data interpretation, and writing the report. Salim Abdulla and Stephen L. Hoffman had full access to all data in the study and final responsibility for decision to submit for publication.

RESULTS

Study population and experience with DVI. Fifty-seven Tanzanian men (Table 1; Figure 1) met the criteria (Supplemental Tables 2 and 3) and received PfSPZ Vaccine (N = 49) or NS (N = 8). All volunteers had AA hemoglobin and normal G6PD activity. Thirty-one volunteers (46%) were heterozygous for α-thalassemia; 21 had evidence of latent tuberculosis infection by Quantiferon testing, but showed no evidence of active tuberculosis. One volunteer (group 2, NS) had *Strongyloides stercoralis* on screening and was successfully treated before vaccination (Table 1).

Of 237 immunizations with PfSPZ Vaccine, 234 were completed with a single injection (98.7%). Two hundred and thirty injections (97.0%) were considered painless by the volunteer. For NS subjects, 39 of 40 immunizations (97.5%) were completed in a single injection and 39 of 40 (97.5%) considered painless by the volunteer. The nurse performing immunizations considered the procedure to be simple in 265 of 273 single injections (97.1%).

One subject in Group 2 received four immunizations. The third immunization was withheld while the subject was evaluated for what was diagnosed as benign ethnic neutropenia.[27,28] One subject in Group 4 missed his second immunization when he left town. All other subjects (other than Group 1 and added infectivity controls) received five immunizations.

Safety. Among 49 volunteers who received 237 doses of PfSPZ Vaccine, there were 17 solicited AEs possibly related to IP (17/237 = 7.2%) in 10 of the 49 vaccinees (20.4%) (Table 2). Among eight volunteers who received 40 doses of NS, there were two solicited AEs possibly related to IP (2/40 = 5.0%) in one of the eight controls (12.5%) (Table 2). There were no AEs considered by the clinicians to be probably or definitely related to IP. There were no local or serious AEs. One episode each of headache and fever were grade 2; all other solicited AEs were grade 1. None of the comparisons of AEs between vaccinees and controls or between Group 2 (1.35×10^5 PfSPZ) and Groups 3 and 4 (2.7×10^5 PfSPZ) showed statistically significant differences (Table 2). Twenty-six of 49 vaccinees (53.1%) experienced 43 unsolicited AEs (0.88/individual) in the 28 days following injections #1–#4 and the 21 days before CHMI after injection #5. Seven of eight controls (87.5%) experienced 14 unsolicited AEs (2/individual) during this period. None of these unsolicited AEs recorded within 28 days of an immunization was considered related to IP.

Laboratory abnormalities occurred at roughly equal rates comparing PfSPZ Vaccine recipients and controls, except for leukocytosis and eosinophilia, which were more frequent in controls (Table 3). There was no apparent explanation for these differences. A cyclic variation in total bilirubin following

TABLE 2

Solicited AEs by group considered possibly* related to administration of the investigational product during the first 7 days post immunization

	Group 1 (dose escalation)	Group 2 (1.35×10^5 PfSPZ)	Group 3 (2.7×10^5 PfSPZ)	Group 4 (2.7×10^5 PfSPZ)	Total PfSPZ vaccine	NS controls
Number of volunteers	3	20	20	6	49	8
Total number of injections	9	99	100	29	237	40
Number of local AEs	0	0	0	0	0	0
Numbers of systemic AEs (% of total immunizations)						
All	1 (11%)	10 (10.1%)	6 (6%)	0	17 (7.2%)	2 (5.0%)
Headache*	1 (11%)	7 (7%)†	2 (2%)	0	10 (4.2%)	1 (2.5%)
Abdominal pain	0	2 (2%)	1 (1%)	0	3 (1.3%)	0
Chills	0	0	1 (1%)	0	1 (0.4%)	0
Fever	0	0	2 (2%)	0	2 (0.8%)	0
Diarrhea	0	0	0	0	0	1 (2.5%)
Chest pain	0	1 (1%)	0	0	1 (0.4%)	0
Other	0	0	0	0	0	0
Systemic AEs - no. volunteers with ≥ 1 event (% of volunteers)						
Any	1 (33%)	7 (35%)	2 (10%)	0	10 (20.4%)	1 (13%)
Headache	1 (33%)	6 (30%)	2 (10%)	0	9 (18.4%)	1 (13%)
Abdominal pain	0	2 (10%)	1 (5%)	0	3 (6.1%)	0
Chills	0	0	1 (5%)	0	1 (2.0%)	0
Fever	0	0	2 (10%)	0	2 (4.1%)	0
Diarrhea	0	0	0	0	0	1 (13%)
Chest pain	0	1 (5%)	0	0	1 (2.0%)	0
All other	0	0	0	0	0	0

AEs = adverse events; PfSPZ = *Plasmodium falciparum* sporozoites. There were no significant differences between vaccinees as compared with normal saline (NS) controls for any or all AEs. All AEs were grade 1, except one headache and one fever. Local solicited AEs: injection site pain, tenderness, erythema, swelling, or induration. Systemic solicited AEs: allergic reaction (rash, pruritus, wheezing, shortness of breath, bronchospasm, allergy-related edema/angioedema, hypotension, and anaphylaxis), abdominal pain, arthralgia, chest pain/discomfort, chills, diarrhea, fatigue, fever, headache, malaise, myalgia, nausea, pain (other), palpitations, shortness of breath, and vomiting.

* All AEs were considered possibly related. None were considered probably or definitely related.

† 4/7 episodes of headache occurred after the third vaccine dose and did not recur with fourth or fifth doses. No factor was identified to account for this apparent clustering of headache.

each immunization was observed equally in volunteers receiving vaccine or NS that was attributed to enriched diet, as the volunteers were transported to Bagamoyo from Dar es Salaam during the periods of immunization and CHMI and were amply fed (see Supplemental Figure 1). In Dar es Salaam, malaria transmission is low. No volunteer had malaria during screening or during the trial other than from CHMI.

Tolerability, safety, and VE during CHMI. Forty-six vaccinees, eight NS controls, and 10 added infectivity controls underwent homologous CHMI. All subjects were negative by TBS and qPCR for Pf infection on the day of CHMI. Two volunteers were excluded from primary analysis—a Group 2 volunteer who left the area 2 days after administration of PfSPZ Challenge and a Group 4 volunteer who left 9 days after. Both volunteers were located and treated preemptively.

Tolerability and safety of administration of PfSPZ challenge. Controlled human malaria infection was well tolerated with no local solicited AEs and three systemic solicited AEs (grade 1 headache in Group 3, grade 2 headache in Group 4, and grade 1 arthralgia in an infectivity control) in the 7 days post-administration of PfSPZ Challenge.

Parasitemia. Controls. The 18 NS and infectivity controls developed Pf infection after CHMI (16 TBS and qPCR positive and two TBS negative and qPCR positive) (Figure 2A–D and Supplemental Table 9). These included four NS and two infectivity controls in CHMI #1, the same in CHMI #2, and six infectivity controls in CHMI #3. All received the same lot of PfSPZ Challenge. One isolate of those positive from CHMI #1, one from CHMI #2, and four from CHMI #3 were genotyped,[24] and all parasites tested were PfNF54. Vaccine efficacy was calculated based on the results of qPCR assays from the six controls in CHMI #1, CHMI #2, and CHMI #3 individually (Figure 2D).

Group 2 (1.35×10^5 PfSPZ). Seventeen of 18 volunteers who received five doses and 1/1 volunteer who received four doses developed parasitemia (Figure 2A), 15 positive by TBS and qPCR, and 3 by qPCR only (CHMI #1) (Supplemental Table 10). One volunteer was negative through day 28 by TBS and qPCR. Vaccine efficacy by proportional analysis was 5.56% (95% confidence interval [CI]: 3.61%, 14.73%; $P > 0.99$, Fisher's exact test, 2-tailed). There was no significant delay in parasitemia by qPCR in the vaccinees as compared with controls ($P = 0.4481$ by log rank).

Group 3 (2.7×10^5 PfSPZ). First CHMI at 3 weeks (CHMI #2): 16/20 volunteers who received five doses developed parasitemia (Figure 2B), all positive by TBS and qPCR; four volunteers were negative through day 28 by TBS and qPCR. Vaccine efficacy by proportional analysis was 20% (95% CI: 4.62%, 35.38%; $P = 0.543$). There was a delay in the onset of parasitemia in vaccinees as compared with controls ($P = 0.015$ by log rank).

Second CHMI at 24 weeks (CHMI #3): The four uninfected volunteers from the first CHMI underwent a second CHMI 24 weeks after the last vaccine dose (Figure 2C). Three were negative by TBS and qPCR through day 28 day. The fourth volunteer, who was asymptomatic, was reported to have a positive TBS on day 12 and treated. The sample with positive TBS was negative by retrospective qPCR. Reevaluation of the TBS indicated an error in slide reading (false-positive). Vaccine efficacy by proportional analysis at this time point was 100% (for 3/3 and 4/4 protected: 95% CI: 43.8%, 100%, and 51.01%, 100%; $P = 0.012$ and 0.005, respectively). However, given the 20% VE at 3 weeks by proportional analysis, overall VE by proportional analysis was considered to be 20%.

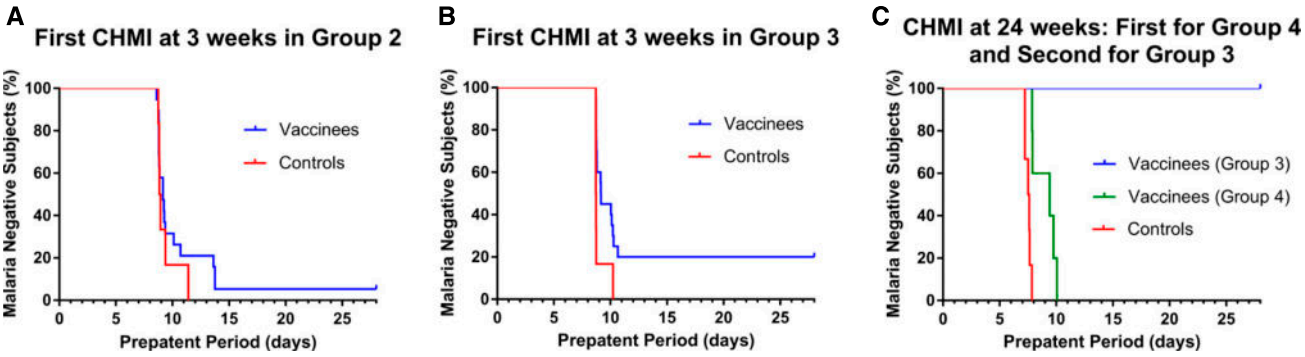
Group 4 (2.7×10^5 PfSPZ). First CHMI at 24 weeks after the last vaccine dose (CHMI #3): 4/5 vaccinees developed parasitemia by TBS and qPCR. The fifth was negative by TBS, but positive by qPCR (see Supplemental Table 10). There was one excluded volunteer (see the previous paragraph). Vaccine efficacy by proportional analysis was 0% ($P > 0.99$%). There was a significant delay in the onset of parasitemia by qPCR in vaccinees as compared with controls ($P = 0.001$ by log rank).

α-thalassemia. Volunteers heterozygous for α-thalassemia were no more likely to be TBS negative and qPCR positive than volunteers without α-thalassemia (three of 27 versus three of 34, $P = 1.0$). Protection from CHMI did not correlate with α-thalassemia status; 3/37 with normal hemoglobin and 2/29 heterozygous for α-thalassemia were protected.

TABLE 3
Summary of abnormal laboratory values and severity grades

Laboratory parameter	Vaccinees in Group 2 (1.35×10^5 PfSPZ) ($N = 20$)		Vaccinees in groups 3 and 4 (2.7×10^5 PfSPZ) ($N = 26$)		NS controls ($N = 8$)		*P* values: vaccinees ($N = 46$) vs. controls ($N = 8$)
	No.	%	No.	%	No.	%	
Leukocytosis	1	5	2	7.7	3	37.5	0.0358
Leukopenia	6	30	7	27	1	12.5	> 0.05
Neutropenia	6	30	5	19	2	25	> 0.05
Lymphopenia	3	15	3	11.5	2	25	> 0.05
Eosinophilia	0	0	2	7.7	3	37.5	0.0194
Decreased hemoglobin	1	5	0	0	0	0	> 0.05
Thrombocytopenia	1	5	0	0	0	0	> 0.05
Elevated creatinine	2	10	4	15.4	2	25	> 0.05
Low total bilirubin	4	20	7	27	1	12.5	> 0.05
Elevated total bilirubin	2	10	2	7.7	2	25	> 0.05
Elevated alkaline phosphatase	1	5	2	7.7	0	0	> 0.05
Elevated alanine aminotransferase	3	15	5	19	2	25	> 0.05
Elevated aspartate aminotransferase	0	0	3	11.5	0	0	> 0.05

PfSPZ = *Plasmodium falciparum* sporozoites. *P* values calculated using Fisher's exact test (2-tailed). One volunteer who received saline developed Grade 3 eosinophilia attributed to *Strongyloides stercoralis* infection, which improved with anthelminthic therapy. This volunteer had a baseline of mild eosinophilia, which persisted throughout the clinical trial. All other laboratory abnormalities were Grade 2 or less. There was no association between laboratory abnormalities and time after a dose or increasing number of doses. Three abnormalities during immunization were deemed clinically significant or Grade 3. One was diagnosed as benign ethnic neutropenia, one was lymphopenia associated with an infected foot laceration, and one was eosinophilia associated with *Fasciolopsis buski* and *S. stercoralis* infection. Lymphopenia and eosinophilia resolved with treatment. Two Group 4 volunteers had asymptomatic hookworm infections diagnosed before controlled human malaria infection; one was coinfected with *Enterobius vermicularis*.



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Group	Timing of CHMI	Number protected/ Number underwent CHMI	Vaccine efficacy by proportional analysis	Vaccine efficacy by proportional analysis 95% CI	Vaccine efficacy by proportional analysis *P* value (Fisher's Exact Test, 2-tailed)	Median prepatent period by qPCR of positive vaccinees (days)****	Median prepatent period by qPCR of controls (days)****	Log rank test
2 (1.35×10^5 PfSPZ)	3 weeks after fifth immunization	1/18	5.56%	(-3.61%, 14.73%)	> 0.999	9.2	8.9	0.45
3 (2.7×10^5 PfSPZ)	3 weeks after fifth immunization	4/20	20%	(4.62%, 35.38%)	0.543	9.0	8.7	**0.02**
3 (2.7×10^5 PfSPZ)	24 weeks after fifth immunization*	3/3 or 4/4**	100% (20% overall)	(43.85%, 100%) or (51.01%, 100%)***	**0.012 or 0.005****	not applicable	7.6	**0.01 or 0.004****
4 (2.7×10^5 PfSPZ)	24 weeks after fifth immunization	0/5	0%	(0.00%, 25.88%)***	> 0.999	8.7	7.6	**< 0.001**

FIGURE 2. Kaplan–Meier survival curves in immunized volunteers vs. controls as assessed by quantitative polymerase chain reaction (qPCR). Kaplan–Meier curves in volunteers undergoing controlled human malaria infection (CHMI) 3 weeks after the last of five doses with 1.35×10^5 (Group 2) (**A**) or 2.7×10^5 (Group 3) (**B**) *Plasmodium falciparum* Sporozoites (PfSPZ) of PfSPZ Vaccine. Panel (**C**) volunteers undergoing either first (Group 4) or second (Group 3) CHMI 24 weeks after the fifth immunization with 2.7×10^5 PfSPZ of PfSPZ Vaccine. (**D**) Vaccine efficacy and prepatent period results. *This was the second CHMI for the 4 volunteers in Group 3 who were protected after the first CHMI at 3 weeks. **One volunteer was inappropriately treated on day 13 for a false positive TBS. Without this volunteer, 3/3 protected. With this volunteer 4/4 were protected. ***Confidence intervals were calculated using Wilson's score interval. ****Volunteers in CHMI #1 and #2 (3 week CHMI in Groups 2 and 3) had specimens first acquired on day9. Volunteers in CHMI #3 (24 week CHMI in Groups 3 and 4) had specimens first acquired on day 8. This figure appears in color at www.ajtmh.org.

Prepatent periods and parasite densities. Although the median prepatent periods by TBS in controls in each CHMI group (12.5, 13.0, and 12.0, respectively) were shorter than in the vaccinees in Groups 2–4 (14.0, 14.0, and 15.3 days, respectively), these differences did not reach the level of statistical significance (P = 0.486, P = 0.491, and P = 0.333, respectively) (Supplemental Table 9). The prepatent periods by qPCR in vaccineees in Group 3 (3 and 24 week CHMIs) and Group 4 (24 week CHMI) were significantly longer than in the respective controls (Figure 2D). The parasite densities by qPCR and TBS at the time of diagnosis for each individual are in Supplemental Table 10. The median parasite density in controls versus vaccinees at the time of first positivity were 0.5 versus 0.4 parasites/μL for qPCR (P = 0.5714) and 11.2 versus 15.0 parasites/μL for TBS (P = 0.1492).

Tolerability and safety of parasitemia during CHMI. Controls. Sixteen controls developed parasitemia by TBS; 9 (56%) never had symptoms (Supplemental Table 11). Headache occurred in 7/7 symptomatic individuals. One of two control volunteers only positive by qPCR did not have any symptoms; the second had headache 8 days after qPCR spontaneously reverted to negative. No volunteer had symptoms at the time of first positive qPCR.

Vaccinees. Thirty-five immunized volunteers developed parasitemia by TBS; 20 (57%) never had symptoms. Three volunteers had temperature > 39.0°C; all other clinical manifestations were grade 1 or 2. Fever (28.6%) and headache (31.4%) were most common. Compared with controls, elevated temperature was more common in vaccinees with positive TBSs (9/35 versus 0/16, P = 0.043). There was no significant difference in the frequency of headache between controls and vaccinees. In the three volunteers in Group 2 who were qPCR positive and TBS negative, one developed headache 3 days after qPCR positivity. No volunteer had symptoms at the time of first positive qPCR.

Clinical laboratories. No unexpected changes were observed following CHMI. Declines in lymphocyte counts were observed in TBS positive controls and vaccinees (mean decline 1,110 ± 720 cells/μL and 1,180 ± 680 cells/μL, respectively) on day of first positive TBS. Absolute lymphocyte

counts less than 1,000 cells/μL were observed in 8/16 and 16/35 TBS positive controls and vaccinees. All lymphocyte counts returned to the baseline by day 28. There were mild decreases in platelet counts in TBS positive subjects, but all platelet counts were > 100 × 10^3 cells/μL.

Treatment. Volunteers with positive TBSs were treated with either atovaquone/proguanil (*N* = 43) or artemether/lumefantrine (*N* = 8) within 24 hours of first positive TBS. Normal saline and infectivity controls who were TBS negative (*N* = 2) were treated at day 28.

Immunogenicity. *Antibody responses.* Pf circumsporozoite protein (PfCSP) and PfSPZ. Antibodies against PfCSP by ELISA 1), PfSPZ by aIFA 2), and PfSPZ by ISI 3) in sera taken 2 weeks after the last vaccine dose and just before CHMI (20–23 days after the last dose) for Groups 2 (CHMI #1) and 3 (CHMI #2) are in Figure 3A–C. The median responses and those uninfected and infected by qPCR are shown.

For all three assays, median antibody responses before first CHMI were higher in uninfected than in infected vaccinees. There was a significant difference in median net aIFA

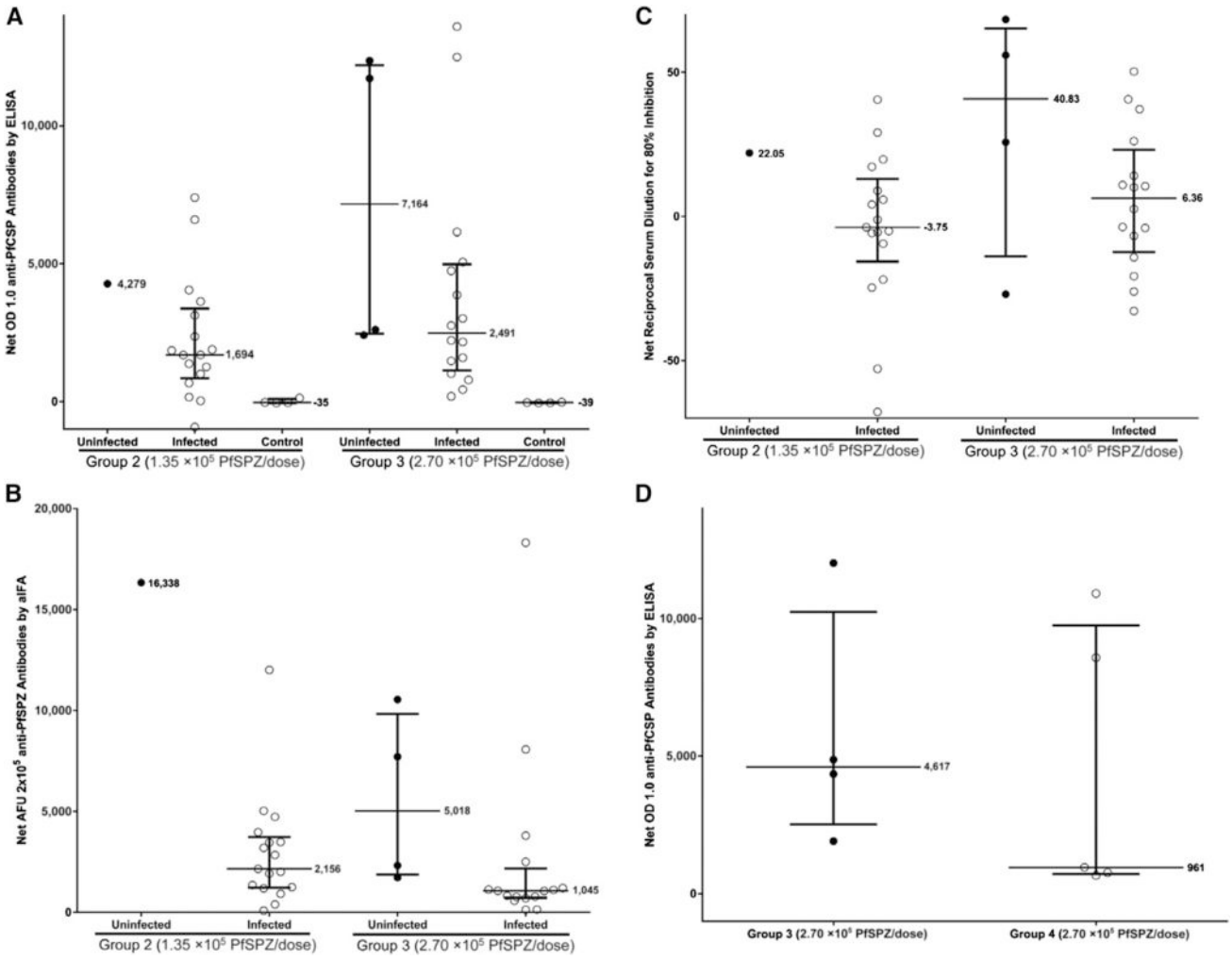

FIGURE 3. Antibody responses to *Plasmodium falciparum* Sporozoites (PfSPZ) and PfCSP before controlled human malaria infection (CHMI). For all assays, uninfected subjects are shown as filled (black) circles and infected subjects are open circles. For each of the defined subject groups, the interquartile ranges and the median values of response of subjects in each group are shown. Assessment of antibodies was performed in sera from subjects before immunization and before CHMI #1 (~2 weeks after the last dose of PfSPZ Vaccine or normal saline [NS]) and/or CHMI #2 (~24 weeks after last dose of PfSPZ or NS) (**A**, **D**). Antibodies to PfCSP by ELISA are reported as net optical density (OD) 1.0 (the difference in OD 1.0 between pre-CHMI and preimmunization sera). (**B**, **E**) Antibodies to PfSPZ by aIFA are reported as net AFU 2 × 10^5, the reciprocal serum dilution at which the fluorescent units were 2 × 10^5 (AFU 2 × 10^5) in pre-CHMI minus preimmunization sera. (**C**, **F**) Results of inhibition of sporozoite invasion (ISI) assay are reported as serum dilution at which there was 80% reduction of the number of PfSPZ that invaded a human hepatocyte line (HC-04) in the presence of pre-CHMI as compared with preimmunization sera from the same subject. Panels **A–C** show groups 2 (five doses of 1.35 × 10^5 PfSPZ) and 3 (five doses of 2.7 × 10^5 PfSPZ) before short-term CHMI (2 weeks after the last dose of PfSPZ or NS) and panels **D–F** show those volunteers in Groups 3 (five doses of 2.7 × 10^5 PfSPZ) and 4 (five doses of 2.7 × 10^5 PfSPZ) who underwent long-term CHMI (24 weeks after the last dose of PfSPZ). Panel **G** shows net optical density (OD) 1.0 anti-PfCSP antibodies by an enzyme-linked immunosorbent assay (ELISA) comparing vaccinated Tanzanian volunteers to volunteers in other trials receiving the same regimen. After five doses of 2.70 × 10^5 PfSPZ/dose, volunteers in bagamoyo sporozoite vaccine 1 (BSPZV1) (*N* = 25) had a 4.3-fold lower median net OD 1.0 than those in the U.S.-based clinical trial Walter Reed Army Institute of Research (WRAIR) 2080 (*N* = 26) but a 6.6-fold higher median OD 1.0 than volunteers in 14-I-N010 in Bamako, Mali (*N* = 42), where malaria transmission rates are higher. There was a significant difference between the results for WRAIR 2080 vs. BSPZV1 (*P* = 0.0012), WRAIR 2080 vs. 14-I-N010 (*P* < 0.0001), and even 14-I-N010 vs. BSPZV1 (*P* = 0.002) (two-tailed *t*-test). AFU = arbitrary fluorescence units; aIFA = antibodies by immunofluorescence assay.

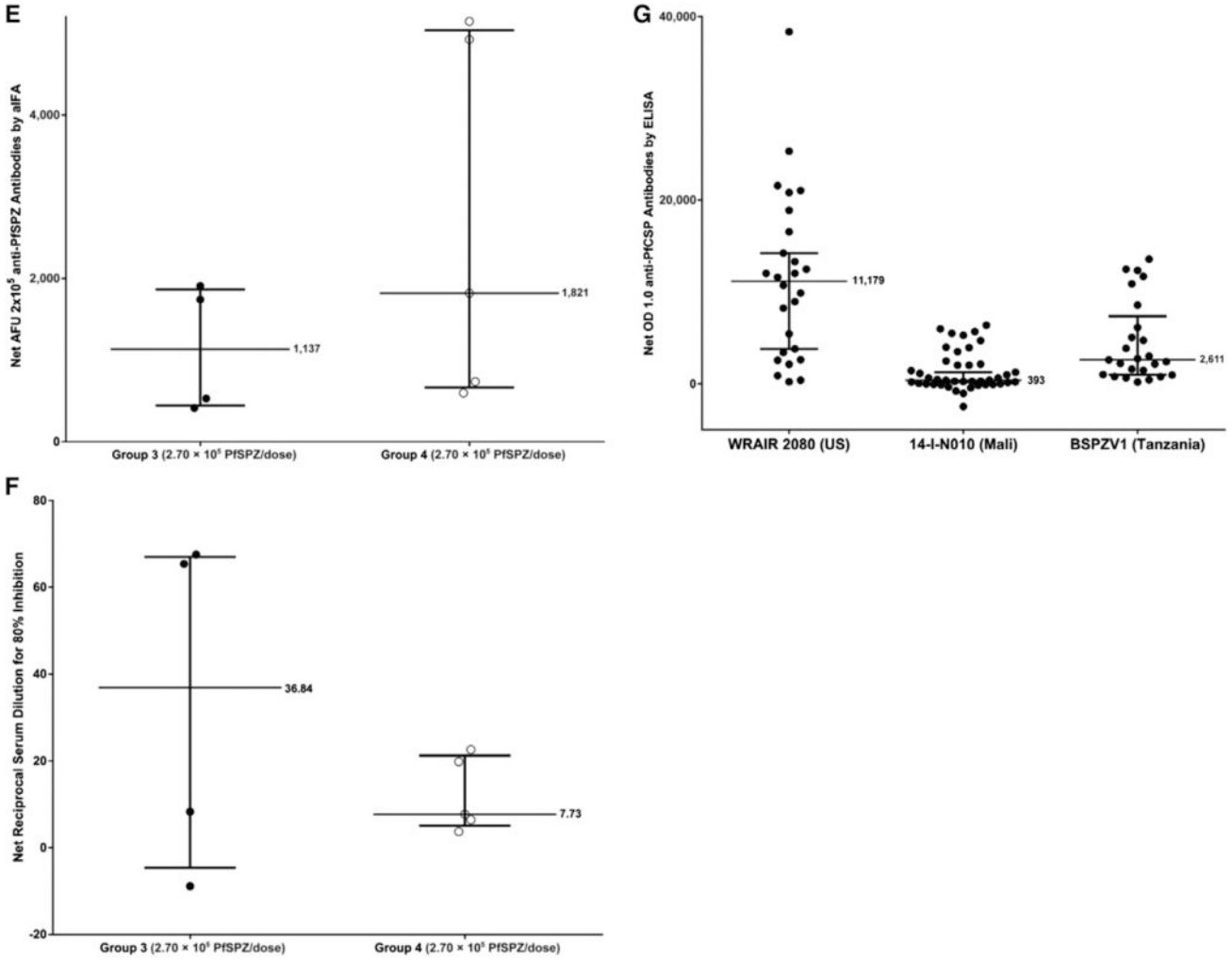

FIGURE 3. (*Continued*)

responses between infected and uninfected volunteers in Group 3 before CHMI #1 ($P = 0.0499$, Wilcoxon Rank-Sum Test), but not PfCSP ($P = 0.290$) or for ISI ($P = 0.249$).

In sera collected before CHMI #3 (170–171 days after the last vaccine dose), antibodies by the three assays for Group 4 and for the four volunteers in Group 3 uninfected in CHMI #1 who underwent CHMI #2 are in Figure 3D–F. All data appear in Supplemental Table 12.

After the fifth dose, in the PfCSP ELISA, volunteers were considered to have seroconverted if their net OD 1.0 and OD 1.0 ratio calculated, respectively, by subtracting or dividing by the prevaccination antibody OD 1.0, were ≥ 50 and ≥ 3.0. By these criteria, 15/18 volunteers (83%) in Group 2, 20/20 (100%) in Group 3, and 5/5 (100%) in Group 4 seroconverted, median net OD 1.0 of positives of 1,189, 2,685, and 961, and median OD 1.0 ratio of positives of 11.50, 21.15, and 37.83, respectively (Supplemental Table 13). In the aIFA, volunteers with a net arbitrary fluorescence unit (AFU) 2×10^5 of ≥ 150 and a ratio of post- to pre-AFU 2×10^5 of ≥ 3.0 were considered positive (Supplemental Table 13). By these criteria, 17/18 volunteers (94%) in Group 2, 18/20 (90%) in Group 3, and 5/5 (100%) in Group 4 seroconverted, median net OD 1.0 of positives of 2,844, 1,165, and 1,820, and median OD 1.0 ratio of positives of 1,193.00, 552.88, and 224.86, respectively (Supplemental Table 13). For the ISI, volunteers with a net ISI activity of ≥ 10% and a ratio of post- to pre-ISI activity of ≥ 3.0 were considered positive. By these criteria, 3/18 volunteers (17%) in Group 2, 8/20 (40%) in Group 3, and 3/5 (60%) in Group 4 were positive, median net OD 1.0 of positives of 22.05, 38.92, and 12.44, and median OD 1.0 ratio of positives of 19.79, 12.53, and 13.44, respectively (Supplemental Table 13).

Other antigens. Two weeks after the fifth dose in Group 2 (1.35×10^5 PfSPZ) and groups 3 and 4 (2.7×10^5 PfSPZ), there were antibodies to PfCSP in 15/18 and 25/25 subjects, respectively. Ten of 25 volunteers immunized with 2.7×10^5 PfSPZ made antibodies to Pf apical membrane antigen 1 and 4–16% responded to PfCelTOS, PfMSP5, PfMSP1, or Pf erythrocyte binding antigen 175 (PfEBA175) (Supplemental Table 14). The presence of antibodies, albeit at low incidence, against proteins first expressed in late liver stages (PfMSP1 and PfEBA175) was unexpected; results were confirmed by repeating the assays. No antibody responses were associated with protection.

T-cell responses. T cells against liver-stage malaria parasites in mice and nonhuman primates immunized with

radiation-attenuated SPZ mediate protection[9,29–31] and it is likely this is the case in humans.[12] CD8 and CD4 T-cell responses generally peak after the first vaccination with PfSPZ Vaccine.[13] In this trial, T-cell responses were measured before immunization, 2 weeks after the first and 2 weeks after the final immunization in Group 2 (1.35×10^5 PfSPZ). For technical reasons (loss of viability), the other groups could not be studied.

After the first vaccination, the percent of Pf red blood cell (PfRBC)-specific and PfSPZ-specific cytokine-producing memory CD4 T-cell responses increased by 0.25 ± 0.06 (mean ± SEM) and 0.24 ± 0.04, respectively (Figure 4A, B). Throughout, "naïve T cell" refers to cells that co-express CCR7 and CD45RA, and "memory T cell" refers to all other T cells. After the final vaccination, at week 22, the CD4 T-cell responses were above prevaccine responses by 0.17 ± 0.05 and 0.18 ± 0.05% points, respectively. These responses were lower than after the same immunization regimen in malaria-naïve U.S. adults.[10]

PfRBC-specific CD8 T cells were not significantly above the prevaccine levels, and PfSPZ-specific CD8 T cells were slightly above background (Figure 4C, D); responses were lower than in U.S. adults.[10,12]

In contrast to other PfSPZ Vaccine trials,[10,12–14] there was negligible change in the frequency of circulating γδ T cells (Figure 4E) or activation as measured by change in expression of the activation markers HLA-DR and CD38 following immunization (Figure 4F). To identify potential explanations for lower cellular immune responses in Tanzanians, we examined frequency of T regulatory (Treg) cells ($CD4^+Foxp3^+CD25^+CD127^-$) expressing the activation marker CD137 (also known as 4-1BB)[32] after stimulation with PfRBC. There was no difference in prevaccine frequency of PfRBC-specific Tregs in the Tanzanians as compared with Americans[10] (Figure 4G). Consistent with CD4 and CD8 T-cell responses, PfRBC-specific Tregs were highest after first immunization (Figure 4H). Last, the prevaccine frequency of total memory T cells relative to total naïve T cells was significantly higher than in Americans (Figure 4I).

DISCUSSION

To our knowledge, this was the first assessment of the VE of a malaria vaccine in Africa against CHMI. *Plasmodium falciparum* SPZ Vaccine was well tolerated and safe but less immunogenic and protective in Tanzanian men than in U.S. volunteers.

In our studies, all 18 controls became infected. Four of 20 (20%) recipients of five doses of 2.7×10^5 PfSPZ did not become infected after homologous CHMI by DVI 3 weeks after the last immunization. By contrast, 12/13 (92.3%) volunteers in the United States who received five doses of 2.7×10^5 PfSPZ were protected after homologous CHMI by mosquito bite 3 weeks after the last vaccine dose.[11] When the four uninfected Tanzanian volunteers underwent repeat homologous CHMI at 24 weeks after the last dose, all four (100%) were protected. In the United States, Seven of 10 previously protected volunteers were protected when they underwent homologous CHMI at 24 weeks[11] and all five volunteers in the United States who were protected at 21 weeks after the last immunization (four doses of 2.7×10^5 PfSPZ) were protected against repeat mosquito-administered CHMI at 59 weeks.[12] This could be due to boosting by the small numbers of PfSPZ administered during the CHMI, or is more likely due to the fact that in these protected individuals, the protective immune responses induced by immunization were sustained.

The same exact immunization regimen was assessed for VE against intense field transmission of heterogeneous Pf in Mali. Vaccine efficacy against infection with Pf on TBS was 52% by time to event and 29% by proportional analysis during 24 weeks after the last vaccine dose.[14] This was higher than the VE by proportional analysis against homologous CHMI in Tanzania. In Tanzania, there was a significant delay in the onset of parasitemia after CHMI at 3 and 24 weeks in subjects who received five doses of 2.7×10^5 PfSPZ and were not fully protected (Figure 2B–D). Nonetheless, the proportional analysis suggests that homologous CHMI by DVI of a 100% infectious dose of homologous PfSPZ Challenge is at least as rigorous as a test of VE and potentially more rigorous than intense field transmission of heterogeneous Pf.

Vaccine-induced antibody and T-cell responses in the Tanzanians were lower than in malaria-naïve Americans who received the exact same dosage regimen. Two weeks after the last dose, the median antibody responses to PfCSP, the major protein on the surface of PfSPZ, were 4.3 times lower in the Tanzanians than those in Americans ($P = 0.0012$, Student's *t*-test, 2-tailed),[11] but significantly higher than in Malians who received the same immunization regimen ($P = 0.002$)[14] (Figure 3G).

The T-cell responses were also lower than in Americans[10,12] (Figure 4), but this could only be assessed in PBMCs from individuals who received the lower dose (five doses of 1.35×10^5 PfSPZ), not in the individuals who received the higher dose (five doses of 2.7×10^5 PfSPZ), the group that had sustained protection for 24 weeks. Thus, it is possible that had PBMCs from the higher dose group been assessed, responses would have been comparable to the responses in nonimmune Americans. The Tanzanians who were assessed had a significantly higher proportion of total memory T cells compared with total naïve T cells at the baseline than did the Americans. This higher frequency of memory cells compared with naïve cells may explain the lower immunogenicity due to less available naïve cells for expansion during the vaccinations. Moreover, the greater frequency of non-Pf–specific memory T cells may compete for infected cell contacts during pathogen surveillance.[33] These data suggest that PfSPZ Vaccine immunogenicity may be dependent on cumulative history of Pf exposure. Another explanation is that an activated immune microenvironment in the Tanzanians as compared with the Americans reduced immune responses.[34] Helminth infections have been associated with reduced immune responses to malaria[35]; however, the paucity of helminth infections in this population does not support helminth infection as a cause of the reduced immune responses.

There were no differences between vaccine and NS placebo recipients in regard to vaccine tolerability or AEs; 97.1% of the DVI administrations were rated painless and no volunteer experienced any local AE. Systemic AEs, most commonly headache, were mild, infrequent, and of short duration, with a similar frequency in NS controls as in vaccinees (no statistically significant differences in rates).

Among the controls, 16 of 18 were positive for Pf by TBS after CHMI. However, all 18 were positive by qPCR. This is consistent with findings in Gabon after CHMI.[21] It is likely that preexisting asexual blood stage immunity limits Pf replication in some individuals. Thus, they never reach the threshold for detection by

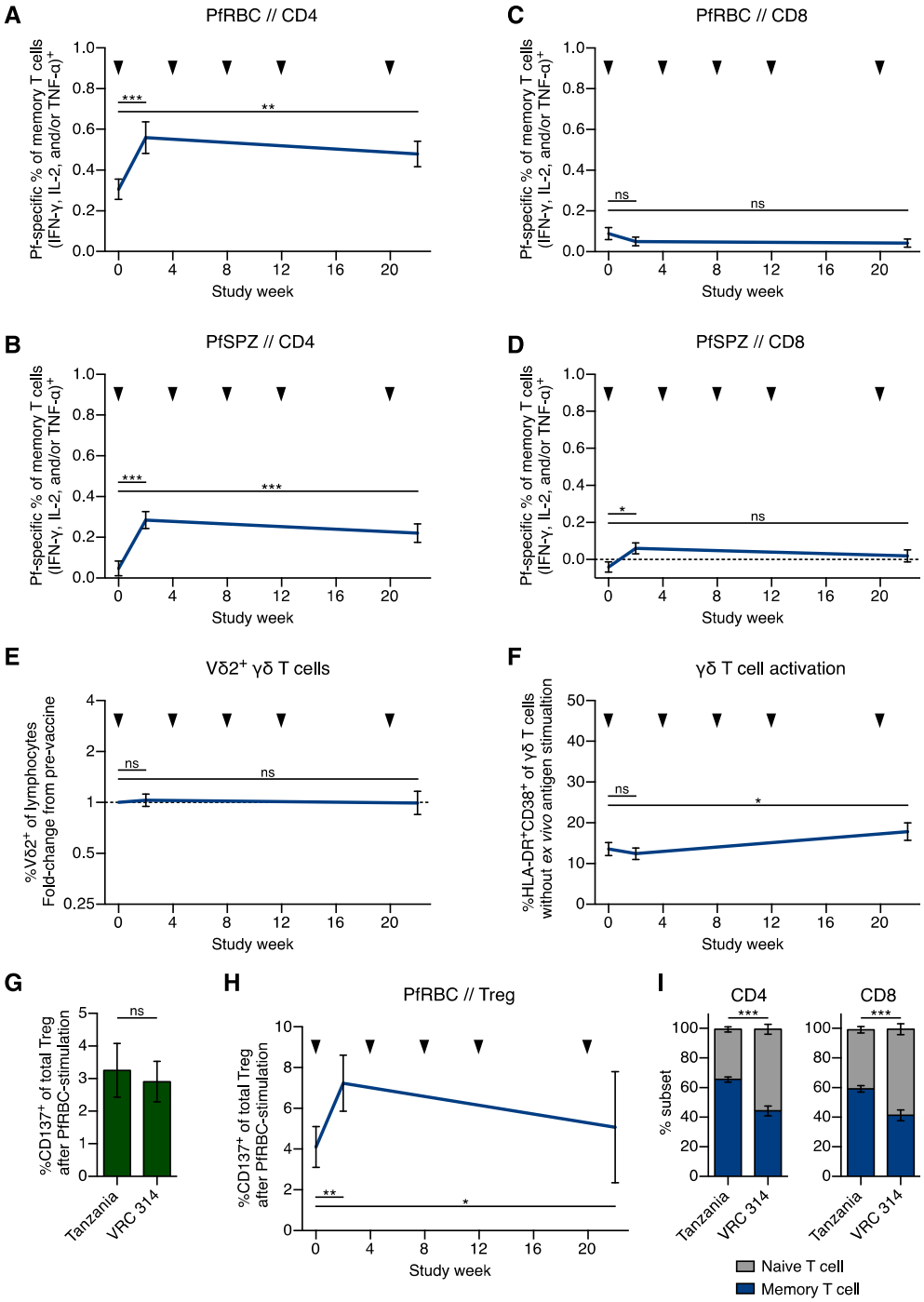

FIGURE 4. *Plasmodium falciparum* Sporozoites (PfSPZ)–specific T-cell responses in vaccine recipients receiving 1.35×10^5 PfSPZ. (**A–D**) PfSPZ-specific T-cell responses. Frequency of cytokine-producing memory CD4 T cells responding to (**A**) PfRBC or (**B**) PfSPZ. Throughout, "naïve T cell" refers to cells that co-express CCR7 and CD45RA, and "memory T cell" refers to all other T cells. Frequency of cytokine-producing memory CD8 T cells responding to (**C**) PfRBC or (**D**) PfSPZ. Results are the percentage of memory T cells producing interferon gamma, interleukin 2, and/or tumor necrosis factor alpha following stimulation minus the percentage of cells following control stimulation. (**E**) Frequency of the $V\delta2^+$ subfamily of γδ T cells of total lymphocytes. Results are expressed as fold-change from the prevaccine frequency. (**F**) γδ T-cell activation in vivo. Data are the percentage of memory γδ T cells expressing HLA-DR and CD38 as measured on PBMCs following incubation with control stimulation (vaccine diluent). (**G**) Prevaccine frequency of PfRBC-specific Tregs in Tanzania compared with malaria-naïve U.S. subjects from the Vaccine Research Center (VRC) 314 study. (**H**) Frequency of PfRBC-specific Treg. Results are the percentage of $CD4^+Foxp3^+CD25^+CD127^-$ T cells expressing CD137 (also known as 4-1BB) after stimulation with Pf red blood cell (PfRBC) minus the percentage of cells following stimulation with uninfected RBC. (**I**) Percentage of total CD4 (left) or CD8 (right) T cells that are naïve (gray bar; $CCR7^+CD45RA^+$) or memory (blue bar; not $CCR7^+CD45RA^+$) phenotype assessed prevaccination in all 48 subjects vaccinated in Tanzania or in 14 healthy U.S. subjects from the VRC 314 study.[13] For **A–F** and **H**, $N = 24$, and statistical difference was measured by using the Wilcoxon matched-pairs signed rank test. For **G** and **I**, statistical difference was measured by using the Mann–Whitney *U* test. *P* values are reported as not significant (ns), < 0.05 (*), < 0.01 (**), or < 0.001 (***). Data are mean ± SEM. Time points are prevaccine, 2 weeks after the first vaccination, and 2 weeks after the final vaccination. Black arrowhead designates PfSPZ Vaccine administration. This figure appears in color at www.ajtmh.org.

TBS. In our CHMI studies in Bagamoyo, we now use qPCR to confirm positive TBS, and retrospectively or in real time, assess parasitemia in all volunteers by qPCR.

We propose that increasing the numbers of PfSPZ per dose and altering intervals between doses will lead to overcoming the downregulation of humoral and cell-mediated immunity most likely because of previous exposure to Pf and thereby increase immune responses to PfSPZ Vaccine and VE. We also hypothesize that immune responses in younger, less malaria-exposed individuals will be of greater magnitude than those in adults.

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Note: Supplemental tables and figure appear at www.ajtmh.org.

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Disclosures: Sanaria Inc. manufactured PfSPZ Vaccine and PfSPZ Challenge, and Protein Potential LLC is affiliated with Sanaria. Thus, all authors associated with Sanaria or Protein Potential have potential conflicts of interest. There are no other conflicts of interest.

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4.4 Paper II: Published Article

Safety and Differential Antibody and T-Cell Responses to the Plasmodium falciparum Sporozoite Malaria Vaccine, PfSPZ Vaccine, by Age in Tanzanian Adults, Adolescents, Children, and Infants: Randomized Controlled Trial

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Safety and Differential Antibody and T-Cell Responses to the *Plasmodium falciparum* Sporozoite Malaria Vaccine, PfSPZ Vaccine, by Age in Tanzanian Adults, Adolescents, Children, and Infants

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Abstract. In 2016, there were more cases and deaths caused by malaria globally than in 2015. An effective vaccine would be an ideal additional tool for reducing malaria's impact. Sanaria® PfSPZ Vaccine, composed of radiation-attenuated, aseptic, purified, cryopreserved *Plasmodium falciparum* (Pf) sporozoites (SPZ) has been well tolerated and safe in malaria-naïve and experienced adults in the United States and Mali and protective against controlled human malaria infection with Pf in the United States and field transmission of Pf in Mali, but had not been assessed in younger age groups. We, therefore, evaluated PfSPZ Vaccine in 93 Tanzanians aged 45 years to 6 months in a randomized, double-blind, normal saline placebo-controlled trial. There were no significant differences in adverse events between vaccinees and controls or between dosage regimens. Because all age groups received three doses of 9.0×10^5 PfSPZ of PfSPZ Vaccine, immune responses were compared at this dosage. Median antibody responses against Pf circumsporozoite protein and PfSPZ were highest in infants and lowest in adults. T-cell responses were highest in 6–10-year olds after one dose and 1–5-year olds after three doses; infants had no significant positive T-cell responses. The safety data were used to support initiation of trials in > 300 infants in Kenya and Equatorial Guinea. Because PfSPZ Vaccine–induced protection is thought to be mediated by T cells, the T-cell data suggest PfSPZ Vaccine may be more protective in children than in adults, whereas infants may not be immunologically mature enough to respond to the PfSPZ Vaccine immunization regimen assessed.

INTRODUCTION

Despite an annual investment of more than $2.7 billion in insecticide-treated bed nets, indoor residual spraying, diagnosis, and treatment, in 2015, 2016, and 2017 there were an estimated 429,000–730,500 deaths each year caused by malaria[1–3]; 90% of the mortality was in children under the age of 5 years. *Plasmodium falciparum* (Pf) was the cause of more than 98% of deaths from malaria and more than 80% of cases of malaria in sub-Saharan Africa. Our goal is to field a vaccine that will prevent infection with Pf and thereby prevent all clinical and pathological manifestations of malaria and halt parasite transmission from humans to mosquitoes.[4]

A number of malaria vaccines are under development, but none have received marketing authorization (licensing) by a regulatory authority. RTS,S/AS01 has completed Phase 3 clinical trials,[5] received a positive opinion (Article 58) from the European Medicines Agency[6] and in 2019, large-scale pilot implementation trials will be initiated in Kenya, Malawi, and Ghana to confirm the level of protective efficacy, demonstrate that the entire immunization regimen can be successfully administered, and assess several safety signals seen in the Phase 3 trial (increased meningitis, febrile seizures, and female mortality in vaccinees as compared with controls).[7,8] A second pre-erthrocytic stage vaccine ChAd63 and MVA ME-TRAP has also been studied in African infants to adults.[9–11]

Sanaria® PfSPZ Vaccine is composed of radiation-attenuated, aseptic, purified, and cryopreserved *Plasmodium falciparum* (Pf) sporozoites (SPZ).[12] The vaccine has been extremely well tolerated and safe in multiple clinical trials.[13–18] In Mali, Equatorial Guinea, and Tanzania, there was no difference in adverse events (AEs) between the PfSPZ Vaccine and normal saline (NS) control in double-blind, placebo-controlled trials.[18–20]

PfSPZ Vaccine has been reported in malaria-naïve adults to have a vaccine efficacy (VE) of > 90% against controlled human malaria infection (CHMI) with homologous Pf parasites (same Pf strain in vaccine and CHMI),[14,16] 80% against CHMI with heterologous Pf parasites (different Pf strain in vaccine and CHMI) 3 weeks after the last vaccine dose,[14,16] 65% and 55% against homologous CHMI 24,[16] and 59[15] weeks and 54% against heterologous CHMI 33 weeks after the last vaccine dose.[17] In Malian adults, VE against Pf infection during the 24 weeks after last vaccine dose was 52% by time to infection analysis and 29% by proportional analysis.[18] Protection by immunization with sporozoites is dependent on T cells in mice and nonhuman primates[13,21–24] and thought to be T cell–dependent in humans.[13] The durable protection demonstrated in the Mali trial was associated with elevated gamma delta T-cell frequencies, providing support for this hypothesis.[25]

However, in Tanzanian adults, five doses of 2.7×10^6 PfSPZ had a VE against 3- and 24-week homologous CHMI of 20%.[20] This was the same immunization regimen used in the Mali trial that gave 52% VE and in a trial in the United States that gave 92% and 65% VE against 3- and 24-week homologous CHMI.[16] In Tanzania, the antibody and T-cell responses to PfSPZ in adults were significantly lower than in adults in the United States[20]; antibody responses in Mali were even

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lower.[18] We hypothesized that the lower immune responses in malaria-exposed African subjects as compared with malaria-naïve U.S. subjects were due to immune dysregulation caused by long-term exposure to malaria parasites[18,20] and that naturally acquired immunity may have reduced the effective PfSPZ inoculum. We, therefore, proposed that injecting larger doses of PfSPZ might partially overcome these effects. This is in part because when humans are immunized with radiation-attenuated PfSPZ administered by mosquito bite,[26] PfSPZ Vaccine[14,15,17,27] and PfSPZ-CVac[27] protection is dose dependent.[26] Thus, increasing immune responses by increasing the dose should increase VE. Thus, in this study, we increased the dose of PfSPZ Vaccine from 2.7×10^5 PfSPZ to 9.0×10^5 PfSPZ and 1.8×10^6 PfSPZ.

All previous studies of PfSPZ Vaccine have been conducted in adults. However, the major burden of malaria is in older infants and children. The present study was the first to assess the tolerability, safety, and immunogenicity of PfSPZ Vaccine in adolescents, children, and infants aged 6 months and older and the first to compare these results with those of adults. Furthermore, we hypothesized that infants and young children with little previous exposure to Pf parasites would have more robust immune responses to the vaccine than adults, recognizing that the infants' immunological systems might not be fully mature, particularly for T-cell responses.[28]

MATERIAL AND METHODS

Study design and population. This single-center, age de-escalation, double-blind, randomized, placebo-controlled trial (ClinicalTrials registration no. NCT02613520) was conducted in Bagamoyo, Tanzania, between December 2015 and March 2017. It had two major components, an age de-escalation, dose escalation component to assess safety, tolerability, and immunogenicity of PfSPZ Vaccine (part A), and a CHMI component to assess VE (part B). Herein, we report the results of part A.

One hundred seventy-three healthy male and female volunteers aged 6 months to 45 years were recruited from the Bagamoyo region through locally presented sensitization meetings. After an initial screening, prospective volunteers were invited to the Bagamoyo Clinical Trial Unit (BCTU) of the Ifakara Health Institute (IHI) to complete the informed consent process and further screening.

Informed consent was obtained from all volunteers or the parents/legal guardians after the nature and risks of the study were explained. Following this, the adult volunteers or the parent/legal guardian of child volunteers were required to complete a 10-question assessment with a 100% correct response rate on the first or second attempt to demonstrate understanding of the study procedures (Supplemental Table 1) to be eligible for enrollment. In addition, all children and adolescents aged 9–18 years provided written assent and children aged 6–8 years provided oral assent. Volunteers were screened using predetermined inclusion and exclusion criteria based on clinical examinations and laboratory tests (Supplemental Tables 2 and 3). Medical history was analyzed to exclude any past or present medical problem in conjunction with a detailed clinical examination. Laboratory testing included hematology, biochemistry, urinalysis, and parasitology testing to include malaria thick blood smear (TBS), stool for intestinal helminth infections, and urine for *Schistosoma haematobium*. Tests for HIV and hepatitis B and C were performed only after pretest counseling was carried out; volunteers were excluded if positive and referred for further evaluation and management. Volunteers were excluded if they had significant abnormalities on electrocardiograms. The complete eligibility criteria are published at https://clinicaltrials.gov/show/NCT02613520.

The trial was performed in accordance with good clinical practices. The protocol was approved by institutional review boards (IRBs) of the IHI (Ref. No. IHI/IRB/ No: 32-2015), the National Institute for Medical Research Tanzania (NIMR/HQ/R.8a/Vol.IX/2049), and the Ethikkommission Nordwest- und Zentralschweiz, Basel, Switzerland (reference number 15/104). The protocol was also approved by the Tanzania Food and Drug Authority (Auth. No. TZ15CT013), registered at ClinicalTrials.gov (NCT02613520) and conducted under a U.S. Food and Drug Administration Investigational New Drug application (FDA IND) application.

Intervention and randomization. Volunteers spanning five age groups were sequentially allocated to 11 different dose groups and randomly assigned to receive PfSPZ Vaccine or NS in a 2:1 ratio. Twelve additional, age-matched, adult volunteers were enrolled as nonimmunized infectivity controls for CHMI studies.

The details of each of five main age groups (Groups 1–5) are outlined in Table 1. Immunization began with the adults (Group 1a, 9.0×10^5 PfSPZ and Group 1b, 1.8×10^6 PfSPZ) and continued progressively to teenagers (11–17 years; Group 2a, 9.0×10^5 PfSPZ and Group 2b, 1.8×10^6 PfSPZ), older children (6–10 years; Group 3a, 9.0×10^5 PfSPZ and Group 3b, 1.8×10^6 PfSPZ), younger children (1–5 years; Group 4a, 4.5×10^5 PfSPZ and Group 4b, 9.0×10^5 PfSPZ), and infants (6–11 months; Group 5a, 2.7×10^5 PfSPZ, Group 5b, 4.5×10^5 PfSPZ, and Group 5c, 9.0×10^5 PfSPZ). Only after the safety of a given PfSPZ dose had been demonstrated in an older age group was the same or a lower dose tested next in a younger age group (age de-escalation). Likewise, within each age group, safety was demonstrated with a lower PfSPZ dose before immunizations began with a higher PfSPZ (dose escalation). At three time points during age de-escalation and dose escalation, a three-member external Safety Monitoring Committee reviewed safety reports and provided a recommendation to proceed to the next study group.

Using this staggered approach, 10 subgroups of nine individuals received three doses of PfSPZ Vaccine at 56-day intervals (days 1, 57, and 113), each comprising six vaccine and three placebo volunteers randomized in a 2:1 ratio to PfSPZ Vaccine or NS control. An exception was Group 5a (Table 1), an 11th subgroup comprising three infants constituting the first ever recipients less than 1 year of age to receive injections with PfSPZ Vaccine. This safety-only pilot group was administered a single reduced dose of PfSPZ Vaccine (2.7×10^5 PfSPZ); there was no randomization and no placebo control. All of the other 90 volunteers and the entire clinical team excluding the pharmacy staff were blinded to treatment assignment, with blinded status maintained throughout the study period.

Investigational product (IP). The IP used in this trial, Sanaria PfSPZ Vaccine,[12–19] consists of aseptic, purified, vialed, metabolically active, nonreplicating (live, radiation attenuated) cryopreserved PfSPZ stored in liquid nitrogen vapor phase at −150 to −196°C. Preparation of IP was done under the supervision of the study pharmacist, who was not blinded

TABLE 1

Vaccine and control groups by age, vaccine dose, dosing schedule, number of doses, and total number of *Plasmodium falciparum* sporozoites (PfSPZ)

Groups	Age (years)	Subgroups details		*N*	PfSPZ/dose	Dosing schedule (weeks)	No. doses	Total PfSPZ
Group 1	18–45	1a	Vaccinees	6	9×10^5	0, 8, 16	3	2.7×10^6
			NS controls	3	NS	0, 8, 16	3	0
		1b	Vaccinees	6	1.8×10^6	0, 8, 16	3	5.4×10^6
			NS controls	3	NS	0, 8, 16	3	0
Group 2	11–17	2a	Vaccinees	6	9×10^5	0, 8, 16	3	2.7×10^6
			NS controls	3	NS	0, 8, 16	3	0
		2b	Vaccinees	6	1.8×10^6	0, 8, 16	3	5.4×10^6
			NS controls	3	NS	0, 8, 16	3	0
Group 3	6–10	3a	Vaccinees	6	9×10^5	0, 8, 16	3	2.7×10^6
			NS controls	3	NS	0, 8, 16	3	0
		3b	Vaccinees	6	1.8×10^6	0, 8, 16	3	5.4×10^6
			NS controls	3	NS	0, 8, 16	3	0
Group 4	1–5	4a	Vaccinees	6	4.5×10^5	0, 8, 16	3	1.35×10^6
			NS controls	3	NS	0, 8, 16	3	0
		4b	Vaccinees	6	9.0×10^5	0, 8, 16	3	2.7×10^6
			NS controls	3	NS	0, 8, 16	3	0
Group 5	6–11 months	5a	Vaccinees	3	2.7×10^5	0	1	2.7×10^5
		5b	Vaccinees	6	4.5×10^5	0, 8, 16	3	1.35×10^6
			NS controls	3	NS	0, 8, 16	3	0
		5c	Vaccinees	6	9×10^5	0, 8, 16	3	2.7×10^6
			NS controls	3	NS	0, 8, 16	3	0
Total				93				

NS = normal saline.

to the study treatment for each volunteer. Vials of PfSPZ Vaccine were thawed and diluted with phosphate-buffered saline containing human serum albumin and the appropriate numbers of PfSPZ in a final volume of 0.5 mL and drawn into a 1-mL syringe. A volume of 0.5 mL of NS was similarly drawn into a 1-mL syringe for placebo recipients. Dilution and syringe preparation were performed under aseptic conditions in a biological safety cabinet. The pharmacist then handed the appropriate syringe for the specific study subject to the blinded nurse through a window. Reconstituted PfSPZ is a clear, odorless, nonviscous liquid indistinguishable from NS. PfSPZ Vaccine or NS was administered by direct venous inoculation (DVI) through a 25 G × 16-mm needle. In infants and young children, there was an option to administer the vaccine through a 24-gauge peripheral intravenous catheter.

Assessment of vaccine safety, tolerability of DVI, and AEs. After vaccination, volunteers under the age of 18 years were observed at the BCTU for approximately 24 hours during which enquiry of AEs and focused physical examinations were performed at 1, 6, and 12 hours after vaccination and at the time of discharge. Adult volunteers were observed at the BCTU for approximately 2 hours during which enquiry of AEs and focused physical examinations were carried out at 1 hour after vaccination and at the time of discharge. Volunteers were given diaries and thermometers for recording of AEs and temperatures thereafter. Volunteers were seen for safety follow-up visits 2, 7, and 14 days after vaccination, with additional follow-up visits by telephone on days 3–6 (and Day 1 for adults). Children and infants were also evaluated again 28 and 56 days after the final immunization.

Local (site of injection) signs and symptoms were solicited in the 2 days following vaccination for adults, teenagers, and older children (groups 1–3) or 7 days following vaccination for younger children and infants (groups 4 and 5). Systemic signs and symptoms were assessed for 7 days for all groups (Supplemental Table 4). In addition, open-ended questioning was used to identify unsolicited AEs through day 28 postimmunization.

During the period of follow-up, all solicited and unsolicited events were recorded and graded by the attending physician as follows: mild (no effect on activities), moderate (some interference with normal activity), severe (prevented normal activity and required medical intervention), life-threatening (hospitalization, immediate medical intervention, or therapy required to prevent death), or death. Axillary temperature was recorded as Grade 1 (> 37.9–38.4°C), Grade 2 (> 38.4–38.9°C), Grade 3 (> 38.9–40.0°C), or Grade 4 (> 40.0°C). Hematological and biochemical abnormalities were also assessed at prespecified intervals as defined in the toxicity table of the study protocol, including prevaccination and 7 days postvaccination.

Malaria parasite diagnosis. All participants were screened for malaria parasites at baseline and before vaccination by TBS microscopy and retrospectively by quantitative polymerase chain reaction (qPCR). Slide preparation and reading for TBS's were performed according to standard procedures.[29] The theoretical limit of detection of TBS was 2 parasites/μL (0.5 μL blood examined) for standard reads and 0.5 parasites/μL (2.0 μL blood examined) for expanded reads done when a volunteer was symptomatic. Quantitative polymerase chain reaction analyses were based on DNA extracted from 180 μL whole blood and amplification of the *pan-Plasmodium* 18S gene[30] and the Pf-specific telomere-associated repetitive element 2[31] following essentially the published procedures. The 18S gene DNA qPCR had a sensitivity of 50 parasites/mL. *Plasmodium malariae* (Pm) cases were identified by a qPCR species identification assay using Pm-specific amplification of plasmepsin 4 as described.[32]

Genotyping of parasites. DNA from Pf-positive samples was used to genotype the parasites based on Pf Merozoite Surface Protein-1 (PfMSP-1) and PfMSP-2 gene polymorphisms[33] as well as seven microsatellite markers.[34] All Pf strains were compared with the Pf vaccine strain (NF54).

Antibody assays. Sera were assessed for antibodies by enzyme-linked immunosorbent assay (ELISA) to the major protein on the surface of sporozoites (Pf circumsporozoite protein [PfCSP]), immunofluorescence assay (aIFA) to air-dried PfSPZ, and inhibition of sporozoite (PfSPZ) invasion (aISI) of HC-04 cells (hepatocytes) as described.[27]

T-cell assays. T-cell responses in cryopreserved peripheral blood mononuclear cells (PBMCs) were assessed by flow cytometry as described.[15]

Statistical analysis. The sample sizes of three to six vaccinees in each age group dosage group, 12–15 vaccinees in each age category, and six controls in each age category were selected to be appropriate for the initial assessment of safety, tolerance, and immunogenicity of an investigational vaccine. Categorical variables were summarized using absolute (*n*) and relative (%) frequencies. Continuous variables were summarized using mean and SD, median, and range. Comparisons of categorical variables between groups were analyzed using Barnard's two-sided exact unconditional test, or a two-sided Mantel–Haenszel test stratifying by age group. No corrections were made for multiple comparisons because of the early phase nature of this trial. Analyses of immunological data are described with the data. A P value < 0.05 was considered significant. All data analyses and statistical computations were conducted with SAS software, version 9.3 or higher (SAS Institute, Inc., Cary, NC) or GraphPad Prism, version 7.02 (GraphPad Software, LaJolla, CA).

RESULTS

Study population, experience with DVI, and tolerability. A total of 105 Tanzanian volunteers (Figure 1) met the criteria (Supplemental Tables 2 and 3) and were enrolled. Ninety-three received either PfSPZ Vaccine ($n = 63$) or NS ($n = 30$) (Table 1). The remaining 12 volunteers participated as infectivity controls in the subsequent challenge portion (CHMI) of the protocol and are not part of the analyses in this article. There were no significant differences between participants in any age group for age, height, weight, or body mass index (BMI) ($P > 0.05$ for all comparisons, one-sided analysis of variance (ANOVA) (Table 2). All 93 volunteers received all scheduled immunizations. One volunteer in group 5b received only a partial vaccine dose with the second immunization; all other volunteers received the complete 0.5-mL injection at all time points.

Of 273 total injections, 234 were completed with a single injection (85.7%); 225 of these (96.2%) were assessed as simple to perform by the nurse performing the injection. In volunteers aged ≤ 5 years old, DVI was successful on the first attempt in 25 of 39 for first dose, 26 of 36 for second dose, and 31 of 36 for the third dose. The option to establish intravenous access with an intravascular catheter was used for 12 first injections in volunteers ≤ 5 years old, and one first injection in a 6–10-year old. An intravenous catheter was used only once for a second injection in an infant and was not used during the third round of injections, consistent with evidence of a learning curve with the DVI technique in infants and young children.

Pain from DVI was assessed in volunteers aged 6–45 years (groups 1a, 1b, 2a, 2b, 3a, and 3b); 157 of 161 injections (97.5%) were associated with mild or no pain (Supplemental Table 5).

Safety. A global summary of solicited AEs is provided in Table 3. Among the 63 volunteers who received 183 doses of PfSPZ Vaccine, one Group 1b adult volunteer who received 1.8×10^6 PfSPZ reported three solicited local AEs (tenderness and pain after first dose and tenderness after second dose); all were Grade 1 in severity and resolved within 2 days. No solicited local AEs were reported among the 30 volunteers after receiving 90 doses of NS. Solicited systemic AEs were detected after 3/183 injections of PfSPZ and 0/90 injections of NS. All 16 AEs occurred in a single Group 2b volunteer (age 11 years) who received a dose of 1.8×10^6 PfSPZ. All AEs were Grade 1 except elevated temperatures, which were Grade 2. Symptoms included chills and feverishness after two of the three injections with fatigue, headache, malaise, and elevated temperature of 38.5°C after all three injections. The AEs occurred 12–24 hours after each immunization and resolved within 24 hours. This individual had no change in total white blood cell or absolute neutrophil counts or biochemistry values 2 days after each immunization but did have a mild decline in total lymphocyte counts that did not go below the lower limit of normal on Day 2 after each immunization. No other vaccine recipient experienced a systemic solicited AE. The local AEs in the one adult and the systemic AEs in the one adolescent are delineated in Figure 2. No significant differences were found between vaccinees and placebo recipients with respect to systemic or local event rates whether assessed as overall rates or specific rates for each type of AE ($P = 0.60$ for all by Barnard's test).

Twenty of 63 vaccinees (31.7%) experienced 30 unsolicited AEs (0.48/individual) during the 28 days after each immunization (Table 3). All unsolicited AEs were identified as unlikely related to administration of IP. Three unsolicited AEs were moderate (Grade 2) and one (the serious adverse event [SAE] described subsequently) severe (Grade 3) in severity; all others were mild (Grade 1). Ten of 30 controls (33.3%) experienced 13 unsolicited mild (Grade 1) AEs (0.43/individual) during this period. Two episodes of fever in one volunteer (Group 4a, age 3 years receiving 4.5×10^5 PfSPZ), occurring 14 and 19 days after the first dose of NS, were determined to be possibly related to study product during the blinded safety assessment. Details of the unsolicited AEs can be found in Supplemental Table 6.

One SAE was reported in a 2-year-old volunteer (Group 4a) receiving 4.5×10^5 PfSPZ Vaccine who was hospitalized with multiple injuries after she was struck by a motorcycle. She subsequently recovered and completed participation in the study.

Four volunteers developed parasitemia during the immunization period. Three infections in adults were detected retrospectively by qPCR (Supplemental Table 7); these individuals were TBS negative throughout this period of the study. Two of the three cases were Pm infections and one case was Pf, the latter confirmed by genotyping to differ from the NF54 strain of Pf used in the vaccine (Supplemental Table 8). The two individuals with Pm were determined retrospectively to be positive by qPCR at the time of first immunization and remained positive during the entire 16-week immunization period until treated after the third immunization. The individual with Pf infection was negative at the time of the first immunization, developed Pf infection before the second immunization, and remained positive until treated after the third immunization. All three volunteers were treated with artesunate–amodiaquine once the polymerase chain reaction results were known. A fourth volunteer, from Group 5 (ages 6–12 months), who received NS, was positive by qPCR for Pf genotypically distinct

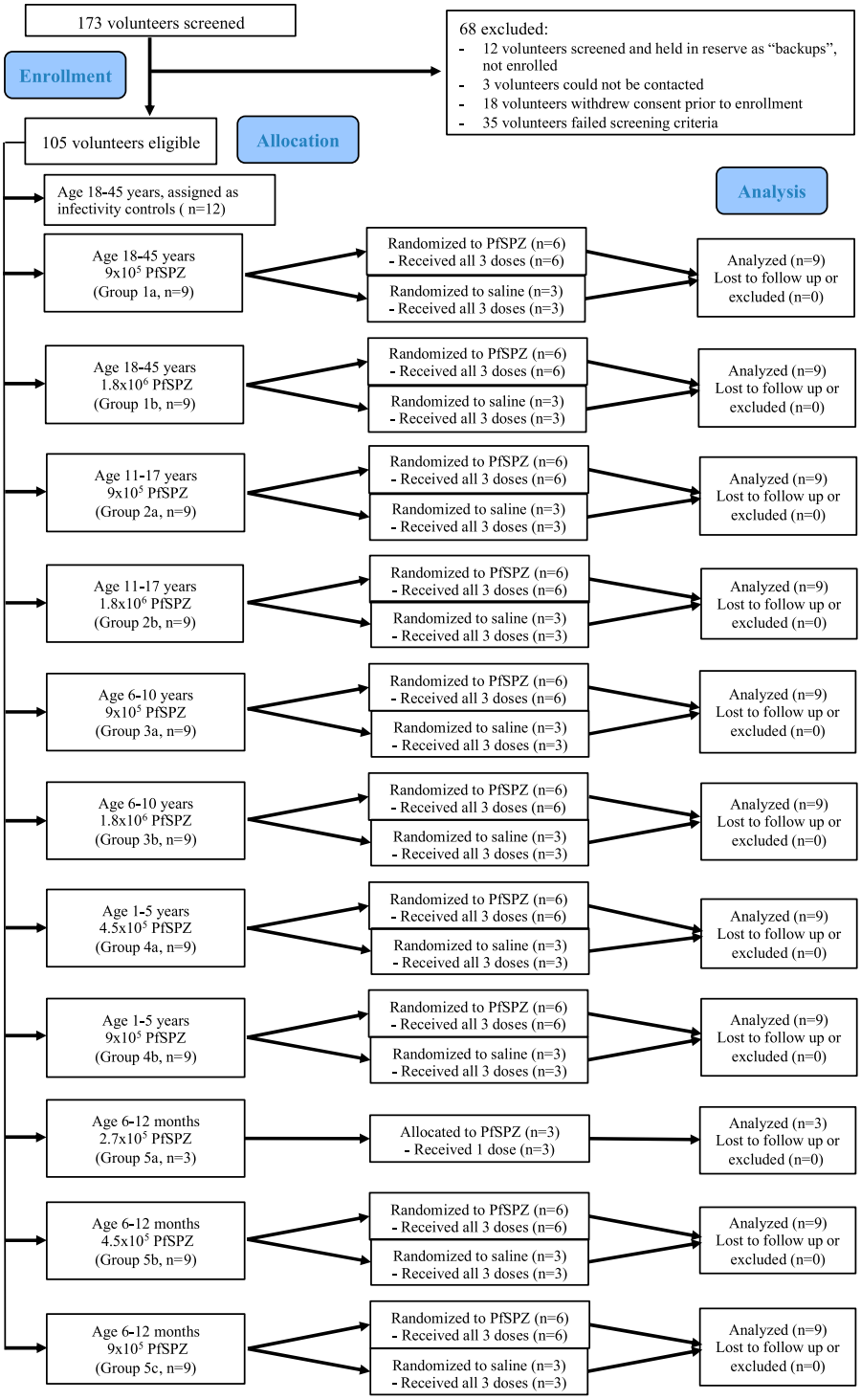

FIGURE 1. Volunteer participation (CONSORT 2010 Diagram). Once allocated, no volunteers were removed, lost to follow-up, or excluded from analysis. This figure appears in color at www.ajtmh.org.

from the NF54 vaccine strain (Supplemental Tables 7 and 8) before the first and second immunizations; parasitemia in this infant was detected in real time by TBS before the second immunization, leading to immediate treatment with artemether–lumefantrine (Supplemental Table 7). No signs of illness were reported by the mother for this infant, who continued in the trial.

No clinically significant laboratory abnormalities were attributed to PfSPZ (Supplemental Table 9). The most commonly identified abnormalities listed in order of prevalence included anemia, lymphopenia, and leukopenia, with no difference in frequency across age groups or between vaccine recipients and NS controls in each age group. Two volunteers experienced a Grade 3 laboratory abnormality: an isolated

TABLE 2
Volunteer characteristics

		Group 1 (18–45 years)				Group 2 (11–17 years)			Group 3 (6–10 years)		
		9×10^5 ($N = 6$)	1.8×10^6 ($N = 6$)	Placebo ($N = 6$)	CHMI controls ($N = 12$)	9×10^5 ($N = 6$)	1.8×10^6 ($N = 6$)	Placebo ($N = 6$)	9×10^5 ($N = 6$)	1.8×10^6 ($N = 6$)	Placebo ($N = 6$)
Age	Units	Years	Years	Years	Years	Years	Years	Years	Years	Years	Years
	Mean (SD)	23.5 (5.7)	24.2 (5.3)	28.7 (7.9)	23.9 (4.8)	11.8 (1.0)	13.2 (1.3)	12.2 (1.3)	7.8 (1.5)	7.8 (1.5)	7.8 (1.5)
	Median	22	24	30	23	12	13	12	8	8	8
	(min, max)	(20, 35)	(18, 33)	(19, 38)	(18, 36)	(11, 13)	(11, 15)	(11, 14)	(6, 10)	(6, 10)	(6, 10)
Sex	Male	4 (66.7%)	4 (66.7%)	5 (83.3%)	7 (58.3%)	3 (50.0%)	2 (33.3%)	3 (50.0%)	3 (50.0%)	4 (66.7%)	3 (50.0%)
	Female	2 (33.3%)	2 (33.3%)	1 (16.7%)	5 (41.7%)	3 (50.0%)	4 (66.7%)	3 (50.0%)	3 (50.0%)	2 (33.3%)	3 (50.0%)
Race	African	6 (100%)	6 (100%)	6 (100%)	12 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)
Height (cm)	Mean (SD)	163.2 (5.0)	166.5 (10.4)	166.3 (8.0)	157.1 (9.0)	139.9 (15.3)	153.5 (9.8)	145.4 (9.2)	123.2 (9.2)	122.0 (7.8)	123.3 (7.1)
	Median	164.0	166.0	166.0	158.3	139.8	154.3	149.8	125.3	120.8	123.0
	(min, max)	(154, 168)	(149, 178)	(153, 175)	(136, 167)	(118, 165)	(138, 165)	(134, 154)	(112, 137)	(114, 135)	(114, 135)
Weight (kg)	Mean (SD)	62.3 (8.1)	65.8 (11.2)	64.3 (3.3)	58.0 (8.4)	33.2 (10.9)	40.6 (9.4)	37.8 (9.3)	23.3 (5.4)	21.2 (4.5)	22.9 (2.4)
	Median	62.5	67.0	65.0	57.5	31.5	41.0	39.3	24.0	20.5	22.5
	(min, max)	(53, 70)	(51, 80)	(60, 68)	(44, 75)	(21, 53)	(28, 55)	(25, 51)	(17, 29)	(16, 29)	(20, 27)
BMI	Mean (SD)	23.6 (4.1)	23.7 (2.9)	23.4 (2.6)	23.6 (3.8)	16.6 (2.6)	17 (2.4)	17.6 (2.7)	15.2 (1.9)	14.1 (1.2)	15.1 (.7)
	Median	23.5	23.6	23.1	23.4	16.1	16.5	17.3	15.1	14.1	15.2
	(min, max)	(19, 30)	(20, 28)	(20, 27)	(19, 31)	(14, 20)	(15, 20)	(14, 22)	(13, 18)	(12, 16)	(14, 16)

		Group 4 (1–5 years)			Group 5 (6–11 months)			
		4.5×10^5 ($N = 6$)	9×10^5 ($N = 6$)	Placebo ($N = 6$)	2.7×10^5 ($N = 3$)	4.5×10^5 ($N = 6$)	9×10^5 ($N = 6$)	Placebo ($N = 6$)
Age	Units	Years	Years	Years	Months	Months	Months	Months
	Mean (SD)	3.3 (1.0)	2.7 (1.9)	3.0 (1.3)	7.3 (1.5)	7.3 (0.8)	8.7 (1.5)	9.3 (1.9)
	Median	3	3	3	7	8	8	10
	(min, max)	(2, 5)	(1, 5)	(2, 5)	(6, 9)	(6, 8)	(7, 11)	(6, 11)
Sex	Male	2 (33.3%)	3 (50.0%)	3 (50.0%)	1 (33.3%)	4 (66.7%)	2 (33.3%)	1 (16.7%)
	Female	4 (66.7%)	3 (50.0%)	3 (50.0%)	2 (66.7%)	2 (33.3%)	4 (66.7%)	5 (83.3%)
Race	African	6 (100%)	6 (100%)	6 (100%)	3 (100%)	6 (100%)	6 (100%)	6 (100%)
Height (cm)	Mean (SD)	99.7 (7.4)	91.6 (16.0)	96.1 (7.1)	66.0 (2.6)	67.7 (2.5)	69.9 (3.8)	66.0 (4.2)
	Median	99.3	92.0	93.8	65.0	68.3	71.8	66.5
	(min, max)	(92, 111)	(72, 113)	(89, 107)	(64, 69)	(63, 70)	(63, 73)	(59, 71)
Weight (kg)	Mean (SD)	14.3 (1.6)	12.3 (3.9)	13.3 (1.4)	7.0 (1.0)	8.0 (0.9)	8.4 (1.2)	7.8 (1.2)
	Median	14.5	11.3	13.0	7.0	8.0	8.8	7.5
	(min, max)	(12, 16)	(9, 18)	(12, 15)	(6, 8)	(7, 9)	(7, 10)	(7, 10)
BMI	Mean (SD)	14.5 (1.4)	14.5 (1.3)	14.5 (1.4)	16.0 (1.2)	17.5 (2.4)	17.2 (1.6)	18 (2.8)
	Median	14.6	14.4	14.5	16.6	17.9	17.2	16.9
	(min, max)	(13, 16)	(13, 17)	(13, 16)	(15, 17)	(14, 20)	(15, 20)	(16, 23)

CHMI = controlled human malaria infection.

TABLE 3
Global adverse event (AE) summary

	Vaccine (*N* = 63)		Placebo (*N* = 30)	
	All AEs	Possibly, probably, or definitely related AEs	All AEs	Possibly, probably, or definitely related AEs
No. of volunteers with at least one solicited AE within 7 days of immunization (%)	2 (3.2%)	2 (3.2%)	0 (0.0%)	0 (0.0%)
Total no. of solicited AEs (maximum severity grade)	19 (Grade 2)*	18 (Grade 2)*	0 (NA)	0 (NA)
No. of volunteers with a solicited Grade 3 AE (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
No. of volunteers with at least one solicited local AE	1 (1.6%)	1 (1.6%)	0 (0.0%)	0 (0.0%)
Total no. of local AEs (maximum severity grade)	3 (Grade 1)	3 (Grade 1)	0 (NA)	0 (NA)
No. of volunteers with at least one solicited systemic AE (%)	1 (1.6%)	1 (1.6%)	0 (0.0%)	0 (0.0%)
Total no. of systemic AEs (maximum severity grade)	16† (Grade 2)*	15† (Grade 2)*	0 (NA)	0 (NA)
No. of volunteers with at least one unsolicited AE within 28 days of immunization (%)	23 (36.5%)	0 (0.0%)	10 (33.3%)	1 (3.3%)
Total no. of unsolicited AEs within 28 days of immunization (maximum severity grade)	34 (Grade 3)	0 (NA)	11 (Grade 1)	2 (Grade 1)
No. of volunteers with an unsolicited Grade 3 AE (%)	1 (1.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
No. of volunteers experiencing an SAE (%)	1 (1.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Total no. of SAEs (maximum severity grade)	1 (Grade 3)	0 (NA)	0 (NA)	0 (NA)

SAE = serious adverse event.
* The only Grade 2 AE was elevated temperature.
† All solicited systemic AEs occurred in a single individual.

occurrence of lymphopenia in a Group 1b adult volunteer 28 days after the first dose of 1.8×10^6 PfSPZ in association with a concomitant viral infection, and neutropenia in a Group 5b volunteer receiving 4.5×10^5 PfSPZ determined to have benign ethnic neutropenia. Both abnormalities resolved without sequelae.

Antibody responses to Pf. Antibodies against PfCSP by ELISA, PfSPZ by aIFA, and PfSPZ by aISI in sera taken before immunization and 2 weeks after last vaccine dose are shown in Supplemental Table 10, Figure 3, and Supplemental Figure 1 for vaccinees and Supplemental Table 11 for controls.

In the PfCSP ELISA, volunteers were considered to have made a positive response if their net optical density (OD) 1.0 (Figure 3A–C) and OD 1.0 ratio (Supplemental Figure 1A–C), calculated, respectively, by subtracting or dividing by the prevaccination antibody OD 1.0, were ≥ 50 and ≥ 3.0, respectively. By these criteria, 59/60 vaccinees developed antibodies to PfCSP. The only volunteer who did not have a positive response to immunization was a 20-year old who received the 9.0×10^5 PfSPZ regimen (Supplemental Table 10). The overall highest responses were in 6–10-year olds who received 1.8×10^6 PfSPZ and had a median net OD 1.0 of 34,793 and median net OD 1.0 ratio of 15,515 (Supplemental Table 8). Only 2/30 of the NS controls, both infants, developed antibodies to PfCSP (Supplemental Table 11).

In the aIFA, volunteers with a net arbitrary fluorescence unit (AFU) 2×10^5 of ≥ 150 (Figure 3D–F) and a ratio of post- to pre-AFU 2×10^5 of ≥ 3.0 (Supplemental Figure 1, panels D-F) were considered to have made a positive response. By these criteria, 57/60 volunteers made a positive response to immunization. The three volunteers who did not make a positive response were 1 year, 7 months, and 9 months of age at the time of first injection and received the 9.0×10^5, 4.5×10^5, and 9.0×10^5 PfSPZ dosing regimens, respectively (Supplemental Table 10). As with the PfCSP ELISA, the overall highest

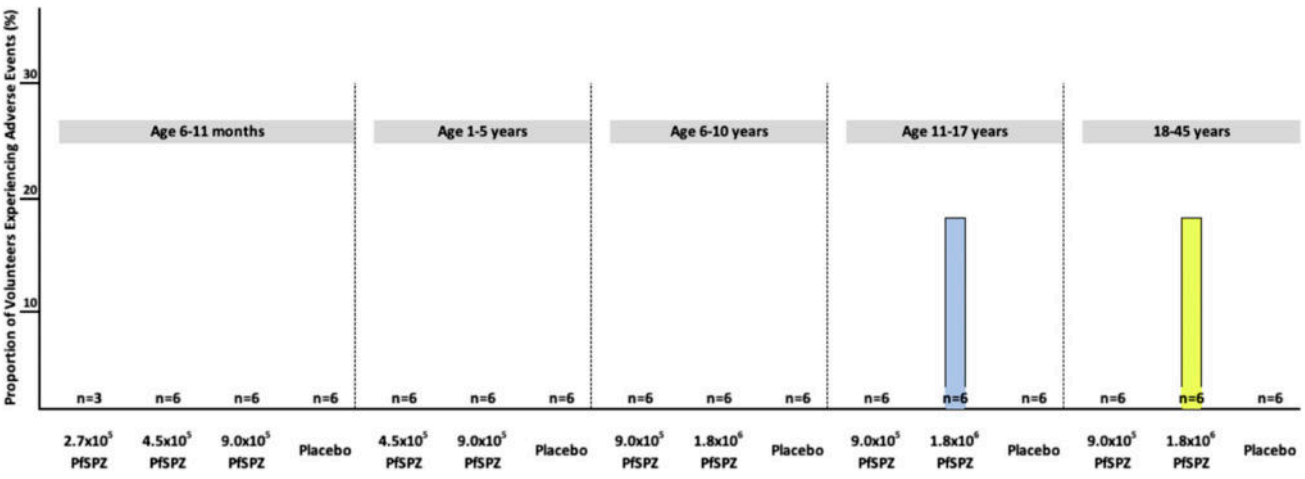

FIGURE 2. Proportion of volunteers experiencing solicited adverse events (AEs) during the 7 days after each immunization. Ninety-one of 93 volunteers injected at least once experienced no solicited AEs during the 7 days after each immunization. One volunteer experienced three Grade 1 local AEs (yellow bar) and one volunteer experienced Grade 2 temperature elevation (38.5°C) after each immunization accompanied by mild (Grade 1) chills, fatigue, headache, and malaise (blue bar). These AEs are further described in the text. This figure appears in color at www.ajtmh.org.

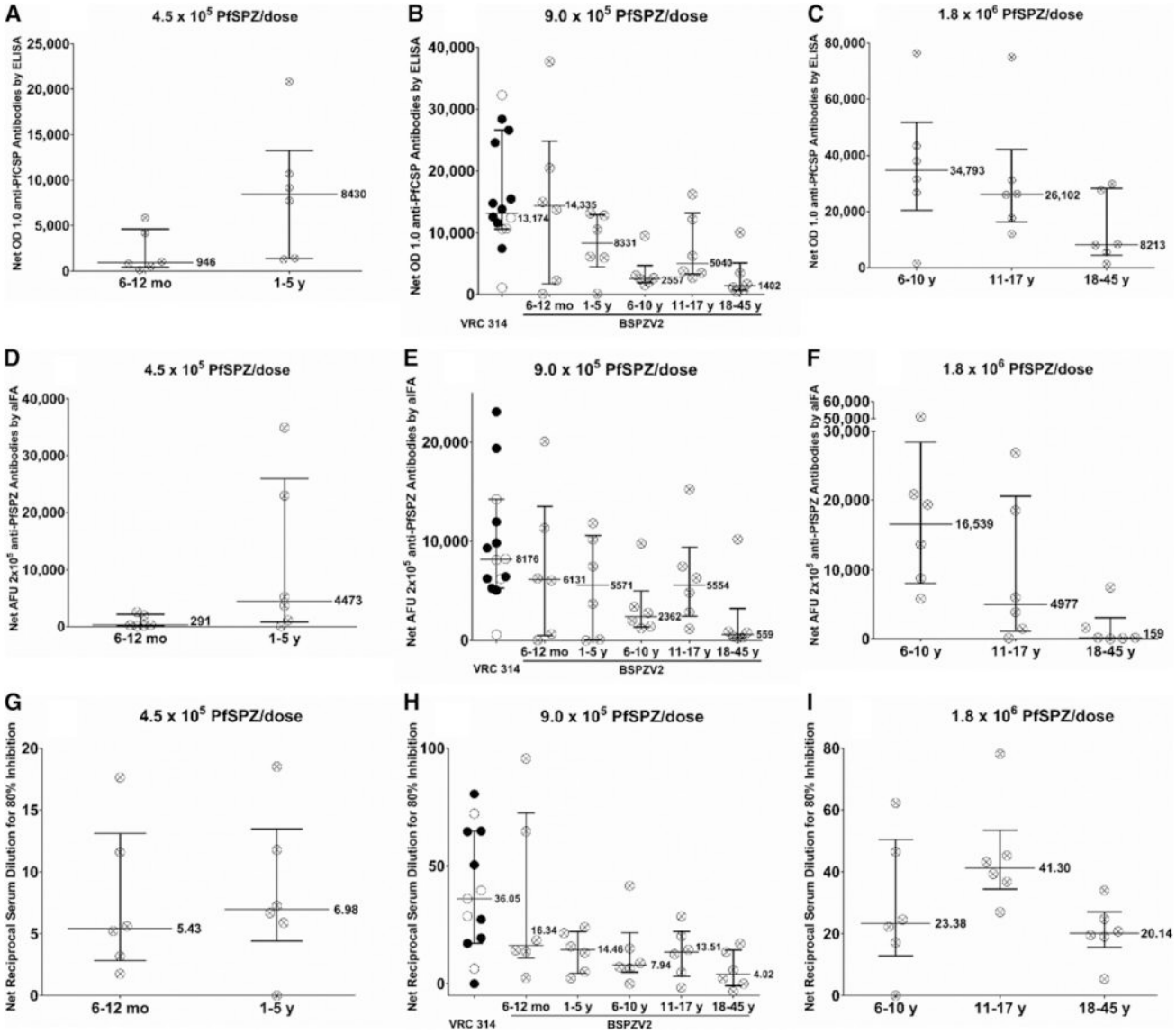

FIGURE 3. Difference between postimmunization and preimmunization antibody results. Antibody assay results by dose in *Plasmodium falciparum* circumsporozoite protein enzyme-linked immunosorbent assay (**A**–**C**), immunofluorescence assay (**D**–**F**), and inhibition of sporozoite invasion (**G**–**I**) assays. Results were obtained by subtracting preimmune values from the values obtained from sera drawn 2 weeks after the third dose. For the 9.0×10^5 *Plasmodium falciparum* sporozoite dose (**B**, **E**, and **H**), previously assessed results from clinical trial VRC 314,[17] conducted in the United States with the same dosage regimen and in the same laboratory with the same assays as in the BSPZV2 trial assays, are shown as a comparison. For the VRC 314 trial[17] the filled in circles indicate protected volunteers and the empty circles the unprotected volunteers. Medians with interquartile ranges are shown.

responses were in 6–10-year olds who received the 1.8×10^6 PfSPZ regimen and had a median net AFU 2×10^5 of 20,099 and median net AFU 2×10^5 ratio of 16,539 (Supplemental Table 10). None of the NS controls developed antibodies to PfSPZ by aIFA (Supplemental Table 11).

In the automated inhibition of sporozoite invasion assay (ISI), volunteers with a net ISI activity of ≥ 10% (Figure 3G–I) and ratio of post to pre-ISI activity of ≥ 3.0 (Supplemental Figure 1, panels G-I) were considered positive. By these criteria, 37/60 volunteers had a positive response to immunization. The only group with a 100% response rate (6/6) was the 11–15-year olds who received the 1.8×10^6 PfSPZ regimen. This group also had the highest median net 80% ISI activity (41.3) and median net 80% ISI activity ratio (26.8). Only 2/30 of the NS controls developed antibodies to PfSPZ by aISI, a 38 and a 13-year old (Supplemental Table 11).

Adults (18–35 years), teenagers (11–15 years), and older children (6–10 years) received the 1.8×10^6 PfSPZ dosage regimen, and these age groups and the younger children (1–5 years) and infants (7–11 months) received the 9.0×10^5 PfSPZ regimen; the younger children and infants also received the 4.5×10^5 the PfSPZ regimen. We, therefore, assessed the effect of age on immunogenicity. This is shown graphically using net values in Figure 3 and for ratios in Supplemental Figure 1 and in Supplemental Tables 10 and 11. For the 1.8×10^6 PfSPZ dosage regimen, the adults had the lowest antibody responses by all assays. The older children had the highest responses in the PfCSP ELISA and aIFA and teenagers

for the aISI. For the 9.0×10^5 PfSPZ dosage regimen, the adults had the lowest median net OD 1.0, median net AFU 2×10^5, and median net 80% ISI responses, and the lowest ratios for PfCSP ELISA and aISI; infants had the highest responses for all of these assays. For the median AFU 2×10^5 ratio, the teenagers had the best response. For the 9.0×10^5 PfSPZ dosage regimen, the median OD 1.0 and median OD 1.0 ratios in the PfSPZ ELISA were 10.2 and 165.7 times higher in the infants as compared with the adults. For the aIFA, they were 11.0 and 3.3 times higher and for aISI, they were 4.1 and 6.3 times higher, respectively (Figure 3, Supplemental Figure 1, Supplemental Table 10). Because of the small sample sizes ($N = 6$) and variability within each group, the differences did not quite reach the level of statistical significance.

Adults, teenagers, and older children received the 1.8×10^6 or 9.0×10^5 regimens. For all three assays, the response to the 1.8×10^6 regimen was higher than the response to the 9.0×10^5 regimen (Figure 3, Supplemental Figure 1, Supplemental Table 10). Younger children and infants received the 9.0×10^5 and 4.5×10^5 regimens. For all three assays, the response to the 9.0×10^5 PfSPZ regimen was higher than the response to the 4.5×10^5 regimen in infants. However, for the younger children, this was not the case (Figure 3, Supplemental Figure 1, Supplemental Table 10).

In previous clinical trials with PfSPZ Vaccine in adults in the United States with no previous exposure to malaria, there has been a significant correlation between the three different antibody assays[14–17] in sera taken 2 weeks after the last dose of vaccine. In this clinical trial, we assessed the correlation between the assays for sera taken 2 weeks after the third (last) dose of vaccine from 60 volunteers. There was a significant correlation between the results of the PfCSP ELISA and the aISI ($R^2 = 0.45$, $P < 0.0001$). The correlations between PfCSP ELISA and aIFA ($R^2 = 0.05$, $P = 0.085$) and aIFA versus aISI ($R^2 = 0.01$, $P = 0.40$) were not significant.

Having demonstrated that median antibody responses in Tanzanian infants were consistently higher than in Tanzanian adults, we compared the Tanzanian responses to those in adults in the United States who received three doses of 9.0×10^5 PfSPZ (Figure 3B, E, and H). Median antibody responses in U.S. adults and Tanzanian infants were, respectively: 1) 13,174 and 14,335 for PfCSP ELISA, 2) 8,176 and 6,131 for aIFA, and 3) 36.05 and 16.34 for aISI.

T-cell responses to PfSPZ. T-cell responses to PfSPZ were assessed by polychromatic flow cytometry on cryopreserved PBMCs acquired before immunization, 2 weeks after the first dose of PfSPZ Vaccine and 2 weeks after the third dose of vaccine in subjects who received three doses of 9.0×10^5 PfSPZ of PfSPZ Vaccine (Figure 4 and Supplemental Table 12). Following the first dose of PfSPZ Vaccine, 18–45-year olds, 11–17-year olds, and 6–10-year olds all had significant increases in the frequency of cytokine-producing memory CD4 T cells compared with the prevaccination time point. However, only the 6–10-year-old group had a 100% response rate to the vaccine. In addition, the responses in the 6–10-year-old group were significantly higher than those in the 1–5-year-old and 6–11-month-old groups, but not significantly different than the responses in the other groups (as assessed by the Kruskal–Wallis test with Dunn's correction for multiple comparisons). Following the third vaccination, only the 1–5-year olds had a significant increase over the prevaccination time point. Infants did not have a significant increase in CD4 T-cell responses at any time point. At no time point were PfSPZ specific memory CD8 T-cell responses detected over background in any group.

DISCUSSION

Before this study, aseptic, purified, cryopreserved PfSPZ-based products (Sanaria PfSPZ Vaccine, PfSPZ Challenge, and PfSPZ-CVac) had only been injected into adults.[13–19,27,29,35–41] This was the first study to assess the safety, tolerability, feasibility, and immunogenicity of any PfSPZ-based product in adolescents, children, or infants.

Because no licensed vaccine against an infectious agent is administered by DVI, there was initially a concern in the vaccinology community about the safety, tolerability, and feasibility of administering PfSPZ-based products by DVI to adults. Clinical trials in the United States, Germany, Spain, Mali, Tanzania, and Gabon established that rapid administration by DVI of PfSPZ products in 0.5 mL of diluent through a 25-gauge needle was safe, extremely well tolerated, straightforward, and protective.[16,18,27,38,39,41] After establishing the safety, tolerability, and feasibility of PfSPZ administration by DVI in adults, there was still concern that DVI administration of PfSPZ might be problematic in younger age groups as no preventative vaccine has ever been administered by intravenous injection (IV)/DVI to these age groups. This concern was not borne out by the findings of the present study.

There were no significant differences in solicited AEs between vaccinees in any age group ($N = 63$) and corresponding controls ($N = 30$) who received NS ($P = 0.6$). Furthermore, there were no differences in AEs between different dosage regimens, no differences between age groups, and no differences between the first, second, and third immunizations. PfSPZ Vaccine was extremely well tolerated. We have no explanation for the symptoms and elevated temperature experienced by the 11-year-old girl after all three injections, which resolved within 24 hours of each immunization. This has not occurred in any subject in any other clinical trial of PfSPZ Vaccine; we will monitor for this pattern in future trials.

Moreover, the administration process itself (DVI) was extremely well tolerated. Six- to 45-year olds were questioned about pain after each injection. Direct venous inoculation was associated with mild or no pain for 157 of 161 injections (97.5%); 129 of 161 injections (80.1%) were associated with no pain (Supplemental Table 5).

There was no significant difference in the feasibility of administration of PfSPZ (or NS placebo) to adults, adolescents, 6–10-year olds, or 1–5-year olds. Administration was achieved with the first needle stick in 53/54 (98%) administrations in 18–45-year olds, 51/54 (94%) administrations in 11–17-year olds, 47/54 (87%) administrations in 6–10-year olds, and 47/54 (87%) administrations in 1–5-year olds (Supplemental Table 5). Administration success with one needle stick decreased to 35/57 (61%) injections in infants (Supplemental Table 5). However, in infants, the learning curve of the nurses administering the vaccine was rapid. In infants, DVI was successful on the first attempt in nine of 21 (43%) for first dose, 11 of 18 (61%) for second dose, and 15 of 18 (83%) for the third dose. The option to establish intravenous access with an intravascular catheter was used for 12 first injections (nine infants), but only once for a second injection (one infant)

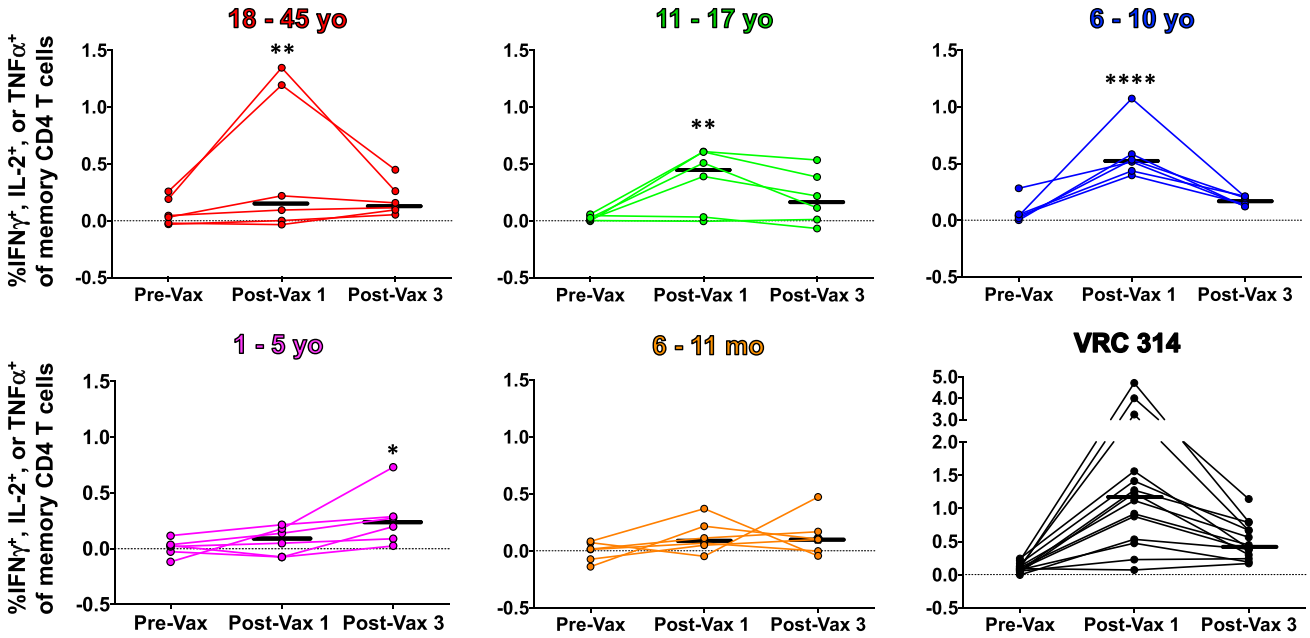

FIGURE 4. *Plasmodium falciparum* sporozoites (PfSPZ)-specific memory CD4 T-cell responses pre- and postvaccination. Percent of memory CD4 T cells in the blood expressing interferon gamma (IFN-γ) interleukin 2 (IL-2) or tumor necrosis factor alpha (TNF-α) at preimmunization or 2 weeks after the first and third doses of PfSPZ Vaccine (9.0×10^5). Results are the percentage of cytokine-producing cells after incubation with PfSPZ minus the percentage of cytokine-producing cells after incubation with vaccine diluent (medium with 1% human serum albumin). Bars indicate median values within each group. Differences within each age group between pre- and postvaccination groups were assessed by two-way ANOVA with Dunnett's correction for multiple comparisons. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$. Previously measured results from clinical trial VRC 314[17] conducted in the United States with the same dosage regimen and the same assay conducted in the same laboratory as for the BSPZV2 trial assays are shown as a comparison. This figure appears in color at www.ajtmh.org.

and was not used during the third round of immunizations, consistent with evidence of a learning curve with the technique when administering to infants.

The levels of antibodies to PfCSP by ELISA were 31 times lower in adults in Mali than in U.S. adults and 4.3 times lower in adults in Tanzania,[20] who received the exact same immunization regimen.[18] We hypothesized that this was due to immunoregulation after long-term exposure to Pf infections and that in malaria-endemic areas, antibody responses would be higher in children and infants who had less exposure to Pf than in adults with long-term exposure. Naturally acquired immunity may also have affected the viability of the sporozoites, and this also is most highly developed in adults with long-term exposure. However, the levels of antibody preimmunization and in the placebo controls were quite low, arguing against this explanation for reduced immunogenicity (Supplemental Tables 10 and 11). Results from this study are consistent with the hypothesis that reduced immune responses in semi-immune African as compared with non-immune American adults was due to immunoregulation after long-term exposure to Pf infections, and that in malaria-endemic areas antibody responses would be higher in children and infants who had less exposure to Pf than in adults with long-term exposure. The median antibody responses by PfCSP ELISA, PSPZ aIFA, and PfSPZ aISI were highest in infants and lowest in adults (Figure 3), and the antibody responses in infants were comparable with the antibody responses seen in adults in the United States who received the identical immunization regimen (Figure 3). However, differences among age groups did not quite reach the level of statistical significance because of the small sample size and the variance. Ongoing studies will establish whether this age effect is consistent and significant.

T-cell responses against PfSPZ have been demonstrated in malaria-naïve adults immunized with PfSPZ Vaccine in the United States[14,16] and in a previous study of PfSPZ Vaccine in adults in Tanzania,[20] but they were much lower in Tanzania than in the United States after administration of the same immunization regimen. In this trial, there were no CD8 T-cell responses against PfSPZ detected. However, significant increases in peripheral CD4 T-cell responses were seen in all age groups except infants after in vitro stimulation with PfSPZ (Supplemental Table 12). The median adult responses were about six times lower than they were after immunization with the same regimen in the United States (Figure 4).[17] Consistent with all of our trials, the best responses in adults, 11–17-year olds, and 6–10-year olds were seen after the first dose of PfSPZ Vaccine[14–17] with the highest responses in 6–10-year olds. We have previously hypothesized that the reason peak T-cell responses are highest after the first dose is that after the first dose the functionally important T cells are resident in the liver and, thus, are not detected in the periphery.[14] However, after the third dose of PfSPZ Vaccine, the only significant results were in 1–5-year olds; this was the best response recorded for this age group. Perhaps, because of immunological immaturity, it took longer to prime 1–5-year olds, and if they were administered more priming doses earlier, they would have better responses. Infants did not have any evidence of induction of T-cell responses. Such poor T-cell responsiveness in infants has also been observed following hepatitis B and oral polio vaccinations.[42,43] This is likely based on the fact that T-cell repertoires in neonates and infants are

skewed toward Th2-type responses.[44–48] Since PfSPZ Vaccine is thought to rely primarily on T-cell responses to mediate protection,[14,15,17,49] the T-cell studies may indicate that children, but not infants, will be protected by the immunization regimen (three doses at 8-week intervals) used in this study. We are now exploring priming regimens in which multiple doses of PfSPZ are administered during the first week,[27] and this may prove to be a more powerful method of priming and could thereby overcome the poor T-cell responsiveness we have identified in infants. However, we recognize that we will be breaking new ground here, as to our knowledge, there are no data in infants for any vaccine that this can be done. In addition, we will explore the impact of booster doses during the first or second years of life, and we are developing an adjuvant that may be useful.

By establishing the safety, tolerability, and feasibility of administration of PfSPZ Vaccine to children and infants, this trial was an important prelude to clinical trials in more than 300 infants being conducted in Kenya (ClinicalTrials.gov NCT02687373) and Equatorial Guinea (ClinicalTrials.gov NCT02859350). It also supported our long-term plans to use PfSPZ Vaccine in mass vaccination programs (MVPs) to focally eliminate malaria. Because high population coverage will be needed to halt transmission, any vaccine intended for this purpose must be extremely safe, easy to administer, and minimally painful for the vaccinee, in all age groups. As there was no difference in the rate of AEs or laboratory abnormalities between any vaccine group and placebo recipients and because 97.2% of volunteers old enough to evaluate pain experienced no pain or only mild pain during administration, PfSPZ Vaccine appeared in the present study to be highly suitable for use in MVPs.

The results demonstrated that 6–10-year olds and 1–5-year olds have the highest CD4 T-cell responses after the first and third doses of PfSPZ Vaccine, respectively. These findings raise hope that the vaccine will be more protective in children than it was in adults in Mali.[18] This will soon be assessed in 1–12-year olds in Gabon. However, because no T-cell responses were identified in infants, we are concerned about infants' immunological capacity to mount protective T-cell responses after this immunization regimen of PfSPZ Vaccine. This is now being assessed in the Kenya study, and if it holds up, we may have to alter the immunization regimen (number of PfSPZ per dose, number of doses, and interval between doses). If this is not successful, we would likely initiate immunization only after the first year of life. PfSPZ Vaccine is intended to be used in MVPs to halt transmission of Pf and not in a routine infant immunization program (Expanded Program for Immunization). In such MVPs, we would cover the infants with antimalarial drugs until they reached 1–2 years of age when immunizations with PfSPZ Vaccine would begin.

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Disclosures: Sanaria, Inc. manufactured PfSPZ Vaccine and PfSPZ Challenge, and Protein Potential LLC is affiliated with Sanaria. Sanaria was the sponsor of the clinical trial. Thus, all authors associated with Sanaria or Protein Potential have potential conflicts of interest.

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4.5 Paper III: Published Article

Increase of Dose Associated with Decrease in Protection Against Controlled Human Malaria Infection by PfSPZ Vaccine in Tanzanian Adults: Randomized Controlled Trial

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Clinical Infectious Diseases

MAJOR ARTICLE

Increase of Dose Associated With Decrease in Protection Against Controlled Human Malaria Infection by PfSPZ Vaccine in Tanzanian Adults

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Background. A vaccine would be an ideal tool for reducing malaria's impact. PfSPZ Vaccine (radiation attenuated, aseptic, purified, cryopreserved *Plasmodium falciparum* [Pf] sporozoites [SPZ]) has been well tolerated and safe in >1526 malaria-naive and experienced 6-month to 65-year-olds in the United States, Europe, and Africa. When vaccine efficacy (VE) of 5 doses of 2.7×10^5 PfSPZ of PfSPZ Vaccine was assessed in adults against controlled human malaria infection (CHMI) in the United States and Tanzania and intense field transmission of heterogeneous Pf in Mali, Tanzanians had the lowest VE (20%).

Methods. To increase VE in Tanzania, we increased PfSPZ/dose (9×10^5 or 1.8×10^6) and decreased numbers of doses to 3 at 8-week intervals in a double blind, placebo-controlled trial.

Results. All 22 CHMIs in controls resulted in parasitemia by quantitative polymerase chain reaction. For the 9×10^5 PfSPZ group, VE was 100% (5/5) at 3 or 11 weeks ($P < .000l$, Barnard test, 2-tailed). For 1.8×10^6 PfSPZ, VE was 33% (2/6) at 7.5 weeks ($P = .028$). VE of dosage groups (100% vs 33%) was significantly different ($P = .022$). Volunteers underwent repeat CHMI at 37–40 weeks after last dose. 6/6 and 5/6 volunteers developed parasitemia, but time to first parasitemia was significantly longer than controls in the 9×10^5 PfSPZ group (10.89 vs 7.80 days) ($P = .039$), indicating a significant reduction in parasites in the liver. Antibody and T-cell responses were higher in the 1.8×10^6 PfSPZ group.

Conclusions. In Tanzania, increasing the dose from 2.7×10^5 to 9×10^5 PfSPZ increased VE from 20% to 100%, but increasing to 1.8×10^6 PfSPZ significantly reduced VE.

Clinical Trials Registration. NCT02613520.

Keywords. malaria; *Plasmodium falciparum*; PfSPZ; vaccine efficacy; controlled human malaria infection.

In 2015–2017 there were 429 000–730 500 deaths caused by malaria annually, most by *Plasmodium falciparum* (Pf) [1–3]. Our goal is to deploy a vaccine that prevents infection with Pf and thereby prevents all manifestations of malaria and halts transmission [4]. Sanaria® PfSPZ Vaccine, composed of radiation-attenuated, aseptic, purified, cryopreserved Pf sporozoites (SPZ), has been well tolerated, safe, and efficacious [5–10].

We are using small trials including controlled human malaria infection (CHMI) to assess vaccine efficacy (VE) to optimize dosage regimens. When the same dosage regimen was assessed against homologous (same parasites in vaccine and challenge) CHMI in adults in the United States and Tanzania, VEs at 3 and 24 weeks against homologous CHMI were 93% and 65% in the United States and 20% and 20% in Tanzania [8, 11]. Protection against heterologous (different strain of Pf in vaccine and challenge) CHMI in the United States was 80% and 8% at 3 and 24 weeks [8]. This same PfSPZ Vaccine regimen gave a VE of 52% by time to event and 29% by proportional analysis over 24 weeks against intense transmission of Pf in Mali [10], suggesting homologous CHMI in Tanzanians at 3 weeks and heterologous CHMI in Americans at 24 weeks are more rigorous tests of VE than natural exposure.

A US study demonstrated 54% VE against heterologous CHMI at 33 weeks after 3 doses at 8-week intervals of 9×10^5 PfSPZ [9]. To improve VE in Tanzania, we assessed 3 doses of 9×10^5 or 1.8×10^6 PfSPZ.

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METHODS

Study Design

A double-blind, randomized, placebo-controlled trial was conducted in Bagamoyo, Tanzania (December 2015 to March 2017). It had an age de-escalation, dose escalation component to assess safety, and immunogenicity of PfSPZ Vaccine (part A) [12], and a controlled human malaria infection (CHMI) component to assess VE (part B), described herein. For part B, 30 healthy males and females aged 18–45 years were recruited from the Bagamoyo region as described [12]. Eligibility criteria are at https://clinicaltrials.gov/show/NCT02613520.

Investigational Products (IP)

Sanaria® PfSPZ Vaccine [5–10, 13], PfSPZ Challenge [14–19], or normal saline (NS) was prepared in 0.5 mL (supervised by study pharmacist). All were administered by direct venous inoculation (DVI) through a 25-gauge needle.

Immunization

Enrolled participants were sequentially entered into the randomization table by the unblinded study pharmacist. Nine volunteers were allocated to group 1a (9×10^5 PfSPZ) and 9 to group 1b (1.8×10^6 PfSPZ) (Figure 1). Within each group volunteers were randomized to receive PfSPZ Vaccine (n = 6) or NS (n = 3) on days 1, 57, and 113.

Vaccine Efficacy

CHMI

VE was assessed by CHMI by DVI of 3.2×10^3 PfSPZ of PfSPZ Challenge [11]. CHMIs were planned for 3 and 24 weeks after last vaccine dose; timing was changed for most. Three additional volunteers were enrolled as infectivity controls for each of the first 2 CHMIs and 6 for the third. Volunteers were observed as inpatients beginning on day 9 after CHMI until diagnosed and treated for malaria or until day 21; daily outpatient monitoring for thick blood smear (TBS) negative volunteers continued until day 28. TBSs were made every 12 hours (days 8–14), then daily until positive or until day 28. Each TBS was paired with a sample for quantitative polymerase chain reaction (qPCR); qPCRs were performed retrospectively except when a TBS was positive, in which case qPCR was performed immediately. TBSs could be performed more frequently, if clinically indicated. Posttreatment, TBSs were assessed until 2 consecutive daily TBSs were negative and on day 28.

Adverse Events (AEs) After CHMI

AEs after CHMI were recorded for 5 days (Table S1) [12]. Volunteers were observed as inpatients for 48 hours after administration of PfSPZ Challenge, discharged with AE diaries and thermometers, and followed up with daily telephone calls. Hematological and biochemical parameters were assessed. AEs were assessed for grade and relatedness to IP (Table S5) through prespecified (Table S1) and open-ended questioning. They were considered related to Pf infection if the event was within 3 days prior to and 7 days after TBS was first positive.

Treatment

Malaria diagnosed prior to CHMI was treated with artesunate-amodiaquine (AS-AQ) or artemether-lumefantrine (AL). Volunteers with positive TBSs confirmed by qPCR in the 28-day interval following CHMI were treated with AL. Volunteers who were TBS negative throughout were treated at day 28.

Detection of Pf Parasites and Parasite DNA

TBSs were prepared and read as described [17]. The theoretical lower limits of detection were 2 and 0.5 parasites/μL blood for asymptomatic and symptomatic subjects respectively. qPCR detecting *Plasmodium spp.* 18S genes had a sensitivity of 0.1 parasites/μL [20]. A second qPCR assay (sensitivity of 0.05 parasites/μL), targeting the Pf specific telomere-associated repetitive element 2 [21] was used to reanalyze samples negative by 18S based qPCR. The time to first positivity by qPCR was the prepatent period.

Drug Levels

Amodiaquine levels were assessed for volunteers who received AS-AQ. Sulfadoxine and lumefantrine levels were assessed for all volunteers in CHMIs no. 1 and no. 2. Plasma samples (−80°C) were shipped to Swiss BioQuant, AG, Reinach, Switzerland for analysis by high performance liquid chromatography coupled to mass spectrometry.

Antibody and T-cell Assays

Methods were previously described [12].

Statistical Analysis

Six vaccinees per dosage group and a minimum of 6 controls for each CHMI was able to show with a power of 80% that a 17% (1/6) Pf infection frequency in vaccinees was different ($\alpha = 0.05$, 2-tailed) than a 100% (6/6) Pf infection frequency in controls. Categorical variables were summarized using absolute (n) and relative (%) frequencies. Continuous variables were summarized using mean, standard deviation, median, and range. Comparisons of categorical variables were analyzed using Barnard's 2-sided exact test, and continuous variables by the Mann-Whitney *U* Test, 2-sided. No corrections were made for multiple comparisons due to the early phase nature of this trial. $P < .05$ was considered significant.

Study Approval

The protocol was approved by institutional review boards (IRBs) of the Ifakara Health Institute (IHI/IRB/ No: 32–2015), the National Institute for Medical Research Tanzania (NIMR/HQ/R.8a/Vol.IX/2049), and the Ethikkommission

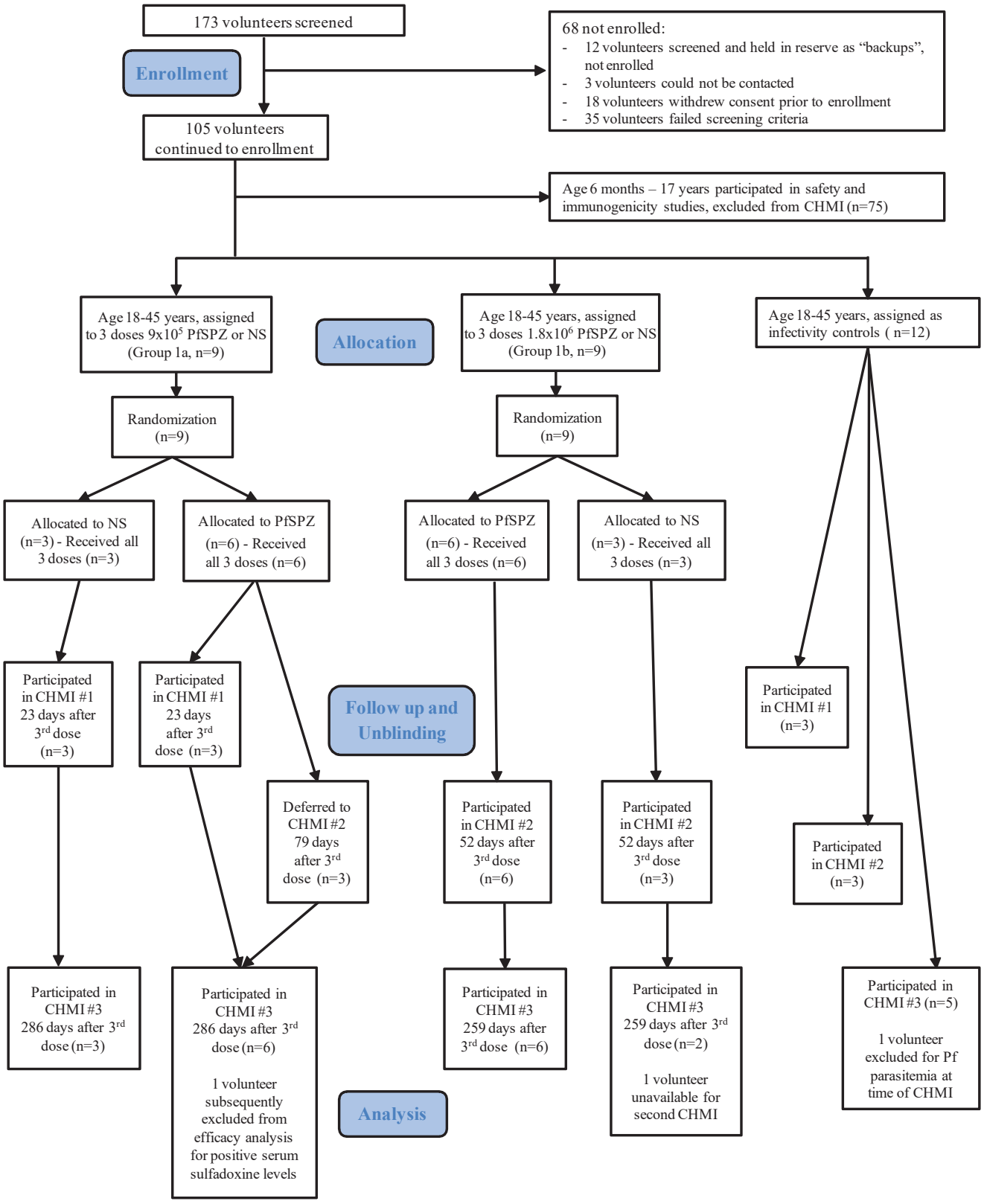

Figure 1. Volunteer participation (CONSORT 2010 Diagram). Abbreviations: CONSORT, Consolidated Standards of Reporting Trials; CHMI, controlled human malaria infection; NS, normal saline; PfSPZ, *Plasmodium falciparum* sporozoite.

Nordwest- und Zentralschweiz (EKNZ), Basel, Switzerland (15/104). The protocol was approved by the Tanzania Food and Drug Authority (TFDA) (TZ15CT013), registered at Clinical Trials.gov (NCT02613520) and conducted under a US Food and Drug Administration (FDA) investigative new drug application. All subjects provided written informed consent.

RESULTS

Twelve vaccinees, 6 NS controls, and 12 added infectivity controls underwent CHMI (Table 1).

Vaccine Efficacy (VE)

Infections by qPCR in Controls

There were 22 injections of PfSPZ Challenge for CHMI in controls in CHMIs no. 1 (6), no. 2 (6), no. 3 (10) (Table 2). All controls developed parasitemia; the median prepatent periods (time to first parasitemia) of controls were similar (CHMIs no. 1, no. 2, no. 3: 7.99, 7.90, 7.79 days). Thus, for assessing VE statistical significance all 22 infections in controls were used (median: 7.90 days).

CHMI#1

Parasitemia was detected by qPCR 7.98–8.00 days after injection of PfSPZ Challenge in controls (Figure 2A). TBSs were positive in all 6; median time to TBS positivity was 16.79 days (range 12.03–17.80 days). Three volunteers immunized with 9×10^5 PfSPZ were negative for parasitemia by TBS and qPCR 18 days after last dose of PfSPZ Vaccine, and underwent CHMI at 23 days (3.3 weeks) (Table 2, Figure 2A). None developed parasitemia. VE was 100% (Table 2) ($P = .0001$, Barnard's exact test, 2-sided).

Three volunteers immunized with 9×10^5 PfSPZ were asymptomatic but positive for Pf (N = 1) or *P. malariae* (Pm) (N = 2) by qPCR 18 days after last vaccine dose. Retrospective qPCR demonstrated Pm in the volunteers at enrollment 4 months previously (Table S2). The volunteer with Pf was qPCR negative at enrollment but positive (35.3 parasites/μL) prior to second immunization (asymptomatic and TBS negative). Four weeks previously she reported fever for 2 days, was diagnosed with gastroenteritis, and treated with ciprofloxacin and metronidazole. The Pf was genotypically distinct from the Pf in PfSPZ Vaccine (Table S3). To provide time for treatment with AS-AQ (confirmed successful by negative qPCR), CHMI for the 3 was deferred to CHMI no. 2.

CHMI no. 2

Parasitemia was detected by qPCR 7.85–10.69 days after injection of PfSPZ Challenge in controls (Figure 2B). TBSs were positive in 5 of 6 controls a median of 14.12 (range 13.67–17.69) days after injection of PfSPZ Challenge.

Six volunteers immunized with 1.8×10^6 PfSPZ (group 1b) were confirmed negative for parasitemia by TBS and qPCR and underwent CHMI at 52 days after 3rd immunization (Table 2, Figure 2B). Four of 6 group 1b volunteers were positive by qPCR after a median of 8.43 (range 7.89–12.18) days. Three of these 4 positive vaccinees were also positive by TBS after a median of 13.92 (range 13.78–19.71) days. VE based on qPCR positivity at this day 52 CHMI (7.4 weeks) was 33% (Table 2) ($P = .028$).

CHMI no. 2 included 3 of the 9×10^5 PfSPZ volunteers who had been positive for Pf or Pm by qPCR at time of first CHMI and treated with AS-AQ. None of the 3 volunteers had detectable AQ (amodiaquine) 58 days after treatment was completed on day 8 of CHMI no. 2. This first CHMI in these 3 group 1a volunteers was 79 days after last vaccine dose (Table 2, Figure 2B). No vaccinee developed parasitemia detectable by TBS or qPCR, but 1 volunteer was excluded from VE analysis because the volunteer had positive serum levels of sulfadoxine on day 8 of CHMI. VE at this CHMI on day 79 (11.3 weeks) was 100% (Table 2) ($P = .001$).

Table 1. Volunteer Characteristics

		Group 1 (18–45 years)			
		9×10^5 (N = 6)	1.8×10^6 (N = 6)	Placebo (N = 6)	CHMI Controls (N = 12)
Age (years)	Mean (SD)	23.5 (5.7)	24.2 (5.3)	28.7 (7.9)	23.9 (4.8)
	Median	22	24	30	23
	(Min, Max)	(20,35)	(18,33)	(19,38)	(18,36)
Sex	Male	4 (66.7%)	4 (66.7%)	5 (83.3%)	7 (58.3%)
	Female	2 (33.3%)	2 (33.3%)	1 (16.7%)	5 (41.7%)
Race	African	6 (100%)	6 (100%)	6 (100%)	12 (100%)
Height (cm)	Mean (SD)	163.2 (5.0)	166.5 (10.4)	166.3 (8.0)	157.1 (9.0)
	Median	164.0	166.0	166.0	158.3
	(Min, Max)	(154,168)	(149,178)	(153,175)	(136,167)
Weight (kg)	Mean (SD)	62.3 (8.1)	65.8 (11.2)	64.3 (3.3)	58.0 (8.4)
	Median	62.5	67.0	65.0	57.5
	(Min, Max)	(53,70)	(51,80)	(60,68)	(44,75)
BMI	Mean (SD)	23.6 (4.1)	23.7 (2.9)	23.4 (2.6)	23.6 (3.8)
	Median	23.5	23.6	23.1	23.4
	(Min, Max)	(19,30)	(20,28)	(20,27)	(19,31)

Abbreviations: BMI, body mass index; CHMI, controlled human malaria infection; SD, standard deviation.

Table 2. Vaccine Efficacy

	CHMI no.1	CHMI no. 2		CHMI no. 3		
	9.0×10^5 PfSPZ (n = 3)	9.0×10^5 PfSPZ (n = 2)	1.8×10^6 PfSPZ (n = 6)	9.0×10^5 PfSPZ (n = 6)	1.8×10^6 PfSPZ (n = 6)	All Controls[a] (n = 22)
Which CHMI	First	First	First	Second	Second	Pooled
Time from last dose of vaccine to CHMI (days)	23	79	52	286	259	…
qPCR + (n)	0	0	4	6	5	22
VE by proportional analysis (*P* value based on all 22 control infections)[b]	100% (0.0001)	100% (0.001)	33% (0.028)	0	16.7% (0.119)	…
95% confidence interval for VE by proportional analysis	[38.3, 100]	[29.0, 100]	[9.3, 70.4]	…	[1.1, 58.2]	…
Prepatent period by qPCR						
median	…	…	8.43	10.89[c]	7.78	7.90
min, max	…	…	7.89, 12.18	7.79, 17.58	7.73, 27.78	7.71, 19.59
TBS+ (n)	0	0	3	5	4	17
Prepatent period by TBS	…	…				
median	…	…	13.92	18.59[c]	14.30	13.64
min, max	…	…	13.78, 19.71	14.09, 22.78	12.92, 27.77	12.03, 17.80

Volunteers were continuously monitored by qPCR until malaria treatment based on TBS positivity. The WHO International Standard for Pf DNA Nucleic Acid Amplification Techniques (NIBSC, Hertfordshire, UK) was used as standard for calculation of parasite densities. DNA was extracted from 100 µL whole blood and eluted with 50 µL Elution Buffer using Quick-gDNA Blood MicroPrep Kit (Zymo Research, Irvine, USA). All TBS negative blood samples were analyzed retrospectively by qPCR after storing at −80°C after the conclusion of CHMIs. To exclude field strain infections, parasite genotyping was performed on samples randomly chosen as described [22]. In all cases in which TBS was negative and qPCR was considered positive, 2 consecutive samples were positive by qPCR.

Abbreviations: CHMI, controlled human malaria infection; PfSPZ, *Plasmodium falciparum* sporozoite; qPCR, quantitative polymerase chain reaction; TBS, thick blood smear; VE, vaccine efficacy.

[a]11 normal saline (NS) and 11 infectivity controls.

[b]*P* value calculated by Barnard's test, 2-tailed.

[c]*P* = .021 and .03, respectively, Mann-Whitney *U* test, compared with prepatent periods of pooled controls.

CHMI no. 3

Five of the 6 NS controls who had previously undergone CHMI, and 6 new infectivity controls underwent CHMI at 259 to 286 days after last NS dose; 1 infectivity control was identified as qPCR positive for Pf at time of CHMI and excluded from analysis. By qPCR, parasitemia was detected 7.79–19.59 days for the 5 NS controls and 7.71–7.99 days for the 5 infectivity controls. By TBS, parasitemia was detected in 1/5 NS controls (13.64 days) and in 5/5 infectivity controls after a median of 13.82 (range 12.03–14.57) days. In the 2 control groups (NS and infectivity) (N = 10), parasitemia was detected by qPCR after a median of 7.79 (range 7.71–19.59) days. By TBS parasitemia was detected in 6/10 with a median prepatent period of 13.73 (range 12.03–14.57) days.

The 6 volunteers immunized with 9×10^5 PfSPZ underwent second CHMI at 286 days (40.9 weeks) after last immunization. (Table 2, Figure 2C). All developed parasitemia by qPCR

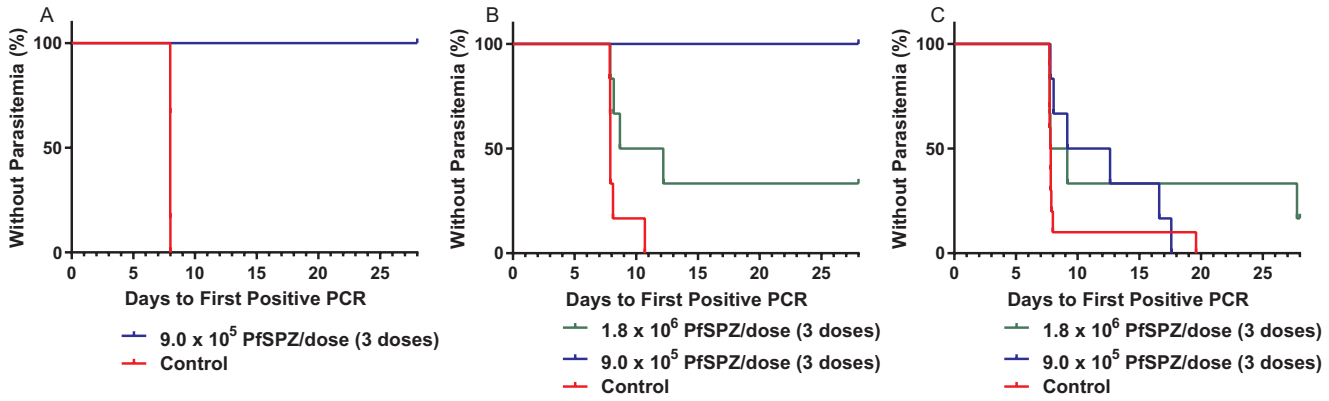

Figure 2. Kaplan-Meier survival curves in immunized volunteers vs controls as assessed by qPCR. Kaplan-Meier curves in volunteers undergoing: *A*, CHMI 23 days after the last of 3 doses with 9.0×10^5 PfSPZ (n = 3) vs NS and infectivity controls (n = 6); *B*, CHMI 79 days after the last of 3 doses with 9.0×10^5 PfSPZ (n = 2) vs CHMI 52 days after the last of 3 doses of 1.8×10^6 PfSPZ (n = 6) vs NS and infectivity controls (n = 6); *C*, volunteers undergoing a second CHMI 259 to 286 days after the last dose of 9.0×10^5 PfSPZ (n = 6) or 1.8×10^6 PfSPZ (n = 6) vs NS and infectivity controls (n = 10). Abbreviations: CHMI, controlled human malaria infection; NS, normal saline; PCR, polymerase chain reaction; PfSPZ, *Plasmodium falciparum* sporozoite.

a median of 10.89 (range 7.79–17.58) days after CHMI, and 5/6 developed parasitemia by TBS a median of 18.59 (range 14.09–22.78) days.

The 6 volunteers immunized with 1.8×10^6 PfSPZ underwent second CHMI at 259 days (37 weeks) after 3rd immunization (Table 2, Figure 2C). Five of the 6 developed parasitemia by qPCR a median of 7.78 (range 7.74–27.78) days after CHMI ($P = .119$). The 1 vaccinee who did not develop parasitemia did not develop parasitemia after first CHMI. Four of 6 developed parasitemia by TBS after a median of 14.3 (range 12.92–27.77) days.

Prepatent periods

The median prepatent period by qPCR for control volunteers in all 3 CHMIs was 7.90 days (Table 2). The median prepatent period for volunteers immunized with 1.8×10^6 PfSPZ was 8.43 days for their first CHMI (CHMI no. 2), and 7.78 days for the second CHMI (CHMI no. 3). In CHMI no. 3 the median prepatent period for the 9×10^5 PfSPZ group was significantly longer (10.89 vs 7.90 days, $P = .021$, Mann-Whitney U test) than in the NS and infectivity controls. In CHMI no. 3 the prepatent period of 10.89 days for the 9×10^5 PfSPZ group was nonsignificantly longer than the 7.78 days for the 1.8×10^6 PfSPZ group ($P = .27$).

Antimalarial Drug Levels

In the 3 group 1a subjects treated with AS-AQ for parasitemia, 58 days after treatment completion (8 days after initiation of CHMI no. 2 and 1 day after parasites would have emerged from the liver), no amodiaquine (AQ) was detectable. One volunteer from group 1a (9.0×10^5 PfSPZ) with a low, but detectable, sulfadoxine level of 781 ng/mL, did not develop parasitemia after CHMI and was excluded from the analysis (Table S4).

Safety of CHMI

There were no local or systemic solicited AEs during the 5 days postadministration of PfSPZ Challenge. One unsolicited AE, an upper respiratory infection, occurred in an infectivity control at 14 days. Symptoms or signs of malaria were recorded in 14/29 subjects with parasitemia detected by TBS and qPCR (Table S5). All 8 infections negative by TBS but positive by qPCR were asymptomatic. Symptom onset was on the same day as the positive TBS in 11 of 14 TBS positive infections with symptoms in the other 3 volunteers preceding the TBS positivity by 1, 2, and 4 days. The qPCR was positive a median of 6 days prior to symptom onset (range 3–14 days). All symptoms were 1–3 days in duration and mild to moderate in severity with headache present in all symptomatic volunteers. Four volunteers had elevated axillary temperatures (all ≤ 39.0°C). There were no unexpected or clinically significant laboratory abnormalities.

Antibodies

For the Pf circumsporozoite protein (PfCSP) enzyme-linked immunosorbent assay (ELISA) (Figure 3A, 3B, Table S6, Figure S1), the serum dilution at which the optical density was 1.0 was determined, and the net optical density (OD) 1.0 and OD 1.0 ratio, calculated, respectively, by subtracting or dividing the OD 1.0 by the prevaccination OD 1.0. At time of the first CHMI the 5 uninfected subjects in the 9×10^5 PfSPZ group had a nonsignificantly lower median net OD 1.0 (1194) than did the 2 uninfected (15 279) and 4 infected (8148) subjects in the 1.8×10^6 group ($P = .19$ in each case, Wilcoxon-Mann-Whitney test, 2-tailed). At time of second CHMI the 6 infected subjects in the 9×10^5 PfSPZ group had a nonsignificantly lower median net OD 1.0 (328) than did the 1 uninfected (4235) and 5 infected (1096) subjects in the 1.8×10^6 group ($P = .29$ and .13).

The results of the automated immunofluorescence assay (aIFA) and the automated inhibition of sporozoite invasion assay (aISI) are shown in Figure 3 and Table S6. The numbers of seroconverters as compared to ELISA were similar for the aIFA but lower for the aISI.

T Cells

There was a significant increase in PfSPZ-specific $CD4^+$ responses at the group level only after the first dose in both dosage groups (Figure 4, Table S7). There were no significant increases in PfSPZ-specific $CD8^+$ T-cell responses at any time point (data not shown). To provide further assessment of PfSPZ-specific $CD4^+$ T-cell responses, we calculated the net percent PfSPZ-specific CD4+ T cell response (% response at a specific time point minus the % response prior to immunization) and the ratio of PfSPZ-specific $CD4^+$ T-cell responses (% response at each time point divided by the % response prior to immunization). For these calculations any negative value was assigned a value 0.001 (Table S8).

The 2 highest net PfSPZ-specific $CD4^+$ T-cell responses (1.155% and 0.936%) were after the first vaccine dose in the 9.0×10^5 PfSPZ group. Both vaccinees were protected against first CHMI and had prolonged prepatent periods by qPCR after the second CHMI (12.62 and 17.58 days, respectively, as compared to median of controls of 7.99 and 7.79 days). However, the 1.8×10^6 PfSPZ group as compared to the 9.0×10^5 PfSPZ group had higher net % specific $CD4^+$ T-cell responses at 3 of the 4 time points and higher ratios of PfSPZ-specific $CD4^+$ T-cell responses at all 4 time points.

Gamma delta (γδ) T cells, especially V delta 2 (Vδ2) cells, have been associated with protection [7, 23]. There were no significant differences in Vδ2 cell frequencies between vaccine groups and no significant changes of Vδ2 frequencies within vaccine groups following each vaccination (data not shown).

DISCUSSION

Previous PfSPZ Vaccine studies indicated increasing numbers of PfSPZ per dose would increase VE (VE) [6–9]. In a study in Bagamoyo, 5 doses of 2.7×10^5 PfSPZ of PfSPZ Vaccine gave 20% VE against homologous CHMI at 3 weeks after last dose. In the present study, a higher dose, 3 doses of 9.0×10^5 PfSPZ had

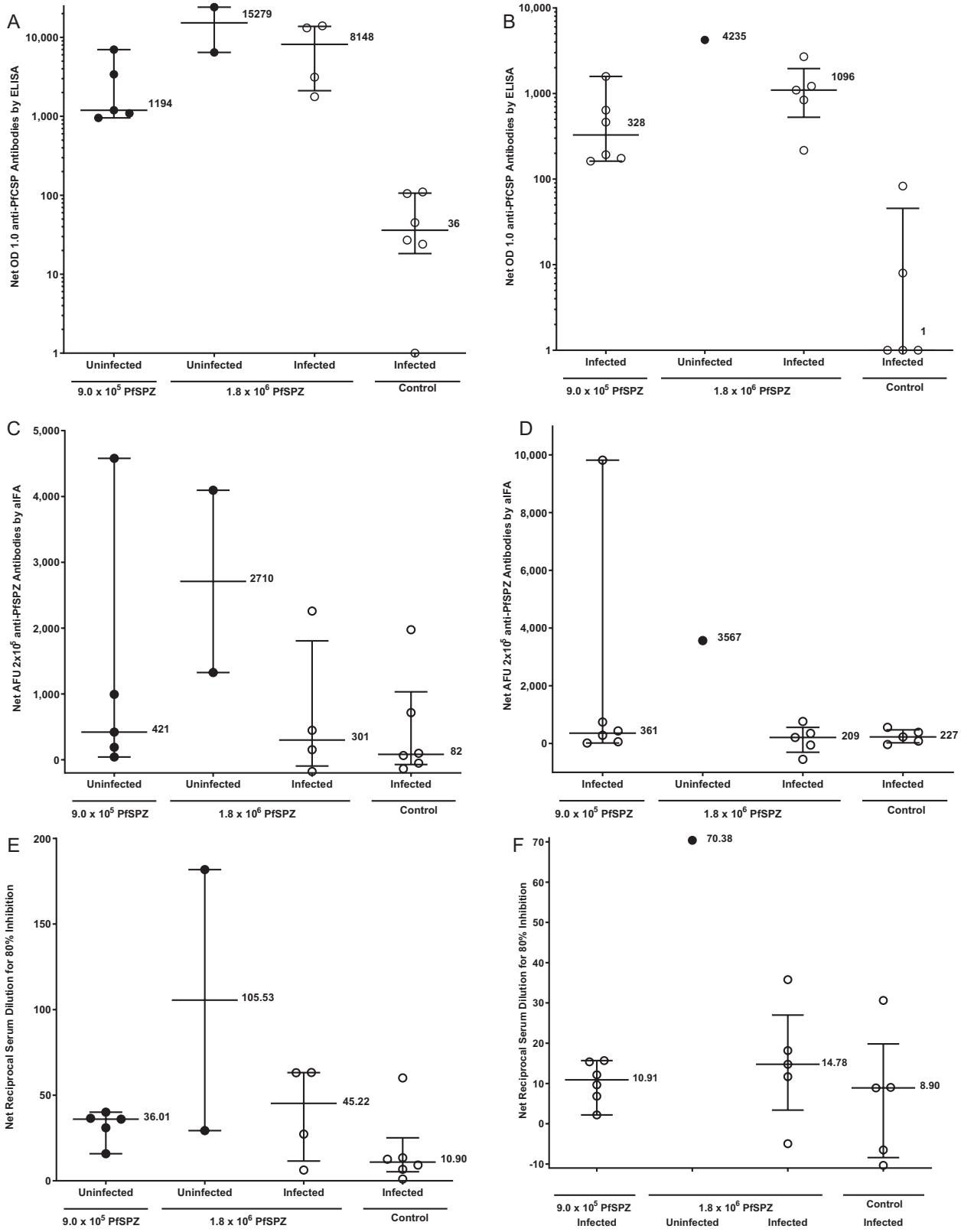

Figure 3. IgG antibodies to PfCSP by ELISA at time of first CHMI (CHMIs no. 1 and no. 2). *A*, IgG antibodies to PfSPZ by aIFA (*C*) and automated inhibition of PfSPZ invasion of hepatoma cells (aISI) (*E*) and at time of second CHMI (CHMI no.3) by ELISA (*B*), aIFA (*D*) and ISI (*F*) in subjects who received 9×10^5 PfSPZ or 1.8×10^6 PfSPZ doses of PfSPZ Vaccine. Filled circles (●) represent volunteers remaining uninfected after CHMI; open circles (○) represent volunteers infected after CHMI. For the PfCSP ELISA vaccinees were considered to have a positive antibody response if their net OD 1.0 and OD 1.0 ratio, calculated, respectively, by subtracting or dividing the OD 1.0 by the prevaccination OD 1.0, were ≥ 50 and ≥ 3.0. By these criteria, in the 9.0×10^5 PfSPZ group, 5/6, 6/6, and 1/6 were positive 2 weeks after third dose, and before their first and second CHMIs. In the 1.8×10^6 PfSPZ group 6/6, 6/6, and 5/6 were positive 2 weeks after third dose, and before their first and second CHMIs. No control volunteers were positive at any time

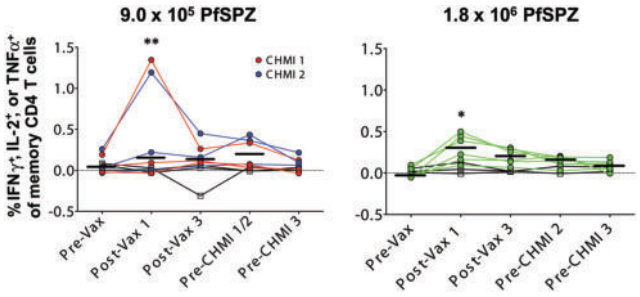

Figure 4. Pf-specific memory CD4 T-cell responses following different PfSPZ Vaccine doses. T-cell responses were assessed by flow cytometry 2 weeks after first and third doses of vaccine, and prior to the first and second CHMIs. We incubated PBMCs from vaccinees and controls with radiation attenuated (150 Gy) aseptic, purified, cryopreserved PfSPZ for ~18 hours and then assessed the percent of T cells that specifically responded to the PfSPZ. The percent of memory CD4 T cells in the blood expressing IFNγ, IL-2, or TNFα preimmunization, 2 weeks after the first and third doses of 9.0×10^5 PfSPZ Vaccine (*left*) or 1.8×10^6 PfSPZ Vaccine (*right*), and before each CHMI time point is shown. Results are the percentage of cytokine-producing cells after incubation with PfSPZ minus the percentage of cytokine-producing cells after incubation with vaccine diluent (medium with 1% human serum albumin). Colored symbols indicate vaccine groups, whereas open symbols represent placebo controls. Red symbols represent individuals challenged at the first CHMI time point, whereas blue and green symbols indicate individuals challenged at the second CHMI time point. Bars indicate median values within each group. Differences within each age group between pre- and post-vaccination groups were assessed by 2-way ANOVA with Dunnett's correction for multiple comparisons. $^*P < .05$, $^{**}P < .01$. For preimmunization T-cell responses, there were no significant differences between infected and noninfected vaccinees (Table S7). Abbreviations: ANOVA, analysis of variance; CHMI, controlled human malaria infection; IFNγ, interferon γ; IL, interleukin; PBMC, peripheral blood mononuclear cell; Pf, *Plasmodium falciparum*; PfSPZ, *Plasmodium falciparum* sporozoite; TNFα, tumor necrosis factor α.

100% VE against homologous CHMI at 3 or 11 weeks after last dose ($P = .0006$).

In this study, increasing from 9.0×10^5 to 1.8×10^6 PfSPZ was associated with reduction of VE from 100% at 23 or 79 days after last vaccine dose to 33% at 52 days after last vaccine dose ($P = .0224$). The results of the second CHMI were consistent with this observation; only the 9.0×10^5 PfSPZ group had a significant delay in prepatent period. Increasing the dose of irradiated *P. yoelii* (Py) SPZ 5-fold decreased VE in mice 4-fold [24]. A similar phenomenon has been described for postexposure immunization against murine tuberculosis [25]. The phenomenon of high-dose tolerance/suppression has been described for T cells but not B cells. Supra-optimal engagement of the T-cell receptor induces checkpoint blockade resulting in reduced proliferation, or ability to secrete tumor necrosis factor α (TNFα) or interferon γ (IFNγ) or cause apoptosis of activated cells [26–31].

In mice and nonhuman primates (NHPs) protection by immunization with radiation attenuated SPZ is eliminated by $CD8^+$ T-cell depletion [5, 32–35]. We think $CD8^+$ T cells against parasite-infected hepatocytes are responsible for the protection induced by radiation attenuated SPZ [33, 36, 37]. Changing administration of PfSPZ Vaccine from intradermal or subcutaneous (SC) [5] to DVI injection [6], was based on demonstration that 4 months after last dose of PfSPZ Vaccine by DVI to NHPs, ~3% of $CD8^+$ T cells in the livers produced IFN γ in response to stimulation with PfSPZ. In contrast, there were minimal PfSPZ-specific $CD8^+$ T cells expressing IFNγ in NHPs immunized SC [5]. Some subsequent clinical trials have shown associations between antibody and T-cell responses and protection [7, 9, 10]. We concluded that it will be difficult to identify immune response signatures that predict VE unless we can assess responses in the liver [4, 38]. This study supports this perspective. We did not identify PfSPZ-specific $CD8^+$ T-cell responses, and antibody and $CD4^+$ T-cell responses were generally higher in nonprotected vaccinees immunized with 1.8×10^6 PfSPZ than in protected vaccinees immunized with 9.0×10^5 PfSPZ.

No vaccines to prevent human parasitic diseases or composed of eukaryotic cells have received marketing authorization (licensure) by the FDA or the European Medicines Agency. We have pursued an empiric development process in which we have altered PfSPZ/dose, and dose numbers and intervals to identify an optimal immunization regimen. We are currently concentrating on 9.0×10^5 PfSPZ/dose and have found that shortening

(Figure 3. continued)
point. For preimmunization anti-PfCSP levels, there were no significant differences between infected and noninfected vaccinees (Table S6). In the aIFA, volunteers with a net arbitrary fluorescence units (AFU) 2×10^5 of ≥150 and a ratio of post to pre AFU 2×10^5 of ≥ 3.0 were considered positive (Table S5). By these criteria, in the 9.0×10^5 PfSPZ group, 5/6, 5/6, and 4/6 were positive 2 weeks after the third dose, and before their first and second CHMIs (Table S5). In the 1.8×10^6 PfSPZ group, 5/6, 5/6, and 4/6 were positive 2 weeks after the third dose, and before their first and second CHMIs (Table S5). Antibodies to PfSPZ at time of first and second CHMIs by aIFA are shown in Figure 3C and 3D Table S5. At the time of the first CHMI the 5 uninfected (protected) subjects in the 9×10^5 PfSPZ group had a lower median net AFU 2×10^5 (421) than did the 2 uninfected (2710) subjects in the 1.8×10^6 group and higher than the 4 infected (301) subjects in the 1.8×10^6 group, but the differences were not significant ($P = .38$ and .73). At the time of the second CHMI the 6 infected subjects in the 9×10^5 PfSPZ group had a lower median net AFU 2×10^5 (361) than did the one uninfected (3567) subject in the 1.8×10^6 group and higher than the 5 infected (209) subjects in the 1.8×10^6 group ($P = .57$ and .43). For the aISI, volunteers with a net ISI reciprocal serum dilution for 80% inhibition of ≥10 and ratio of post to preimmune ISI reciprocal serum dilution for 80% inhibition of ≥ 3.0 were considered positive. By these criteria, in the 9.0×10^5 PfSPZ group, 0/6, 2/6, and 2/6 were positive 2 weeks after the third dose and before their first and second CHMIs (Table S5). In the 1.8×10^6 PfSPZ group, 2/6, 4/6, and 3/6 were positive 2 weeks after the third dose, and before their first and second CHMIs (Table S5). Antibodies to PfSPZ at time of first and second CHMIs by ISI are shown in Figure 3E and 3F and Table S5. At time of the first CHMI the 5 uninfected (protected) subjects in the 9×10^5 PfSPZ group had a nonsignificant lower median net reciprocal serum dilution for 80% inhibition (22.57) than did the 2 uninfected (95.19) and 4 infected (34.12) subjects in the 1.8×10^6 group, but the differences did not reach the level of statistical significance ($P = .095$ and .90). At the time of the second CHMI the 6 infected subjects in the 9×10^5 PfSPZ group had a nonsignificant lower median net reciprocal serum dilution for 80% inhibition (10.91) than did the one uninfected (70.38) subject in the 1.8×10^6 group and the 5 infected (14.78) subjects in the 1.8×10^6 group ($P = .29$ and .54). Abbreviations: aIFA, automated immunofluorescence assay; CHMI, controlled human malaria infection; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; ISI, inhibition of sporozoite invasion assay; PfCSP, Pf circumsporozoite protein; PfSPZ, *Plasmodium falciparum* sporozoite.

the interval between doses is associated with increased VE against heterologous CHMI (Mordmüller unpublished).

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. Sanaria Inc. manufactured PfSPZ Vaccine and Protein Potential LLC is affiliated with Sanaria. Sanaria was the Sponsor of the clinical trial. L. W. P. C., S. C., A. J. R., E. R. J., Y. A., N. K. C., E. S., P. F. B., B. K. L. S., T. L. R., and S. L. H. are salaried, full-time employees of Sanaria Inc., the developer and sponsor of Sanaria PfSPZ Vaccine. Thus, all authors associated with Sanaria or Protein Potential have potential conflicts of interest. D. S. reports that The Emmes Company received payments for support of this and other Sanaria-sponsored studies. S. L. H. and B. K. L. S. are named inventors on patents related to PfSPZ Vaccine. M. T. reports contribution to travel costs from Sanaria Corp, board membership from Optimus Foundation, Novartis Institute for Tropical Disease, Botnar Foundation Basel, and University Hospital Basel. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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4.6 Paper IV: Published Article

Immunogenicity and Protective Efficacy of Radiation-Attenuated and Chemo-Attenuated PfSPZ Vaccines in Equatoguinean Adults

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Immunogenicity and Protective Efficacy of Radiation-Attenuated and Chemo-Attenuated PfSPZ Vaccines in Equatoguinean Adults

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Abstract. *Plasmodium falciparum* sporozoite (PfSPZ) Vaccine (radiation-attenuated, aseptic, purified, cryopreserved PfSPZ) and PfSPZ-CVac (infectious, aseptic, purified, cryopreserved PfSPZ administered to subjects taking weekly chloroquine chemoprophylaxis) have shown vaccine efficacies (VEs) of 100% against homologous controlled human malaria infection (CHMI) in nonimmune adults. *Plasmodium falciparum* sporozoite-CVac has never been assessed against CHMI in African vaccinees. We assessed the safety, immunogenicity, and VE against homologous CHMI of three doses of 2.7×10^6 PfSPZ of PfSPZ Vaccine at 8-week intervals and three doses of 1.0×10^5 PfSPZ of PfSPZ-CVac at 4-week intervals with each arm randomized, double-blind, placebo-controlled, and conducted in parallel. There were no differences in solicited adverse events between vaccinees and normal saline controls, or between PfSPZ Vaccine and PfSPZ-CVac recipients during the 6 days after administration of investigational product. However, from days 7–13, PfSPZ-CVac recipients had significantly more AEs, probably because of Pf parasitemia. Antibody responses were 2.9 times higher in PfSPZ Vaccine recipients than PfSPZ-CVac recipients at time of CHMI. Vaccine efficacy at a median of 14 weeks after last PfSPZ-CVac dose was 55% (8 of 13, $P = 0.051$) and at a median of 15 weeks after last PfSPZ Vaccine dose was 27% (5 of 15, $P = 0.32$). The higher VE in PfSPZ-CVac recipients of 55% with a 27-fold lower dose was likely a result of later stage parasite maturation in the liver, leading to induction of cellular immunity against a greater quantity and broader array of antigens.

INTRODUCTION

Despite an international investment in malaria control of more than $4 billion annually, the numbers of deaths and clinical cases of malaria were essentially unchanged from 2015 to 2018.[1,2] Depending on the estimate,[1,3] there are 16,730–28,000 deaths from malaria every 2 weeks. The Bioko Island Malaria Elimination Program has been working to reduce the impact of malaria on Bioko Island, Equatorial Guinea, for 15 years. During that period, the prevalence of malaria in 2- to 14-year-olds and the deaths attributed to malaria have been reduced by 73% and 85%, respectively.[4] However, despite an annual investment of ~$30 per capita in malaria control efforts by this team of Equatoguineans and international experts, the prevalence of malaria in 2- to 14-year-olds has been unchanged for the past 6 years, paralleling the international situation (G. A., Garcia, personal communication). New tools are required.[5] We believe introduction of an effective malaria vaccine would be the most efficient way to decrease and eventually halt malaria transmission and eliminate the disease from Bioko Island.[6]

We have been assessing Sanaria's whole *Plasmodium falciparum* sporozoite (PfSPZ) vaccines for more than 9 years.[7–19] There are no vaccines with marketing authorization (licensure) against diseases caused by parasites in humans, and there have previously been no vaccines against human infectious diseases composed of eukaryotic cells. With little to no human experience to draw on, the optimization of vaccination regimens with PfSPZ vaccines has been empirical. Here, we report the safety, immunogenicity, and vaccine efficacies (VE) against controlled human malaria infection (CHMI) of Sanaria® PfSPZ Vaccine (radiation-attenuated PfSPZ)[7,8,10–12,14–19] and PfSPZ-CVac (infectious PfSPZ Challenge administered to subjects taking chloroquine chemoprophylaxis)[9,13] in healthy 18- to 35-year-old Equatoguinean adults.

MATERIALS AND METHODS

Study design and population. This age de-escalation, double-blind, randomized, placebo-controlled trial was conducted in Baney, Equatorial Guinea, between October 2016 and January 2018. It had two major components: an age de-escalation and age escalation component to assess safety and immunogenicity of PfSPZ Vaccine in 6 months to 17-year-olds and 36- to 65-year-olds (part A) and a safety, immunogenicity, and CHMI component to assess VE in 18- to 35-year-olds of PfSPZ Vaccine and PfSPZ-CVac (part B); part B is described in this report.

For part B, healthy male and female subjects aged 18–35 years were recruited from the Baney district and city of Malabo on Bioko Island. Fifty subjects who met inclusion and exclusion criteria (Supplemental Appendix, Tables S1 and S2) and successfully completed a test of understanding were

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consented and enrolled. The eligibility criteria are available at https://clinicaltrials.gov/show/NCT02859350. Subjects were allocated to either the PfSPZ Vaccine arm or the PfSPZ-CVac arm; within each arm, they were randomized to either vaccine or normal saline (NS). Controls (placebo subjects) in the PfSPZ-CVac arm also received chloroquine on the same schedule as did vaccinees.

Investigational products (IP). Sanaria PfSPZ Vaccine comprised radiation attenuated, aseptic, purified, vialed, cryopreserved PfSPZ.[7,8,10–12,14–20] Sanaria PfSPZ Challenge is identical to PfSPZ Vaccine, except it is not attenuated.[9,13,21–29] Normal saline was the placebo. Chloroquine phosphate (Resochín, Kern Pharma, Barcelona, Spain), administered weekly beginning 2 days before the first dose through to 12 days after the final dose, was used to chemo-attenuate PfSPZ Challenge for PfSPZ-CVac.

Randomization and intervention. Group 1a subjects were randomized to receive PfSPZ Vaccine (2.7×10^6 PfSPZ) (n = 20) or NS (n = 6) at 0, 8, and 16 weeks. This dose, which was also being assessed at the same time in Burkina Faso (NCT02663700), was chosen assuming higher doses would be associated with increased immunogenicity and protection. Group 1b, PfSPZ-CVac, subjects were randomized to receive PfSPZ Challenge (1.0×10^5 PfSPZ) (n = 19) or NS (n = 5) at 0, 4, and 8 weeks; PfSPZ Challenge and corresponding NS recipients received chloroquine. The dosing intervals for both groups were the same as in previous trials of PfSPZ Vaccine[12,16–18,30] and PfSPZ-CVac.[9,13] The study team was blinded to treatment assignment within each group. *Plasmodium falciparum* sporozoite Vaccine, PfSPZ Challenge, or NS in 0.5 mL was administered by DVI through a 25-gauge needle. Chloroquine was administered orally under direct observation 2 days before the first dose of PfSPZChallenge or NS in the PfSPZ-CVac group and weekly thereafter through 5 days after the final injection of PfSPZChallenge or NS (Figure 1, Supplemental Figure S1); the first dose was 600 mg chloroquine base, and subsequent doses were 300 mg chloroquine base.

Vaccine efficacy. Vaccine efficacy was assessed by CHMI by DVI of 3.2×10^3 PfSPZ of PfSPZ Challenge and calculated based on the first positive quantitative PCR result. Controlled human malaria infection were planned for 10–14 weeks after last immunization, although for several subjects, the CHMI was delayed (Figure 1 and Supplemental Figure S1). Subjects were observed as inpatients beginning 8 days after PfSPZ Challenge injection until diagnosed by thick blood smear (TBS) and treated, or until day 21. Thick blood smear-negative subjects continued with every other day outpatient monitoring until day 28. After initiation of treatment, TBSs were assessed until two consecutive daily TBSs were negative. A qPCR specimen was obtained at each study visit during CHMI and at the final scheduled study visit (56 days after CHMI). All qPCR samples were run retrospectively, unless to confirm a positive TBS, in which case they were run within 24 hours.

Adverse events (AE). Solicited local (Supplemental Table S3) AEs were collected for 3 days after each immunization. Solicited systemic (Supplemental Table S3) and unsolicited AEs were collected for 7 and 28 days, respectively, after each immunization in Group 1a. In Group 1b to account for AEs that might be related to chloroquine administration or the transient parasitemia associated with PfSPZ-CVac, solicited AEs were collected from the first day of chloroquine administration (2 days before the first immunization) through 12 days after final immunization and 7 days after final chloroquine dose (Supplemental Table S4). Solicited AEs after injection of PfSPZ Challenge for CHMI were recorded for 5 days. Subjects were observed for 2 hours after administration of PfSPZ Vaccine or PfSPZ Challenge, then followed with daily home or

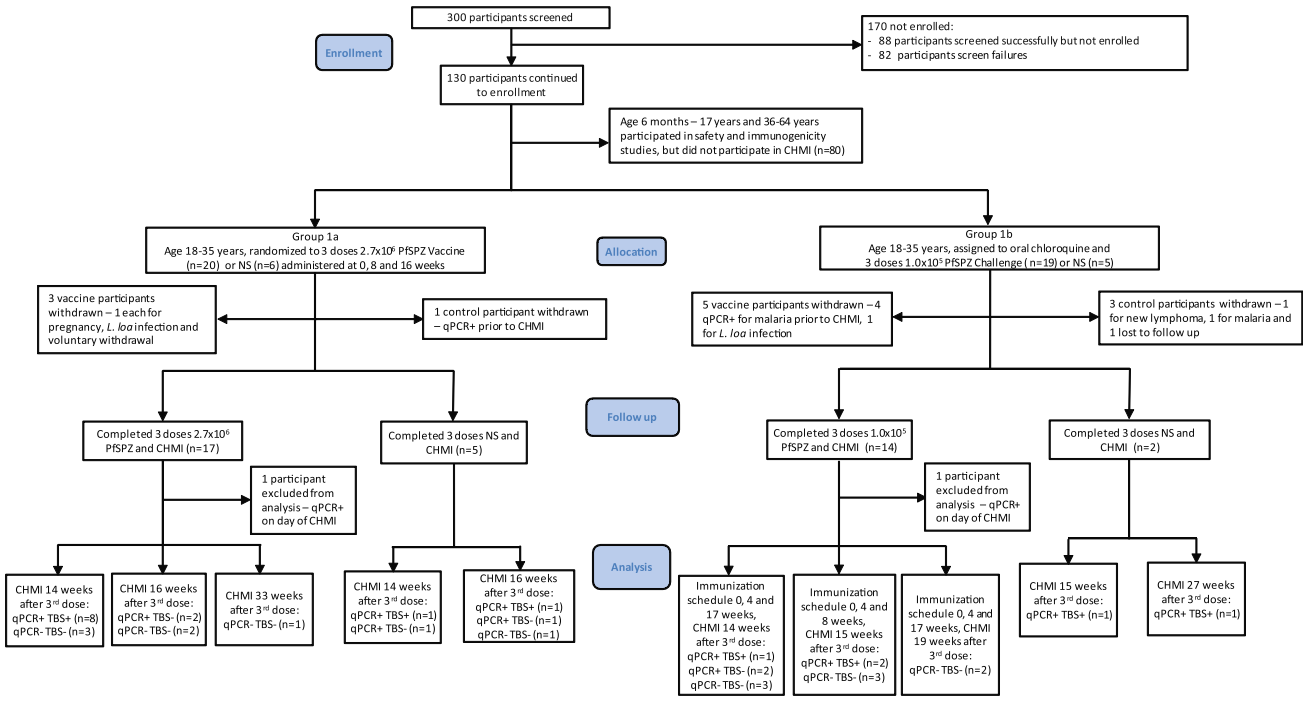


FIGURE 1. CONSORT diagram: Adult subjects aged 18–35 years. This figure appears in color at www.ajtmh.org.

clinic visits. Any subject who reported AEs at home was referred to the clinic. Grading of severity of AEs and relatedness to IP were carried out according to a prespecified system (Supplemental Table S3). Subjects were admitted to a hotel 8 days after PfSPZ Challenge administration for CHMI to be observed and treated for Pf malaria as needed. Symptoms and signs identified through prespecified (Supplemental Table S3) and open-ended questioning during the 8- to 28-day period were assessed for relationship to Pf infection and considered related if the event was within 3 days before and 7 days after the TBS was first positive.

Treatment. *Plasmodium* sp. infections diagnosed in subjects before CHMI were treated according to national guidelines with artesunate–amodiaquine or artemether–lumefantrine. Subjects with positive TBSs in the 28-day interval following CHMI were treated with AL within 24 hours of first positive TBS confirmed by qPCR. Subjects who were TBS negative were treated at the final study visit on day 56 after CHMI, regardless of qPCR findings.

Detection of Pf parasites and parasite DNA. After each immunizing dose in the PfSPZ-CVac arm of the study, parasitemia was monitored daily on days 6–10 by TBS and qPCR. During CHMI, samples were assessed by TBS and qPCR twice daily on days 8–14 after injection of PfSPZ Challenge, daily thereafter until positive or until day 20 and on days 22, 24, 26, and 28. Thick blood smears could be performed more frequently, if subjects had symptoms or signs consistent with malaria.

Slide preparation and reading for TBSs were performed as described.[24] In brief, 10 µL of blood collected in EDTA was placed on a 10-mm by 20-mm rectangle on a glass slide, dried, and stained. For asymptomatic individuals, ~0.5 µL of blood was assessed. For symptomatic individuals, ~2.0 µL of blood was assessed. Two asexual erythrocytic stage Pf parasites had to be identified for a slide to be considered positive, yielding a lower limit of detection for a positive slide of four parasites/µL blood when ~0.5 µL of blood was assessed.

Parasites were quantified by qPCR using the PlasQ qPCR assay as described.[31] The lower limit of detection for this qPCR assay was 50 copies/mL.

A single positive time point was considered positive for infection with Pf. After the start of CHMI, the time of the first blood sample positivity by qPCR was used to determine infection status and calculation of the prepatent period. During CHMI, all samples were analyzed by qPCR in real time as they were continuously collected from the subjects. Samples collected during the immunization period were analyzed retrospectively.

We used molecular approaches to discriminate between NF54, the vaccine, and CHMI strain and naturally acquired infections. First, *Plasmodium* species differentiation by qPCR was conducted.[31] Samples positive for Pf were genotyped by assessing polymorphisms for merozoite surface protein 1/merozoite surface protein 2[32] and selected microsatellite markers.[33] In addition, two widespread markers of sulphadoxine–pyrimethamine, dihydrofolate reductase and deoxyhypusine synthase, were amplified and sequenced.[34]

Chloroquine levels. Whole blood stored at −80°C was shipped to the Clinical Pathology Department, Noguchi Memorial Institute for Medical Research, University of Ghana. Samples collected on the day of first administration of IP, which was 2 days after administration of the loading dose of chloroquine (600 mg base), were analyzed in a blinded fashion for chloroquine, using high-performance liquid chromatography. The samples were run in two lots, corresponding to the two different cohorts enrolled in the PfSPZ-CVac arm of the trial. For the second cohort, plasma samples from the same time point stored at −80°C were also analyzed by Swiss BioQuant AG, Reinach, Switzerland, using HPLC.

Antibody assays. Blood for immunogenicity testing was drawn before the first immunization, 2 weeks after final immunization, and before CHMI. Serum was separated and frozen at −80°C within 4 hours of collection. IgG antibodies to Pf circumsporozoite protein (PfCSP) and Pf merozoite surface protein 1 (PfMSP1) were assessed by ELISA as described.[17] The serum dilution at which optical density was 1.0 (OD 1.0), the difference between the post-OD 1.0 and pre-OD 1.0 (net OD 1.0), and the ratio of post-OD 1.0 to pre-OD 1.0 (OD 1.0 ratio) were calculated. An individual was considered to have seroconverted if the net OD 1.0 was ≥ 50 and the OD 1.0 ratio was ≥ 3.0.

Statistical analysis. The sample size of 20 vaccinees per dosage group with six controls was chosen to show with a power of 80% that a 40% Pf infection frequency in vaccinees was significantly different from a 99% Pf infection frequency in controls ($\alpha = 0.05$, two tailed), with allowance for loss of up to two vaccinees and one control. Categorical variables were summarized using absolute (*n*) and relative (%) frequencies. Continuous variables were summarized using mean and SD, median, and range. Comparisons of categorical variables between groups were analyzed using Barnard's two-sided exact unconditional test; for comparisons of continuous variables, the Mann–Whitney two-sided test was used. Time to event was assessed by Kaplan–Meier curves and logrank for trend. Time to event data was analyzed from CHMI injection until positive qPCR result. No corrections were made for multiple comparisons because of the early phase nature of this trial. A P value < 0.05 was considered significant.

RESULTS

Vaccine efficacy. *Normal saline controls.* Four of 11 subjects did not undergo CHMI (Figure 1). Two subjects developed malaria before CHMI, one was withdrawn with a new diagnosis of non-Hodgkin lymphoma and one was lost to follow-up. Four of the remaining seven saline controls were positive by both TBS and qPCR after CHMI, two were negative by TBS but positive by qPCR, and one was negative by both tests after CHMI (Table 1). This last individual did not receive chloroquine or any other antimalarial during the study.

PfSPZ vaccine. Three of 20 subjects did not undergo CHMI. One subject became pregnant during immunization, one did not respond to initial treatment for incidental *Loa loa* infection, and one withdrew. Seventeen subjects immunized with 2.7×10^6 PfSPZ of PfSPZ Vaccine underwent CHMI. One subject was excluded from analysis after he was retrospectively determined to be qPCR-positive for Pf on the day of CHMI. Fifteen PfSPZ Vaccine subjects underwent CHMI 14–16 (median 14) weeks after last vaccine dose. Eight were positive by both TBS and qPCR, two were negative by TBS but positive by qPCR, and five were negative by both TBS and qPCR. The 16th subject immunized with PfSPZ Vaccine underwent first CHMI 33 weeks after last immunization and was negative by both TBS and qPCR (Table 1). Vaccine efficacies at a median of 14 weeks after last dose of vaccine was 27% (6 of 16

TABLE 1
Vaccine efficacy against homologous CHMI

	# Undergoing CHMI	Median time from last vaccine dose to CHMI (range)	# Without parasitemia at 28 days by TBS	# Without parasitemia at 28 days by qPCR	VE by qPCR
PfSPZ vaccine	17*	14 weeks (14–33 weeks)	8	6	27% (P = 0.32)†
PfSPZ-CVac	14*	15 weeks (14–19 weeks)	10	8	55% (P = 0.051)†
Controls (pooled)	7	–	3	1	–

CHMI = controlled human malaria infection; VE = vaccine efficacies.
* One subject was excluded from analysis in the PfSPZ Vaccine arm and one was excluded in the PfSPZ-CVac arm after they were found to have naturally acquired Pf parasitemia by qPCR on the day of CHMI.
† P-values calculated using Barnard's test, two tailed.

vaccinees versus one of seven controls negative by qPCR, P = 0.32, Barnard's test, two tailed).

PfSPZ-CVac. Five of 19 subjects did not undergo CHMI. One subject developed *L. loa* infection during immunization, and four were positive for Pf by qPCR before CHMI. Fourteen subjects immunized with PfSPZ-CVac underwent CHMI. One subject was excluded from analysis because he was retrospectively determined to be qPCR-positive for Pf on the day of CHMI. Thirteen PfSPZ-CVac subjects underwent CHMI 14–19 (median, 15) weeks after last vaccine dose. Three were positive by both TBS and qPCR, two were negative by TBS but positive by qPCR, and eight were negative by both TBS and PCR (Table 1). Vaccine efficacies at a median of 15 weeks after last vaccine dose was 55% (8 of 13 vaccinees negative versus one of seven controls negative by qPCR, P = 0.051). By time-to-event analysis (Figure 2), there was a significant trend toward improved VE (P = 0.033, logrank test for trend) from saline controls to PfSPZ Vaccine to PfSPZ-CVac. The VEs of PfSPZ-CVac (55%) and PfSPZ Vaccine (27%) were not significantly different (P = 0.27).

Prepatent periods. The prepatent periods for qPCR-positive and TBS-positive subjects are presented in Table 2. There were no significant differences in the prepatent periods by qPCR, although the results for qPCR may be skewed because several subjects were positive on the first qPCR measurement taken on day 8 after CHMI and may have been positive earlier. By TBS, the prepatent periods were significantly longer for PfSPZ Vaccine than controls (P = 0.02), but not for PfSPZ-CVac recipients who became parasitemic.

Antibody responses. Antibodies against PfCSP were assessed in subjects from all groups 14 days after the third immunization and the day before CHMI, which was 98–231 days after last immunization. We also assessed pre-CHMI sera for antibodies to the late liver stage/asexual erythrocytic stage protein PfMSP1 by ELISA (Figure 3, Supplemental Figure S2, Table S5).

Antibodies to PfCSP. PfSPZ vaccine versus PfSPZ-CVac versus NS placebo. Antibody responses to PfCSP 2 weeks after the third dose (Figure 3A, Supplemental Figure S2A, Table S5) were significantly higher in the PfSPZ Vaccine group (median net OD 1.0 = 2,936 and median OD 1.0 ratio = 40.35) than that in the PfSPZ-CVac group (median net OD 1.0 = 258 and median OD 1.0 ratio = 2.98) (net P < 0.0001 and ratio P < 0.0001, Mann–Whitney test, two tailed). The PfSPZ-CVac group had higher antibody levels than NS controls (median net OD 1.0 = 1 and median OD 1.0 ratio = 1.02) (net P < 0.0001 and ratio P = 0.0003). Antibody responses to PfCSP the day before CHMI (Figure 3B and Supplemental Figure S2B) were significantly higher in the PfSPZ Vaccine group (median net OD 1.0 = 1,407 and median OD 1.0 ratio 45.37) than in the PfSPZ-CVac group (median net OD 1.0 = 520 and median OD 1.0 ratio 4.07) (net P = 0.0093 and ratio P < 0.0001). The PfSPZ-CVac group had higher antibody levels than NS controls (median net OD 1.0 = 26 and median OD 1.0 ratio = 1.27) (net P = 0.0002 and ratio P < 0.0001).

Uninfected versus infected 2 weeks after third dose. There was no significant difference in antibody levels 14 days after the third dose between subjects who received PfSPZ Vaccine and did not become infected, versus those who became infected (median net OD 1.0 4,268 versus 2,600, P = 0.180 and

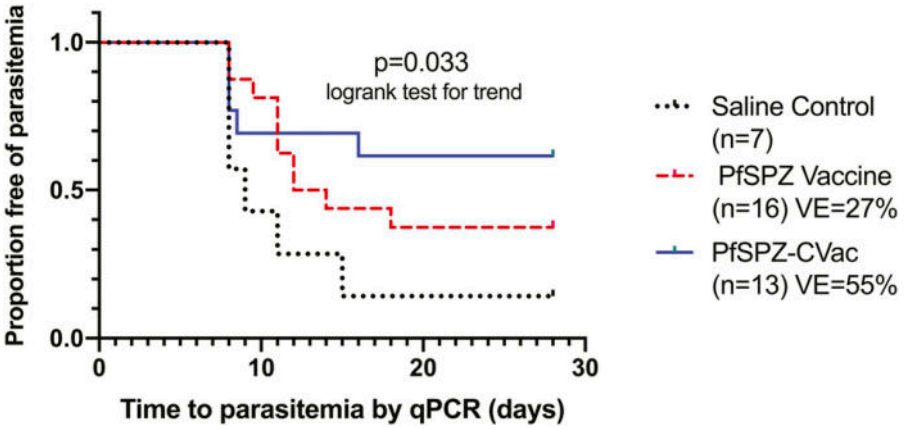


FIGURE 2. Kaplan–Meier survival curves in vaccinees and controls as assessed by qPCR. Kaplan–Meier curves in subjects undergoing controlled human malaria infection (CHMI) after the last of three doses with 2.7×10^6 PfSPZ (n = 16) or 1.0×10^5 PfSPZ Challenge (n = 13) vs. pooled saline (n = 5) and oral chloroquine plus saline controls (n = 2). The median time from the last dose to CHMI was 14 weeks for PfSPZ Vaccine and 15 weeks for PfSPZ-CVac. This figure appears in color at www.ajtmh.org.

TABLE 2
Prepatent periods by qPCR and TBS

	PfSPZ vaccine	PfSPZ-CVac	Controls
Controlled human malaria infection (*n*), evaluable	16	13	7
qPCR + (*n*)	10	5	6
Prepatent period, qPCR (days)			
Median	11.0	8.0	8.5
Minimum, maximum	8, 18	8, 16	8, 15
P-value (vs. control)	$P = 0.21$	$P = 0.84$	–
TBS+ (*n*)	8	3	4
Prepatent period, TBS (days)			
Median	17	14	14.5
Minimum, maximum	15, 19	14, 26	13, 16
P-value (vs. control)	$P = 0.02$	$P = 0.89$	–

PfSPZ = *Plasmodium falciparum* sporozoite; TBS = thick blood smear.

median OD 1.0 ratio 67.57 versus 40.35, $P = 0.591$) (Figure 3C, Supplemental Figure S2C). Likewise, there was no significant difference in antibody levels 14 days after the third dose between subjects who received PfSPZ-CVac who were not infected, versus those who became infected (median net OD 411 versus 258, $P = 0.833$ and median OD 1.0 ratio 3.76 versus 2.48, $P = 0.943$). Antibody levels were higher in subjects who received PfSPZ Vaccine and became infected, versus those who received PfSPZ-CVac and did not become infected (median net OD 2600 versus 411, $P = 0.0012$ and median OD 1.0 ratio 40.35 versus 3.76, $P = 0.0008$).

Uninfected versus infected before CHMI. There was a significant difference in net OD 1.0 antibody levels before CHMI between subjects who received PfSPZ Vaccine who were uninfected versus those who were infected (median net OD 1.0 2,936 versus 1,012, $P = 0.031$) (Figure 3D and Supplemental Figure S2D). The median OD 1.0 ratio was also higher in uninfected vaccinees but did not reach the level of statistical significance (median OD 1.0 ratio 60.28 versus 30.97, $P = 0.219$). In subjects who received PfSPZ-CVac who were uninfected or infected, the difference in the net OD 1.0 and OD 1.0 ratios was higher in the uninfected, but not significant (median net OD 618 versus 293, $P = 0.126$, and median OD 1.0 ratio 6.04 versus 2.49, $P = 0.247$).

Antibodies to PfMSP1. Uninfected versus *infected before CHMI*. In subjects who received PfSPZ Vaccine who were uninfected, the PfMSP1 median OD 1.0 measured before CHMI was higher than that of infected subjects (median OD 1.0 = 899 versus 55), but not significantly ($P = 0.515$) (Supplemental Figure S2E and Table S5). Uninfected subjects who received PfSPZ-CVac also had higher antibodies to PfMSP-1 before CHMI than the infected subjects (median OD 1.0 = 3,000 versus 423), but the difference was not significant ($P = 0.178$).

Safety. *Solicited AEs following immunization.* PfSPZ vaccine. There were no significant differences between solicited AEs in vaccinees and controls (Figure 4) collected 6 days following each immunization. Ten of 56 injections (in nine of 20 subjects receiving 2.7×10^6 PfSPZ of PfSPZ Vaccine) were associated with 17 systemic solicited AEs (five reports of fatigue and three each of arthralgias, headache, myalgias, and subjective fever) compared with one of 18 injections (one report of arthralgias) in the six subjects receiving NS ($P = 0.215$, Barnard's test two tailed). One event (headache in a vaccine recipient) was grade 2, and all others were grade 1. All events were considered related to IP (Supplemental Table S6). One subject in the PfSPZ Vaccine group experienced one local AE (tenderness) during immunization.

PfSPZ-CVac. There were no significant differences between systemic solicited AEs in vaccinees and controls collected throughout the 70-day immunization period (Figure 4). Seventeen of 55 injections (in 12 of 19 subjects receiving PfSPZ-CVac) were associated with 30 systemic solicited AEs and six local solicited AEs, all grade 2 or less; five of 14 injections (in four of five subjects receiving NS with chloroquine) experienced 10 systemic solicited AEs and no local solicited AEs ($P = 0.822$) (Supplemental Table S6). Seven systemic solicited AEs in five subjects were temporally associated with a positive qPCR for Pf, suggesting these AEs represented symptomatic parasitemia; no solicited systemic AEs were documented in the control subjects during this time period (Figure 4).

Comparison of PfSPZ vaccine and PfSPZ-CVac. Systemic solicited AEs were more frequent after each dose in the PfSPZ-CVac group (occurring in 17 of 55 immunizations) than the PfSPZ Vaccine group (occurring in 10 of 56 immunizations) but were collected over a longer period of time, and the difference was not significant ($P = 0.128$). There was no difference when the first 6 days of solicited AEs for PfSPZ-CVac (occurring in 12 of 55 immunizations) were compared with the first 6 days for PfSPZ Vaccine (occurring in 10 of 56 immunizations) ($P = 0.676$), implying that the excess AEs observed in the PfSPZ-CVac group were associated with the transient parasitemia occurring on day 7–10 or to the continuous use of chloroquine. The role for chloroquine was supported by the comparison of systemic solicited AEs in the two control arms of the study, demonstrating more frequent events in the NS + chloroquine controls (five of 14 injections) than the NS controls (one of 18 injections, $P = 0.028$). Pruritus, a frequently cited adverse effect of chloroquine in African populations, was only noted in two subjects after the first dose with PfSPZ-CVac, was mild, resolved spontaneously, and did not reoccur, despite ongoing chloroquine administration.

Laboratory abnormalities following immunization. There was no significant difference in the number of subjects experiencing laboratory abnormalities between PfSPZ Vaccine and NS control (Supplemental Table S7). All laboratory abnormalities were grade 2 or less. Likewise, there were no differences in vaccinees and controls in the PfSPZ-CVac arms, except that significantly more subjects receiving PfSPZ-CVac experienced grade 1 or 2 neutropenia than controls ($P = 0.0089$) (Supplemental Table S7), although none of the episodes were clinically significant. One subject had an unexplained, unrelated grade 3 elevation of total bilirubin 14 days after the third dose, with all prior and subsequent sample results in the normal range.

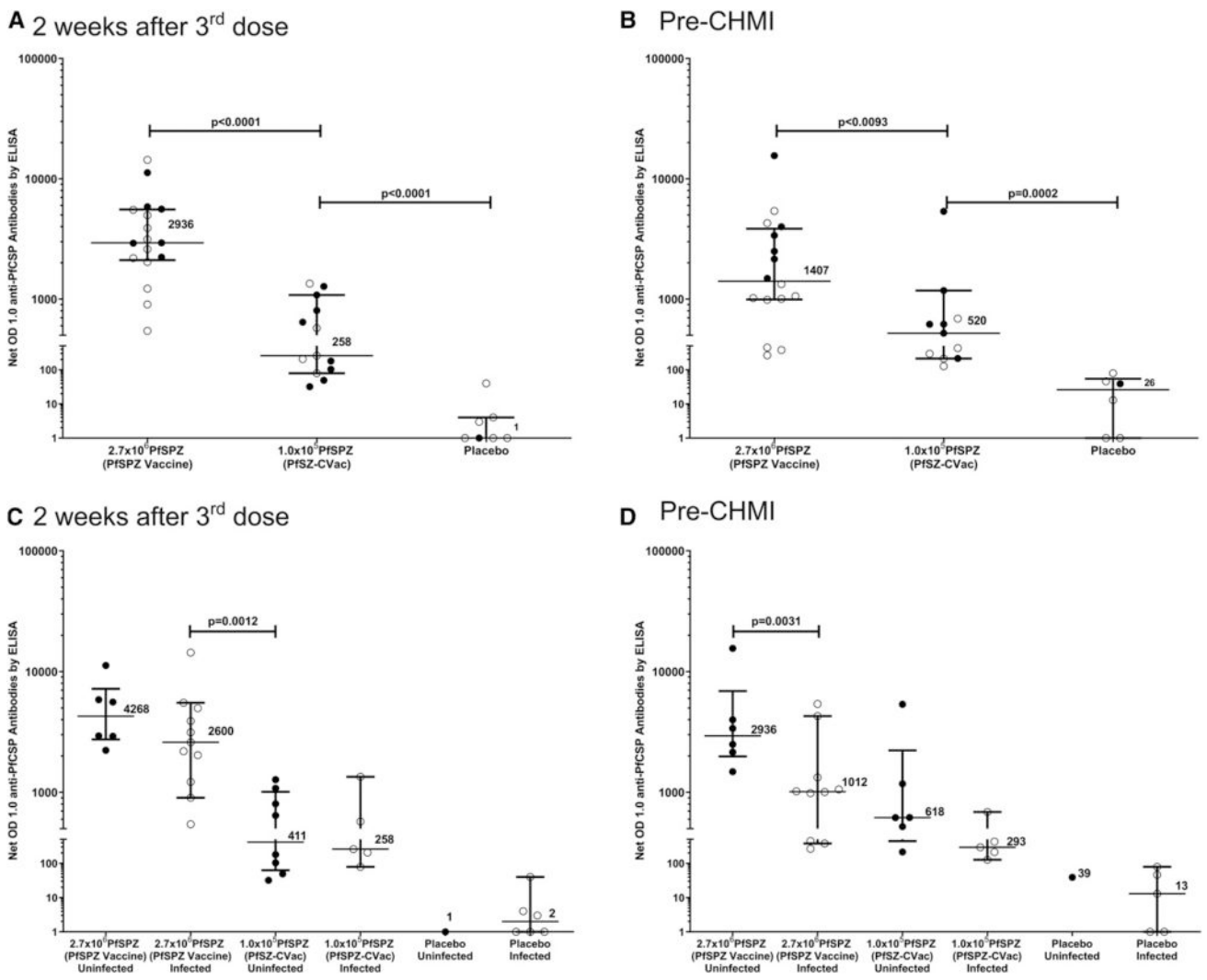


FIGURE 3. Antibodies to *Plasmodium falciparum* circumsporozoite protein (PfCSP) ELISA. IgG antibodies to Pf circumsporozoite protein PfCSP by ELISA 2 weeks after the third dose (**A**) and at the time of controlled human malaria infection (CHMI) (**B**) comparing PfSPZ Vaccine and PfSPZ-CVac. IgG antibodies to Pf circumsporozoite protein PfCSP by ELISA 2 weeks after the third dose (**C**) and at the time of CHMI (**D**) comparing infected and uninfected subjects in PfSPZ Vaccine and PfSPZ-CVac. Filled circles (•) represent subjects remaining uninfected after CHMI; open circles (x̂) represent subjects infected after CHMI. Additional figures for antibodies to PfCSP and the antibody results for MSP-1 are found in the Supplemental Appendix.In the PfSPZ Vaccine group, 18/18 (100%), and in PfSPZ-CVac group, 7/17 (41.2%) subjects seroconverted (P = 0.00012) when measured 2 weeks after the third dose. When PfCSP antibodies were measured before CHMI, 15/16 (93.8%) had positive antibody response in the PfSPZ Vaccine group and 8/13 (61.5%) in the PfSPZ-CVac group (P = 0.038) (Supplemental Table 5). Antibody responses to PfCSP 2 weeks after the third dose (**A**) were significantly higher in the PfSPZ Vaccine group (median net OD 1.0 = 2,936) than in the PfSPZ-CVac group (median net OD 1.0 = 258) (P < 0.0001, Wilcoxon signed-rank test, two tailed). The PfSPZ-CVac group had higher antibody levels than normal saline (NS) controls 2 weeks after the third dose (median net OD 1.0 = 1) (net P < 0.0001, Wilcoxon signed-rank test, two tailed). Antibody responses to PfCSP the day before CHMI (**B**) were significantly higher in the PfSPZ Vaccine group (median net OD 1.0 = 1,407) than in the PfSPZ-CVac group (median net OD 1.0 = 520) (net P = 0.0093, Mann–Whitney test, two tailed). The PfSPZ-CVac group had higher antibody levels than NS controls before CHMI (**B**, median net OD 1.0 = 26, P = 0.0002, Wilcoxon signed-rank test, two tailed). Median net OD 1.0 of PfCSP antibodies measured 2 weeks after the third dose (**C**) in the PfSPZ Vaccine group were higher in uninfected vs. that in infected subjects (median net OD 1.0 4,268 vs. 2,600, P = 0.180, Wilcoxon signed-rank test, two tailed), but the difference was not significant. Likewise, there was no significant difference in antibody levels 2 weeks after the third dose between subjects who received PfSPZ-CVac who were not infected, vs. those who became infected (median net OD 411 vs. 258, P = 0.833). There was a significant difference in net OD 1.0 anti-PfCSP antibody levels before CHMI (**D**) between subjects who received PfSPZ Vaccine who were uninfected vs. those who were infected (median net OD 1.0 2,936 vs. 1,012, P = 0.031, Wilcoxon signed-rank test, two tailed). Net OD 1.0 anti-PfCSP antibody levels before CHMI in subjects who received PfSPZ-CVac who were uninfected were higher than infected subjects (median net OD 618 vs. 293, P = 0.126), but not significantly.

Unsolicited AEs following immunization. Nine unsolicited AEs were reported in eight of the 20 subjects receiving PfSPZ Vaccine; one AE was considered probably related to vaccine (acute gastritis, grade 2). One event was considered grade 3 (toothache); all others were grade 2 or less. One AE was reported in controls. Twenty-five unsolicited AEs were reported in 12 of the 19 subjects immunized with PfSPZ-CVac, with five AEs reported in three of the five chloroquine controls; all AEs were grade 2 or less, and none were considered related to IP. The most common unsolicited AEs included toothache, upper respiratory tract infections, and musculoskeletal pain.

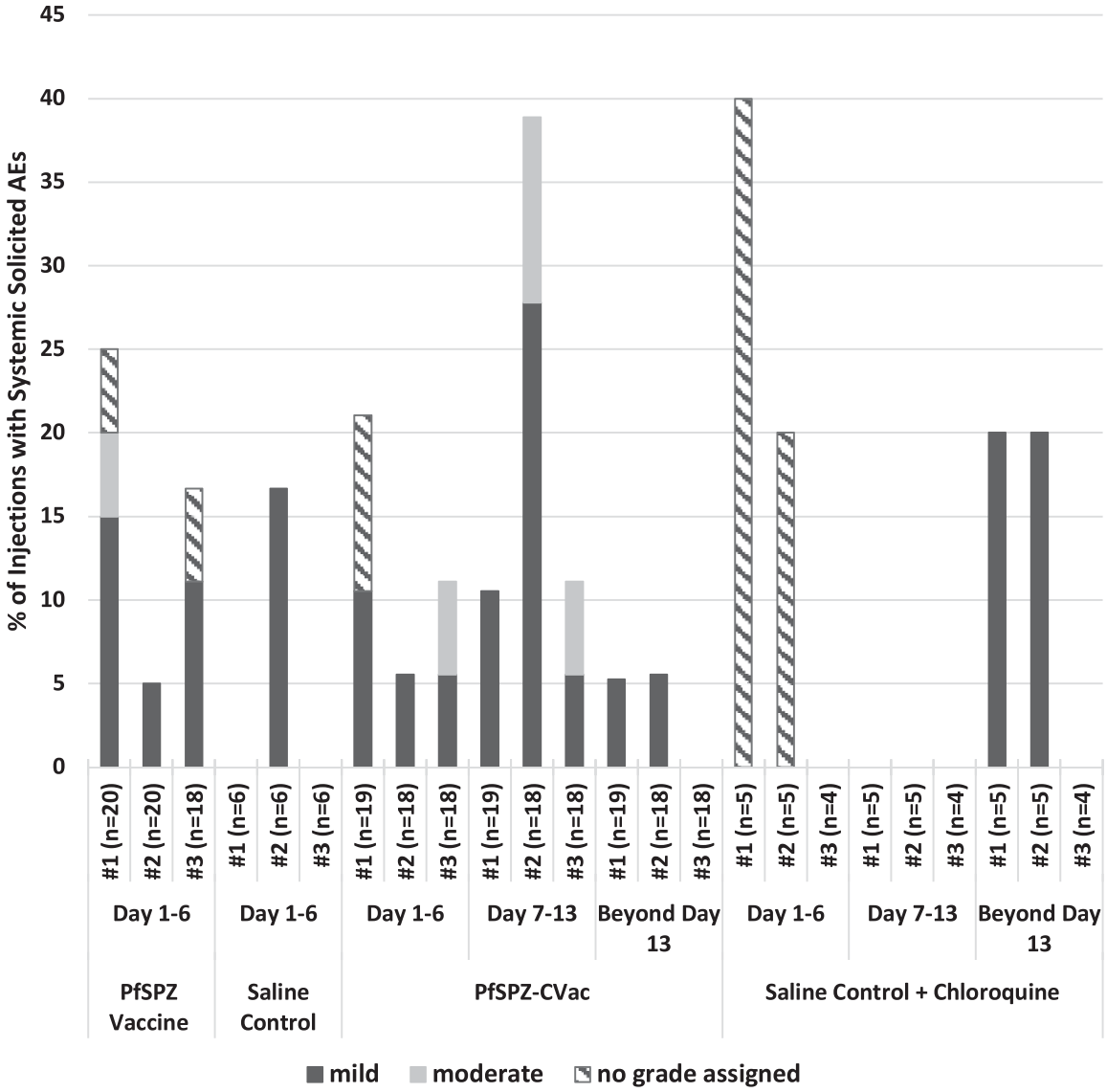


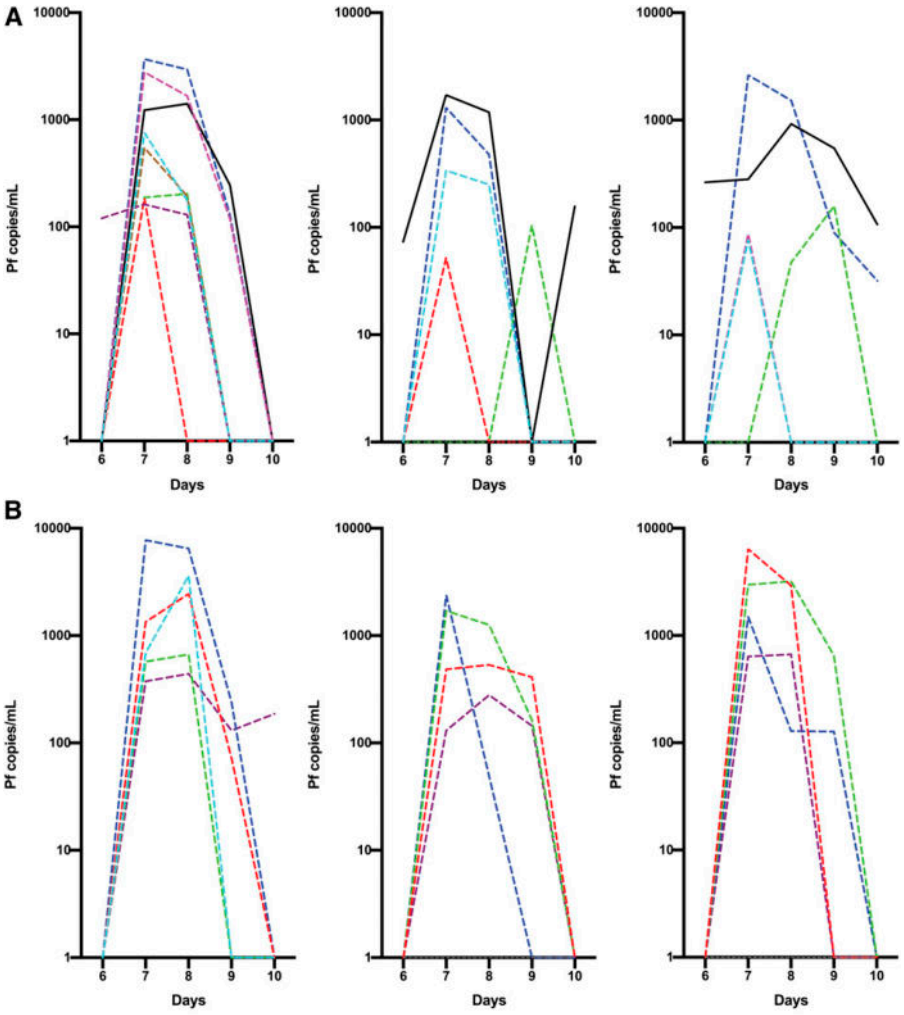
FIGURE 4. Solicited systemic adverse events as a percentage of doses administered. For subjects in the PfSPZ Vaccine arm, solicited adverse events (AEs) are reported for 6 days after each immunization. In the PfSPZ-CVac arm, solicited AEs are collected at the time of the first chloroquine administration (2 days before the first immunization) and continued for 12 days after the third immunization (70 days total). These AE are further categorized into predetermined intervals (days 1–6, 7–13, and beyond day 13) following each immunization (#1, #2, or #3) for PfSPZ-CVac. During days 1–6, reactions to vaccination were assessed, while during days 7–13 the impact of Pf parasitemia in PfSPZ-CVac recipients. The period after day 13 was to assess the additional impact of continued chloroquine administration. There was no significant difference in AEs between PfSPZ Vaccine and normal saline (NS) ($P = 0.215$), between PfSPZ-CVac and NS + chloroquine ($P = 0.822$), or between PfSPZ Vaccine and PfSPZ-CVac on days 1–6 ($P = 0.676$) or overall ($P = 0.128$). On days 7–13, more AEs occurred in the subjects receiving PfSPZ-CVac than subjects receiving NS + chloroquine ($P = 0.073$, Barnard's test, two-sided).

Serious AEs. Three serious AEs occurred in three study subjects. An 18-year-old woman was hospitalized for hyperemesis gravidarum. Symptom onset was 19 weeks after her last dose of PfSPZ Vaccine. The remainder of her pregnancy was uneventful, and she delivered a healthy girl at 37 ½ weeks.

A 19-year-old woman was found to have intrauterine fetal demise 9 weeks into her third pregnancy and 9 weeks after her first and only dose of PfSPZ Vaccine. Pregnancy loss before 20 weeks approaches 20% in sub-Saharan Africa[35]; however, the temporal relationship to immunization led the team to consider the event possibly related to vaccine.

A 29-year-old man in the chloroquine + placebo arm of the PfSPZ-CVac group developed non-Hodgkin's lymphoma. This serious adverse event was considered unrelated to immunization; the details of this case are reported elsewhere (S. Manock et al., manuscript in accepted for publication in AJTMH).

Plasmodium *parasitemia during PfSPZ-CVac immunizations and before CHMI.* Six to 10 days after each immunization, 17/18 subjects who received three doses of PfSPZ-CVac developed parasitemia by qPCR after the first dose, 13/18 after the second, and third doses (Figure 5). Median parasitemia at each time point was lower in subjects protected during CHMI (Figure 5C). There was no significant correlation between parasitemia with dose 1 and pre-immunization antibody levels to PfCSP ($r^2 = 0.10$) or PfMSP1 ($r^2 = 0.002$). There was no significant difference in peak parasitemia between subjects infected and subjects not infected after CHMI (Figure 5C).



C

Group	Immunization #1 (V1) No. pos (%)	Immunization #1 (V1) Median Pf copies/mL (range)	Immunization #1 (V1) Median days to peak parasitemia (range)	Immunization #2 (V2) No. pos (%)	Immunization #2 (V2) Median Pf copies/mL (range)	Immunization #2 (V2) Median days to peak parasitemia (range)	Immunization #3 (V3) No. pos (%)	Immunization #3 (V3) Median Pf copies/mL (range)	Immunization #3 (V3) Median days to peak parasitemia (range)
All n=18	17 (94)	713 (0-7746)	7 (7-8)	13 (72)	297 (0-2385)	7 (7-9)	13 (72)	296 (0-6393)	7 (7-9)
Protected during CHMI n=8	8 (100)	653 (162-3686)	7 (7-8)	5 (63)	78 (0-1710)	7 (7-9)	5 (63)	82 (0-2626)	7 (7-9)
Infected during CHMI n=5	5 (100)	2447 (440-7746)	8 (7-8)	4 (80)	533 (0-2385)	7.5 (7-8)	4 (80)	1495 (0-6393)	7.5 (7-8)
		p=0.28*			p=0.29*			p=0.13*	

*peak parasitemia, protected at CHMI vs. infected at CHMI, Wilcoxon rank-sum test

FIGURE 5. Parasitemia by qPCR after first, second, and third PfSPZ-CVac immunizations. (**A**) Transient parasitemia with each successive dose of PfSPZ Challenge in vaccinees protected after controlled human malaria infection (CHMI). (**B**) Transient parasitemia with each successive dose of PfSPZ Challenge in vaccinees not protected after CHMI. (**C**) Median Pf parasitemia overall and in subjects protected and not protected during CHMI. Only subjects completing all three doses and participating in CHMI ($n = 13$) represented. One subject (#525, solid black line in **A**) was persistently qPCR positive on day 10 after the second and third immunizations and subsequently found to have naturally acquired Pf infection. Two additional subjects positive 10 days after the third (**A**) or first (**B**) dose were qPCR negative at the next measurement 18 days later. This figure appears in color at www.ajtmh.org.

During the immunization phase, six subjects had asymptomatic parasitemia outside the 6- to 10-day window during scheduled PCR sampling in two subjects each receiving PfSPZ Vaccine, PfSPZ-CVac, and NS control (Supplemental Table S8). Genotype analysis excluded the PfNF54 strain identified as the cause of parasitemia in three subjects and suggested this was not the etiology in two additional subjects (Supplemental Table S9). Four additional subjects in the PfSPZ-CVac arm (three receiving PfSPZ Challenge and one NS) had asymptomatic Pf parasitemia in the interval between immunization and CHMI. A fifth subject in this group had symptomatic Pf parasitemia with an oral temperature of 38.2°C 14 weeks after third immunization.

Adverse events after CHMI. Solicited AEs were assessed for 5 days after administration of PfSPZ Challenge for CHMI. Controlled human malaria infection was well-tolerated (Supplemental Table S10). One subject in Group 1a had mild pain after injection. One subject in Group 1b had arthralgias 2 days after CHMI which were attributed to physical activity; no other local or systemic AEs were reported during the 5 days after administration of PfSPZ Challenge. Nine unsolicited AEs were reported in eight subjects during the 28 days after CHMI, none of which were deemed related to IP or malaria in blinded assessments.

Chloroquine levels during administration of PfSPZ-CVac. Whole blood chloroquine levels 2 days after the initial loading dose ranged from 15.4 to 129.9 ng/mL; corresponding plasma levels for a subset of samples were uniformly higher than the corresponding whole blood level. All levels were above IC_{50} for the NF54 strain to chloroquine (8.7 ng/mL).[36]

Clinical manifestations of malaria after CHMI. Symptoms or signs of malaria were recorded in 9/21 subjects who developed parasitemia: 4/11, 2/5, and 3/7 who received PfSPZ Vaccine, PfSPZ-CVac, and NS, respectively (Supplemental Table S10). Eight of the nine with symptoms were qPCR-positive and TBS-positive; 1/9 was only qPCR-positive. The median interval between qPCR positivity and symptom onset was 7 days (range, 5–13), and the median interval between TBS positivity and symptom onset was 1 day (range, −1 to +3). All symptoms were mild to moderate; two subjects had elevated temperature (38.2, 38.3°C).

DISCUSSION

This is the first trial to directly compare the VE of PfSPZ-CVac and PfSPZ Vaccine. At 14–15 weeks after the last dose, a three-dose PfSPZ-CVac regimen of 1.0×10^5 PfSPZ/dose had a VE of 55% (eight of 13, $P = 0.051$), whereas a three-dose PfSPZ Vaccine regimen of 2.7×10^6 PfSPZ/dose had a VE of 27% (five of 15, $P = 0.32$), both against homologous CHMI. The VE of PfSPZ-CVac versus that of PfSPZ Vaccine occurred, despite the fact that 27 times fewer PfSPZ were included in the PfSPZ-CVac regimen. Subjects who received PfSPZ Vaccine and became infected had significantly longer prepatent periods by TBS than did the controls, a finding consistent with previous studies.[18] Subjects who received PfSPZ-CVac and became infected did not have significantly longer prepatent periods than did controls. We will investigate this unexpected finding in subsequent studies.

During the week after inoculation, the chemo-attenuated PfSPZ in PfSPZ-CVac replicated up to 5×10^4 times in hepatocytes of the vaccinees and expressed ~3,000 proteins not expressed by the nonreplicating PfSPZ of PfSPZ Vaccine.[13] Three doses of 9×10^5 PfSPZ of PfSPZ Vaccine protected 72% of vaccinees against homologous CHMI at 9.5 weeks after the last dose in Germany (B. Mordmüller, personal communication) and 64% of vaccinees in the United States against homologous CHMI at ~19 weeks after the last vaccine dose,[12] and three doses of 5×10^4 PfSPZ of PfSPZ-CVac protected 100% of vaccinees in Germany against homologous CHMI at 10 weeks after the last dose.[13] Thus, the better VE with a much lower dose of PfSPZ-CVac in our trial was expected.

Data from mice and nonhuman primates indicate the VE of radiation-attenuated and chemo-attenuated PfSPZ is dependent on $CD8^+$ T cells; antibodies alone are not adequate.[7,37–39] Nonetheless, in a previous study of PfSPZ Vaccine, protected vaccinees had significantly higher levels of antibodies to PfCSP than unprotected vaccinees.[8] In this study, at the time of CHMI, uninfected PfSPZ Vaccine vaccinees had 2.9 times higher levels of antibodies to PfCSP than did infected vaccinees (2936 versus 1012, $P = 0.031$), and uninfected PfSPZ-CVac vaccinees (618) had 2.7 times higher levels of antibodies to PfCSP than did infected vaccinees (293) ($P = 0.126$). Most strikingly, infected (non-protected) PfSPZ Vaccine vaccinees had significantly higher levels of antibodies than uninfected (protected) PfSPZ-CVac vaccinees. Within a particular group (e.g., PfSPZ Vaccine or PfSPZ-CVac), there was an association between levels of antibodies to PfCSP and protection, but between groups, this was not the case. Within a group, levels of antibodies to PfCSP are a biomarker for protection and may contribute marginally to protection; on the other hand, the complete lack of association when antibodies are compared between PfSPZ Vaccine and PfSPZ-CVac is consistent with $CD8^+$ T cells being the primary mediator of protection.

Conducting this second ever clinical trial of an IP in Equatorial Guinea introduced challenges and potential limitations to the interpretation of the results. Serious AEs that led to halting of the trial disrupted the schedule for immunizations and CHMI in some of the subjects, leading to differences in the time between the second and third doses and the intervals between the third dose and CHMI. Naturally acquired, asymptomatic malaria was discovered in some of the subjects before undergoing CHMI, and this had to be treated, which led to a delay in the CHMIs or, in some cases, the subjects not undergoing CHMI. The investigative team was also challenged by several unrelated SAEs that required substantial amounts of their time (e.g., lymphoma in a young adult).

One of the seven control subjects who participated in CHMI did not develop Pf parasitemia by either TBS or qPCR. This substantially contributed to lower VE calculations in both arms of the study. Although 100% of nonimmune control subjects in the United States and Europe (73/73)[13,26,27,29,40,41] and Tanzania (34/34)[15,18] have been infected with this dose, in other settings in Africa, including Gabon (20/25),[27,42] Gambia (17/19),[28] and unpublished data from Mali (8/15) and Kenya (137/170) (submitted), this has not been the case.

The doses of PfSPZ Vaccine and PfSPZ-CVac were probably not optimal, based on the results of concurrent studies in similar populations. In studies of the VE against homologous CHMI of PfSPZ Vaccine in Tanzania, it has been shown that three immunizations of 9.0×10^5 PfSPZ administered at 8-week intervals resulted in VE of 100% against CHMI conducted 3–11 weeks after the third immunization. However, when the dose was increased to 1.8×10^6 PfSPZ administered at 8-week intervals, VE against CHMI conducted 7 weeks after the third

immunization was reduced to 33%, suggesting doses can be too high.[18] When the EGSPZV2 trial was designed, it was thought that higher doses would be better, thus the choice of 2.7×10^6 PfSPZ per dose. The resulting VE of 27% is consistent with the findings in Tanzania, increasing the dose beyond 9.0×10^5 apparently reduces VE against CHMI. Thus, in our next studies, we plan to immunize with 9.0×10^5 PfSPZ.

The dose of PfSPZ-CVac, 1.0×10^5 PfSPZ, was two times higher than the dose that achieved 100% VE in malaria-naive adults in Tubingen, Germany.[13] A regimen of three doses of 2.0×10^5 PfSPZ at 1-month intervals was tested in Mali for protection against naturally transmitted malaria and gave suboptimal results (Thera and Laurens, unpublished). The results indicated that higher doses of PfSPZ-CVac are needed in Africa because of naturally acquired immunity and an associated immune hyporesponsiveness to malaria immunogens. Immune hyporesponsiveness could also explain the limited decrease in parasitemia seen after each successive immunization in this study, unlike PfSPZ-CVac trials in malaria-naive adults, where a consistent decrease in mean parasitemia is seen in all subjects with each successive dose.[13,43] A subsequent study in Mali assessing the VE of PfSPZ-CVac against naturally transmitted malaria is using three doses of 4.0×10^5 PfSPZ (NCT03952650).

For subjects immunized with radiation-attenuated PfSPZ Vaccine, there were no significant differences in the number of solicited systemic AEs between PfSPZ Vaccine and NS recipients. Likewise, there was no significant difference between vaccinees and controls in the first 5 days after each dose of PfSPZ-CVac. However, solicited AEs were more frequent in vaccinees receiving PfSPZ-CVac than controls during days 7–13 after each immunization ($P = 0.073$), which we attribute to symptoms related to parasitemia in the 6–12 days after immunization. In addition, AEs attributed to chloroquine accounted for 1/3 of the AEs reported in vaccinees and controls in the PfSPZ-CVac group and account for the differences in the numbers of AEs between the NS + chlorquine control group and the NS control group. Despite parasitemia-associated AEs occurring exclusively in vaccinees, there was no significant difference in the number of solicited systemic AEs between those receiving PfSPZ-CVac (PfSPZ Challenge + chloroquine) and those receiving NS and chloroquine. It was noteworthy that only two of 19 individuals experienced pruritus during 9 weeks of chloroquine administration; in both cases, it was mild, resolved spontaneously, and did not reoccur, despite ongoing chloroquine dosing.

In summary, as part of an ongoing effort to optimize the dosage regimens for PfSPZ Vaccine and PfSPZ-CVac, we conducted a trial comparing a single dosage regimen of each. *Plasmodium falciparum* sporozoites-CVac provided higher VE than did PfSPZ Vaccine at a much lower dose. However, neither regimen was optimal. Higher doses of PfSPZ-CVac and lower doses of PfSPZ Vaccine will be assessed next.

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Disclosures: Sanaria Inc. manufactured PfSPZ Vaccine and Protein Potential LLC is affiliated with Sanaria. Sanaria is the sponsor of the clinical trial. L. W. P. C., N. K. C., E. R. J., T. S., Y. A., T. M., P. F. B., B. K. L. S., T. L. R., and S. L. H. are salaried, full-time employees of Sanaria Inc., the developer and sponsor of Sanaria PfSPZ Vaccine. SRM and E.S. were a salaried, full-time employee of Sanaria Inc. at the time the trial was conducted. Thus, all authors associated with Sanaria or Protein Potential have potential conflicts of interest.

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4.7 Paper V: Published Article

Safety and protective efficacy of PfSPZ Vaccine administered to HIV-negative and -positive Tanzanian adults

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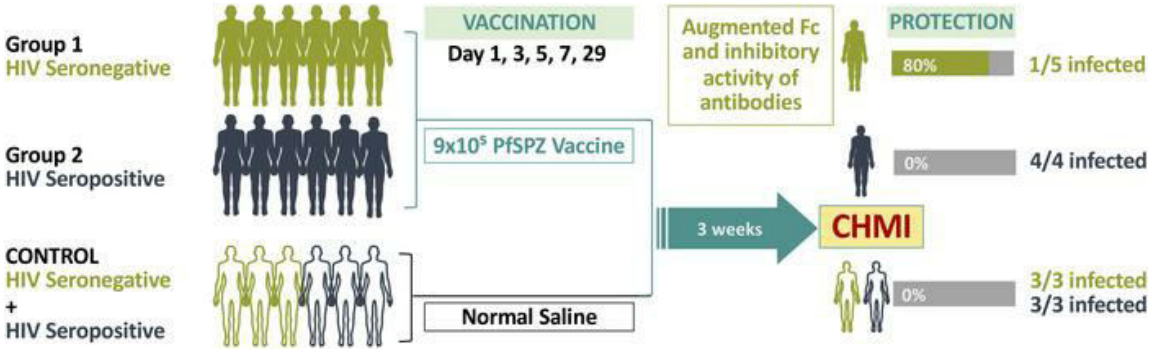
Clinical Medicine | Infectious disease | Vaccines

Graphical abstract



Safety and protective efficacy of PfSPZ VACCINE administered to HIV-negative and -positive Tanzanian adults

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BACKGROUND. Sanaria PfSPZ Vaccine, composed of attenuated *Plasmodium falciparum* (Pf) sporozoites (SPZ), protects against malaria. We conducted this clinical trial to assess the safety and efficacy of PfSPZ Vaccine in HIV-positive (HIV^+) individuals, since the HIV-infection status of participants in mass vaccination programs may be unknown.

METHODS. This randomized, double-blind, placebo-controlled trial enrolled 18- to 45-year-old HIV-negative (HIV^-) and well-controlled HIV^+ Tanzanians (HIV viral load <40 copies/mL, CD4 counts >500 cells/μL). Participants received 5 doses of PfSPZ Vaccine or normal saline (NS) over 28 days, followed by controlled human malaria infection (CHMI) 3 weeks later.

RESULTS. There were no solicited adverse events in the 9 HIV^- and 12 HIV^+ participants. After CHMI, 6 of 6 NS controls, 1 of 5 HIV^- vaccinees, and 4 of 4 HIV^+ vaccinees were Pf positive by quantitative PCR (qPCR). After immunization, anti–Pf circumsporozoite protein (anti-PfCSP) (isotype and IgG subclass) and anti-PfSPZ antibodies, anti-PfSPZ $CD4^+$ T cell responses, and $V\delta2^+$ $\gamma\delta$ $CD3^+$ T cells were nonsignificantly higher in HIV^- than in HIV^+ vaccinees. Sera from HIV^- vaccinees had significantly higher inhibition of PfSPZ invasion of hepatocytes in vitro and antibody-dependent complement deposition (ADCD) and Fcγ3B binding by anti-PfCSP and ADCD by anti–cell-traversal protein for ookinetes and SPZ (anti-PfCelTOS) antibodies.

CONCLUSIONS. PfSPZ Vaccine was safe and well tolerated in HIV^+ vaccinees, but not protective. Vaccine efficacy was 80% in HIV^- vaccinees ($P = 0.012$), whose sera had significantly higher inhibition of PfSPZ invasion of hepatocytes and enrichment of multifunctional PfCSP antibodies. A more potent PfSPZ vaccine or regimen is needed to protect those living with HIV against Pf infection in Africa.

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Introduction

Our long-term goal is the development of a malaria vaccine against *Plasmodium falciparum* (Pf) malaria that can be used in endemic areas for mass vaccination programs (MVPs) to immunize the entire population, halt transmission, and eliminate the parasite (1). To be appropriate for use in MVPs, the vaccine must be well tolerated and safe for all recipients, even those who are immunocompromised, and ideally administered over a short interval with a high level of protective efficacy.

Sanaria PfSPZ Vaccine, composed of radiation-attenuated, aseptic, purified, cryopreserved Pf sporozoites (SPZ), is a candidate for MVPs. It has been assessed in more than 2,000 research subjects 5 months to 61 years old in 22 clinical trials in the US, Europe, 6 countries in Africa, and Indonesia (2–21). PfSPZ Vaccine has been well tolerated and safe at all doses and inoculation intervals tested. A metaanalysis of 13 double-blind, placebo-controlled

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trials of PfSPZ Vaccine, 11 in Africa, showed no significant difference in adverse event (AE) profiles between vaccinees and controls who received normal saline (NS) (22). Vaccine efficacy (VE) has reached 100% against homologous (same Pf strain as the vaccine, NF54) controlled human malaria infection (CHMI) at 3 to 7 weeks after the last dose of vaccine (3, 14, 19) and 78% to 80% against heterologous (Pf7G8 strain) CHMI at 3 and 9 to 10 weeks (6, 20), with protection lasting for at least 14 months against homologous (5) and 8 months against heterologous (7) CHMI in malaria-naive participants. VE against Pf infection in field studies in African adults, who mount less robust vaccine-induced immune responses, has been demonstrated to last for at least 18 months and range from 47% to 86% (8, 21, 23). Multidose priming of malaria-naive participants, in whom 2 or 4 doses of PfSPZ Vaccine were administered in the first week, provided better VE than other immunization regimens (15, 18, 20) and allowed shortened administration regimens, improving practicality for use in MVPs.

Our primary target is fielding a vaccine for malaria elimination in Africa. An estimated 25.4 million people living with HIV reside in the WHO African region (24), where 98% of Pf infections also occur (25). Demonstrating safety in HIV-infected individuals is critical to the PfSPZ Vaccine–development program, as it would allow immunization of an entire population without screening for HIV-infection status. HIV-infected individuals, however, frequently demonstrate impaired cellular immune responses, even when $CD4^+$ T cell counts are indistinguishable from those found in those without HIV infection (26). It is unclear whether PfSPZ Vaccine, even if safe and well tolerated, would offer benefit to persons living with HIV, since cellular immunity is thought to underlie the protective immunity induced by this vaccine (2). Antibody responses, although associated with protection (8, 17, 19) and potentially contributory, have not been considered critical for durable immunity (1, 2). The current clinical trial was designed to assess the safety, tolerability, immunogenicity, and VE of a multidose prime regimen of PfSPZ Vaccine administered over 28 days in HIV-positive (HIV^+) compared with HIV-negative (HIV^-) individuals.

Results

Study participants

Study participants were scheduled to receive a 5-dose regimen of Sanaria PfSPZ Vaccine (9.0×10^5 PfSPZ of PfSPZ Vaccine per dose) or NS administered by direct venous inoculation (DVI) on days 1, 3, 5, 7, and 29. Both products were clear, colorless, odor free, administered as 0.5 mL, and indistinguishable by study staff or participants, enabling a randomized, double-blind design. Nine HIV^- participants (6 male, 3 female; median age, 25 years) were enrolled first into group 1 and randomized to receive PfSPZ Vaccine ($n = 6$) or NS ($n = 3$) (Figure 1). After vaccine safety was demonstrated, 12 HIV^+ participants were enrolled. Three participants were allocated to a reduced-dose, open-label pilot safety cohort (group 2a) receiving 4.5×10^5 PfSPZ of PfSPZ Vaccine, after which 9 participants (6 male, 3 female; median age, 40 years) were randomized to receive 9.0×10^5 PfSPZ of PfSPZ Vaccine ($n = 6$) or NS ($n = 3$) (group 2b). Further details on participant demographics, the medical history for each HIV^+ participant, and the reasons for screening failure are presented in Supplemental Tables 1–3 (supplemental material available online with this article; https://doi.org/10.1172/JCI169060DS1). HIV^- participants receiving the 9.0×10^5 PfSPZ dose were younger (median age, 25.5; range, 18–33 years) than the HIV^+ participants receiving the same dose (median age, 41.5; range, 30–43 years).

Parasitemia during vaccination

Study participants were recruited from the town of Bagamoyo in northern, coastal Tanzania. The surrounding Bagamoyo district is considered an area of moderate Pf malaria transmission, although risk is uneven throughout the district, with substantial variability observed in malaria risk indicators such as antenatal clinic prevalence rate (2.0%), annual parasite incidence (7.24%), and Pf prevalence in 5- to 16-year-old schoolchildren (73.6%) (27). Participants were screened by thick blood smear (TBS) and quantitative PCR (qPCR) for asexual blood-stage parasitemia prior to immunization, and none were positive. Immunizations were initiated without presumptive malaria treatment of the study participants to clear any latent, undetected parasitemia. Two IV^+ participants were diagnosed with Pf infection during the immunization period. One was a control participant with asymptomatic parasitemia by qPCR 2 days after the fourth dose of NS, who was treated with artemether/lumefantrine (AL) and confirmed negative by qPCR at the time of the fifth dose of NS and before CHMI (Figure 2, participant M). The second was a vaccinee who presented with asymptomatic parasitemia by TBS (257 Pf/μL) prior to the fifth dose of PfSPZ Vaccine. The participant was treated with AL, was not administered the fifth dose, and underwent CHMI on schedule 3 weeks later (Figure 2, participant N). This sample was not available for microsatellite testing (Supplemental Table 4), although a subsequent qPCR-positive sample obtained during the CHMI period did not match NF54.

Safety

AEs following immunization. Solicited local AEs at the site of injection were recorded for 2 days and solicited systemic AEs for 7 days after each immunization (Supplemental Table 5). No solicited local or systemic AEs were reported in any of the participants receiving PfSPZ Vaccine or NS. Unsolicited AEs were recorded for 2 weeks after each immunization. Three grade-1 and 2 grade-2 unsolicited AEs were reported in 5 participants receiving PfSPZ Vaccine: 4 HIV^- participants experienced abdominal pain, a superficial burn, contact dermatitis, and vaginal bleeding, respectively, the latter developing into a serious AE (SAE) (see below), and 1 HIV^+ participant experienced grade 2 diarrhea. One grade 1 unsolicited AE (lower abdominal pain) was reported in an HIV^- control (Supplemental Table 6). All unsolicited AEs were deemed unrelated to the study product.

One SAE was reported in an HIV^- participant who was hospitalized for vaginal bleeding and incomplete abortion after the self-administration of misoprostol. The participant had been withdrawn from further immunization a few days prior for a positive serum pregnancy test prior to the scheduled fifth immunization. The participant recovered without complications, and

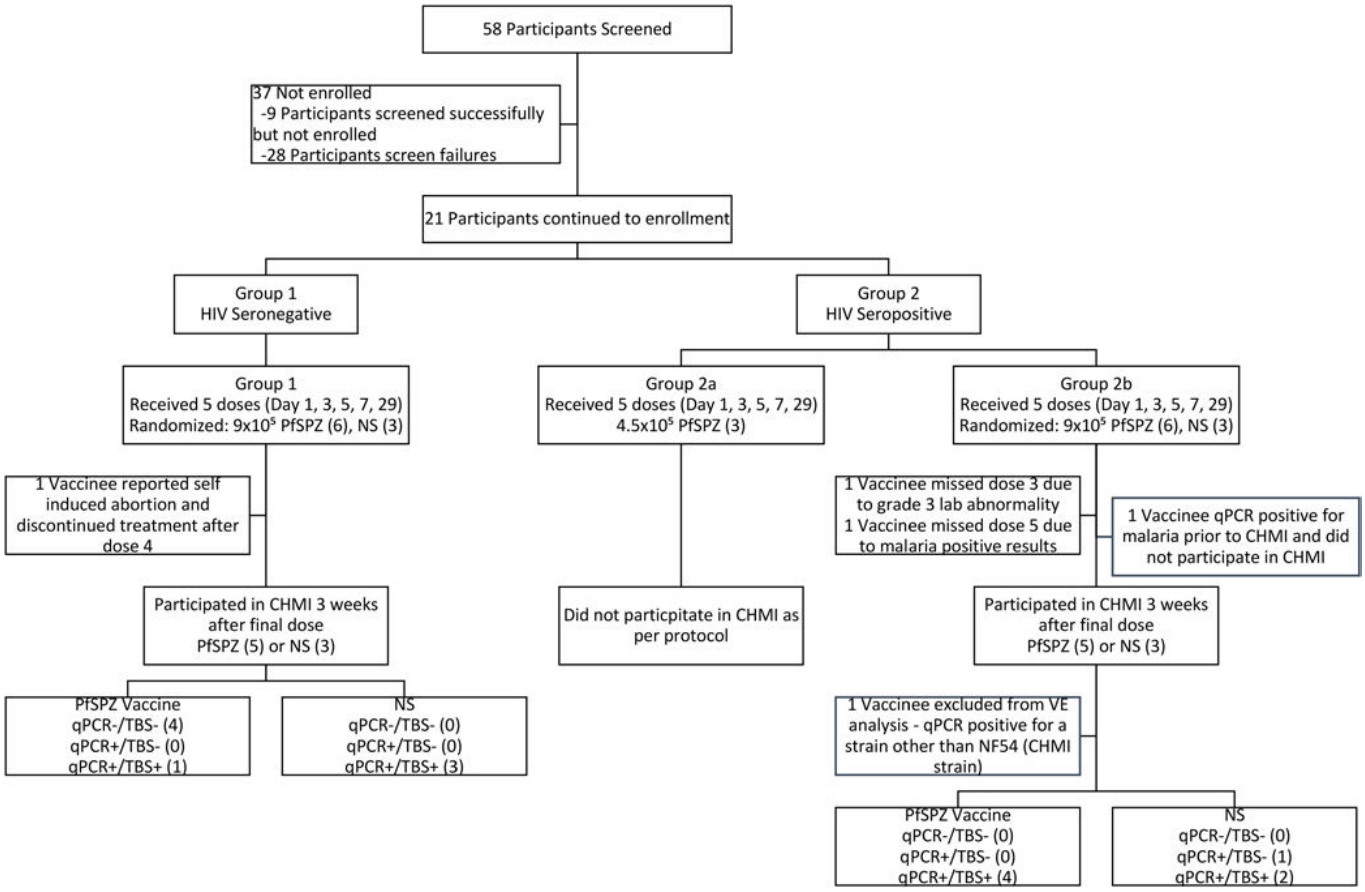

Figure 1. CONSORT flow diagram. Reasons for screen failure can be found in Supplemental Table 3.

the SAE was deemed unrelated to study participation. Based upon this history, the participant likely conceived 4 days prior to the informed consent process and screening evaluation (6 days prior to first immunization), at which time serum pregnancy test had been negative.

Laboratory abnormalities following immunization. Hematology and biochemistry (alanine transaminase [ALT], aspartate aminotransferase [AST], total bilirubin, and serum creatinine) were obtained prior to each of the 5 immunizations, 2 and 7 days after the fourth immunization and the fifth immunization. Although grade-1 and -2 leukopenia and neutropenia were observed more frequently in the HIV$^+$ vaccinees and controls, there were no significant differences in the number of participants experiencing laboratory abnormalities between HIV$^-$ and HIV$^+$ vaccinees who received the 9×10^5 PfSPZ dose (Supplemental Table 7). Grade 3 neutropenia (580 cells/μL) was observed in 1 HIV$^+$ participant 2 days after the second dose of PfSPZ Vaccine. The third dose was not administered due to this finding, but as the neutrophil count rose, the fourth dose was administered 2 days later. Two days after the fourth dose of PfSPZ Vaccine, the absolute neutrophil count was 1,030 cells/μL (lower limit of normal 1,180 cells/μL) and remained above 1,000 cells/μL for the duration of the trial.

HIV viral load remained below the lower limits of quantitation (40 copies/mL) for all participants at all time points throughout immunizations and CHMI. No changes were noted in the CD4$^+$ lymphocyte counts 3 weeks after the priming series of immunizations or 29 days after the booster among the 3 HIV$^+$ participants who served as a pilot safety group (Supplemental Figure 1A). Among the 9 HIV$^+$ participants from group 2b who received sequential doses of 9.0×10^5 PfSPZ or NS, there was no consistent pattern to suggest CD4$^+$ lymphocyte counts or percentages declined in response to immunization (Supplemental Figure 1, B and C).

AEs following administration of PfSPZ Challenge (through day +6). CHMI was performed by DVI of 3.2×10^3 PfSPZ of Sanaria PfSPZ Challenge, a product identical to PfSPZ Vaccine using the same West African–derived parasite strain (NF54), except that the parasites are not irradiated and are therefore fully infectious (4). The PfSPZ Challenge parasites develop in the liver during the first 5 to 6 days after injection, before breaking out into the blood. One HIV$^+$ participant reported solicited AEs (arthralgias, fatigue, and headache) attributed to PfSPZ Challenge 6 days after administration (Supplemental Table 8). This participant was consistently qPCR negative until 15 days after CHMI (Figure 2, participant Q).

VE

PfSPZ Challenge was administered by DVI 3 weeks after the last booster dose of vaccine (except for 1 participant who missed the pre-CHMI booster dose but received CHMI on schedule; see below). Participants were followed daily by TBS starting 7 days (day 8) after injection. If TBS was positive, qPCR was run to confirm the result prior to treatment, and if qPCR was positive,

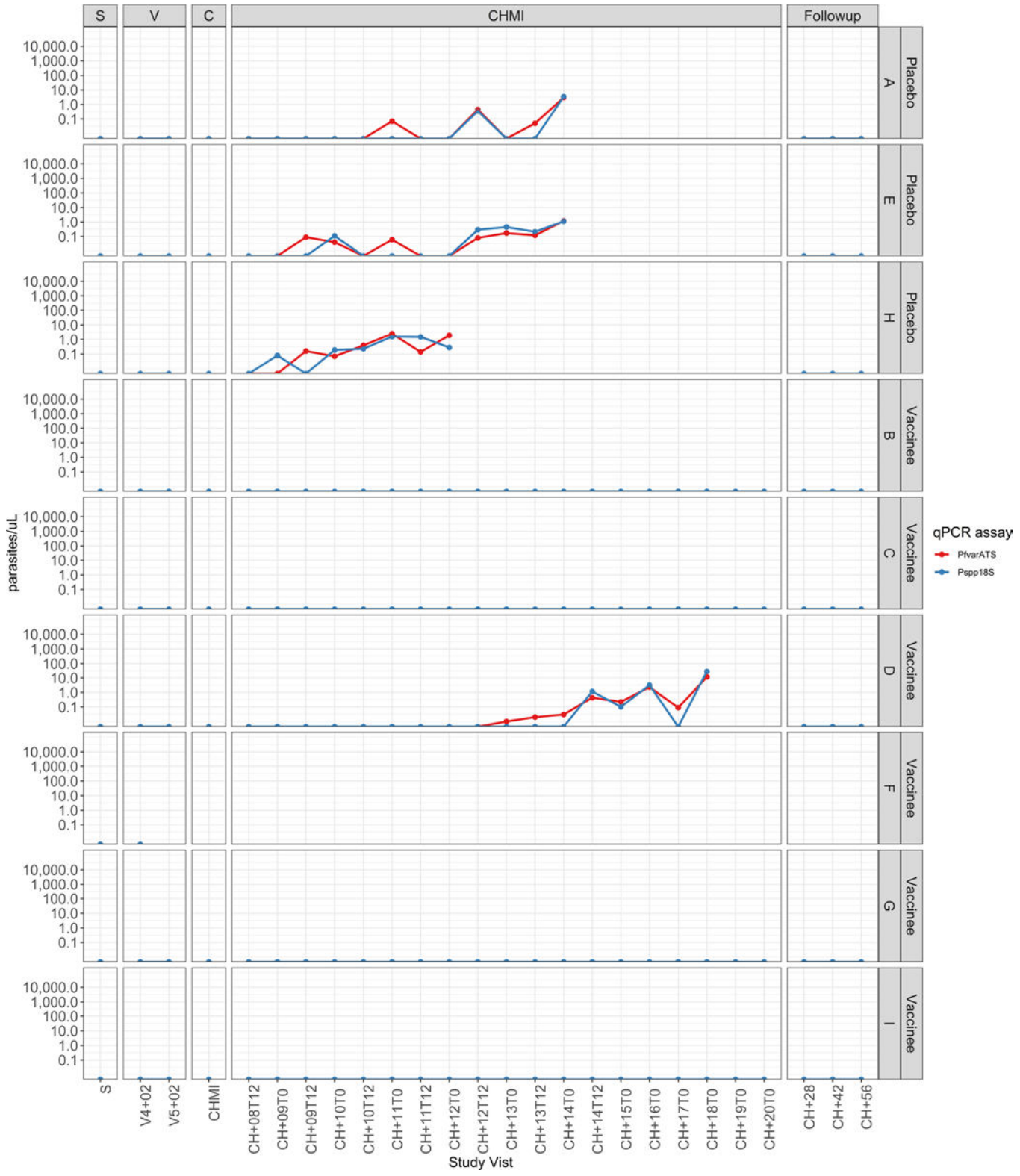

Figure 2. Detection of parasitemia by qPCR at screening, during vaccination and before, during, and after CHMI in HIV⁺ participants. Three HIV⁺ participants (J, K, and L) did not undergo CHMI and are not included in this figure. Participant S withdrew prior to CHMI. Participant N was excluded from analysis after genotyping demonstrated the parasitemia detected during the CHMI period was not NF54, the challenge strain. S, screening; V, vaccination; C, CHMI,

participants were then treated with a 3-day course of AL using directly observed treatment (DOT). VE was defined as 1-relative risk and computed by comparing the number of vaccinees developing parasitemia to the combined control groups.

NS controls. Three of 3 HIV⁻ and 3 of 3 HIV⁺ controls participated in CHMI (Figure 1). Six of 6 developed parasitemia by qPCR (Figure 2, Figure 3, and Figure 4) at a median of 10 days (9, 9, and 11 days for HIV⁻ and 8, 11, and 18 days for HIV⁺) after CHMI. Five

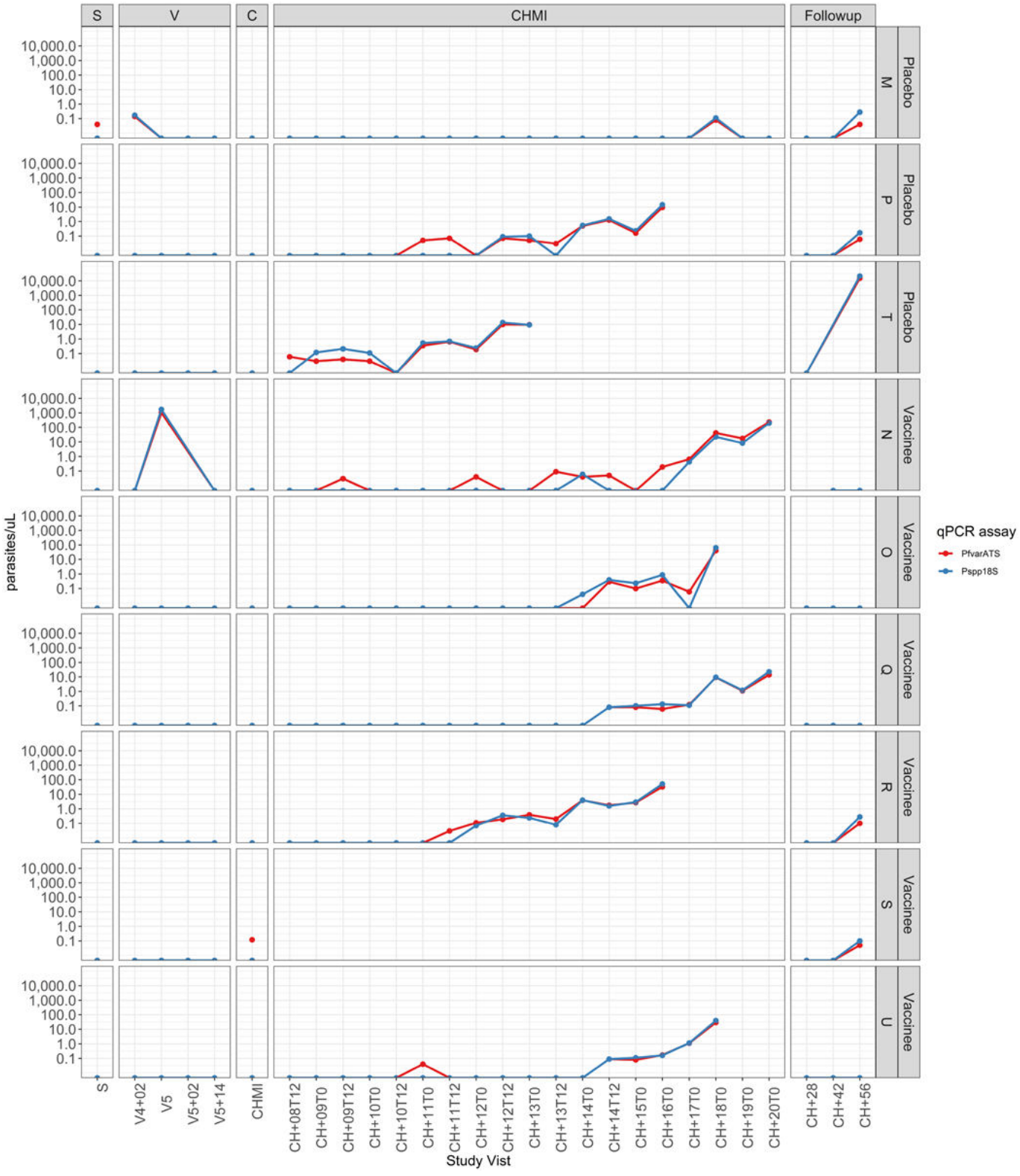

Figure 3. Detection of parasitemia by qPCR at screening, during vaccination, and before, during, and after CHMI in HIV⁻ participants. Results for the PlasQ qPCR target PfvarATS are in red; results for pan-plasmodium 18s rDNA are in blue.

of 6 were positive by TBS on days 12, 12, and 14 for HIV⁻ participants and on days 13 and 16 for the HIV⁺ participants.

Group 1: HIV⁻ PfSPZ Vaccine recipients. Five of 6 PfSPZ Vaccine recipients received 5 immunizations with 9.0×10^5 PfSPZ on days 1, 3, 5, 7, and 29 and participated in CHMI (Figure 1). Four of 5 were negative by both qPCR and TBS (Figure 3 and Figure 4). One of 5 was positive by qPCR (13 days after CHMI) and TBS (18 days after CHMI). VE 3 weeks after the last dose of vaccine was 80% (4 of 5 vaccinees versus 0 of 6 controls negative by qPCR, $P = 0.012$, Barnard's test, 2-tailed).

Group 2b: HIV⁺ PfSPZ Vaccine recipients. Four of 6 participants received 5 immunizations with 9.0×10^5 PfSPZ on days 1, 3, 5, 7, and 29 (Figure 1). One participant was qPCR positive for Pf just prior to the scheduled CHMI and did not participate in CHMI; the other 3 participants all developed parasitemia by qPCR and TBS (Figure 4). One participant received 4 immunizations with 9.0×10^5 PfSPZ on days 1, 3, 7, and 29 (the third dose was held for asymptomatic grade 3 neutropenia), participated in CHMI, and was subsequently positive by qPCR and TBS (Figure 4). One participant received 4 immunizations with 9.0×10^5 PfSPZ on days 1, 3, 5, and 7, was TBS positive (257 Pf/μL) without symptoms prior to the fifth dose, received a full course of AL, and proceeded to CHMI without the fifth dose. This participant developed parasitemia by qPCR on day 9 (Figure 2, participant N) and was TBS positive on day 20. The Pf genotype of this infection (Supplemental Table 4), however, did not match NF54, and this participant was excluded from the VE analysis. The median prepatent period for the remaining 4 participants by qPCR (Figure 4) was 12.5 (range 11, 14) days after CHMI. VE 3 weeks after the last dose of vaccine was 0% (0 of 4 vaccinees versus 0 of 6 controls negative by qPCR, $P = 1.0$, Barnard's test, 2-tailed). VE was also significantly different between HIV⁻ and HIV⁺ vaccinees (4 of 5 versus 0 of 4 negative by qPCR, $P = 0.014$, Barnard's test, 2-tailed).

Parasitemia and symptoms during and after CHMI (day +7 onwards)

Seven to 28 days after administration of PfSPZ Challenge, clinical manifestations attributable to malaria (fever, chills, headache, fatigue, malaise, myalgia, arthralgia, nausea, or vomiting) were reported in 6 of 12 participants positive by qPCR (6 of 11 positive by TBS), including 1 HIV⁻ participant (NS control) and 5 HIV⁺ participants (3 PfSPZ and 2 NS). The most frequently reported symptom was headache, occurring in all 6 participants (Supplemental Table 8). Grade 3 fever was reported in 1 HIV⁻ control, while all other events were grade 2 or less. All events resolved within 12 hours after the start of antimalarial treatment. None of the qPCR-negative participants reported symptoms.

One day prior to the last scheduled study visit (55 days after CHMI and 40 days after completion of AL administered by DOT), 1 of the HIV⁺ NS control participants presented with fever (axillary temperature 38.0°C) and was TBS positive (23,951 Pf/μL) (Figure 2, participant T). Parasite genotyping strongly suggested that this participant had PfNF54 parasitemia 12.5 days after CHMI (which was subsequently treated) and acquired new, unrelated Pf infection 55 days after CHMI (Supplemental Table 4). The other 2 HIV⁺ NS controls and 1 of the 5 HIV⁺ vaccinees, all of whom had been treated for PfNF54 parasitemia during CHMI, were determined to have Pf parasitemia by qPCR without symptoms on day 56 after CHMI (Figure 2, participants M, P, and R). One additional HIV⁺ vaccinee was qPCR positive at the same study visit (Figure 2, participant S); this participant was qPCR positive just prior to CHMI, was treated with AL, and did not undergo CHMI, but was followed to the end of the study. These 4 participants were retreated with 6 doses of AL administered by DOT, followed for 55 to 60 additional days (to day 111 or 116 after CHMI), then confirmed negative by qPCR. While it is presumed that all 4 infections were naturally acquired, each had very low estimated parasite densities by the more sensitive PlasQ qPCR (range 0.040 to 0.1 copies/μL) and it was not possible to perform parasite genotyping successfully.

Antibody responses

Antibodies were assessed in sera from participants from all groups prior to the first immunization and 2 weeks after the final immunization (Supplemental Table 9).

IgG and IgM antibodies to PfCSP by ELISA. All 10 participants (5 HIV⁻ who received all 5 doses and 5 HIV⁺, 2 of whom only received 4 doses) who received PfSPZ Vaccine followed by CHMI met our criteria for development of IgG and IgM antibodies to Pf circumsporozoite protein (PfCSP) (seroconversion) (Supplemental Table 10); none of the 6 NS control participants met these criteria. Net anti-PfCSP IgG and IgM antibodies were substantially higher for vaccinees than placebo controls for HIV⁻ and HIV⁺ participants for each of the assays. IgG antibodies to PfCSP trended lower in the HIV⁺ vaccinees than in the HIV⁻ vaccinees, whereas IgM antibodies trended higher in the HIV⁺ vaccinees compared with the HIV⁻ vaccinees, but the differences were not significant ($P = 0.86$ for both comparisons) (Figure 5, A and B).

IgG antibodies to PfSPZ by automated immunofluorescence assay. All (5 of 5) HIV⁻ (100%) and 4 of 5 HIV⁺ (80%) participants who received PfSPZ Vaccine, but none of the 6 NS control participants, met our criteria for development of antibodies to PfSPZ (Supplemental Table 10). Net anti-PfSPZ IgG antibodies were higher for vaccinees than for the placebo controls for HIV⁻ and HIV⁺ participants. IgG antibodies to PfSPZ trended lower in the HIV⁺ vaccinees than in the HIV⁻ vaccinees, but the differences were not significant ($P = 0.86$) (Figure 5C).

Functional activity of sera by automated inhibition of sporozoite invasion assay. All (5 of 5) HIV⁻ (100%) and 4 of 5 HIV⁺ (80%) participants who received PfSPZ Vaccine, but none of the 6 NS control participants, met our criteria for developing inhibition of sporozoite invasion (ISI) activity (Supplemental Table 10). Net serum dilutions at which there was 80% inhibition were higher for vaccinees than for controls for HIV⁻ and HIV⁺ participants. The net serum dilutions for 80% inhibition in the ISI, adjusted for multiple comparisons, was significantly higher in the HIV⁻ vaccinees (21) than in the HIV⁺ vaccinees (11) ($P = 0.047$) (Figure 5D).

IgG antibodies to PfMSP5 by ELISA. Only 1 participant met our criteria for development of antibodies to Pf merozoite protein 5 (PfMSP5). This HIV⁺ individual received 4 doses of PfSPZ Vaccine, on days 1, 3, 5, and 7. The subject did not receive the fifth dose because naturally acquired Pf parasitemia was detected on day 29 (Supplemental Table 10 and Supplemental Figure 2). Antibodies to PfMSP5 were detected on day 41.

Systems serology analysis. Since we saw a difference in the automated ISI (aISI) between HIV⁺ and HIV⁻ vaccinees, but failed to find significant differences in levels of IgG or IgM antibodies to PfCSP (Figure 5, A and B) or PfSPZ (Figure 5C), we hypothesized that non–titer-driven differences in antibody profiles might be implicated in the lack of protection of HIV⁺ subjects. To determine whether the HIV status of vaccinees altered the anti-Pf humoral immune responses and potentially explained the differences in VE, we measured antibody isotype, IgG subclass, Fc receptor–binding (FcR-binding) profiles, and complement activity using 7 Pf proteins expressed in SPZ and/or liver-stage parasites (Supplemental Figure 4). The antigens were PfCSP, sporozoite surface protein 2/thrombospondin-related anonymous protein (PfSSP2/PfTRAP), apical membrane

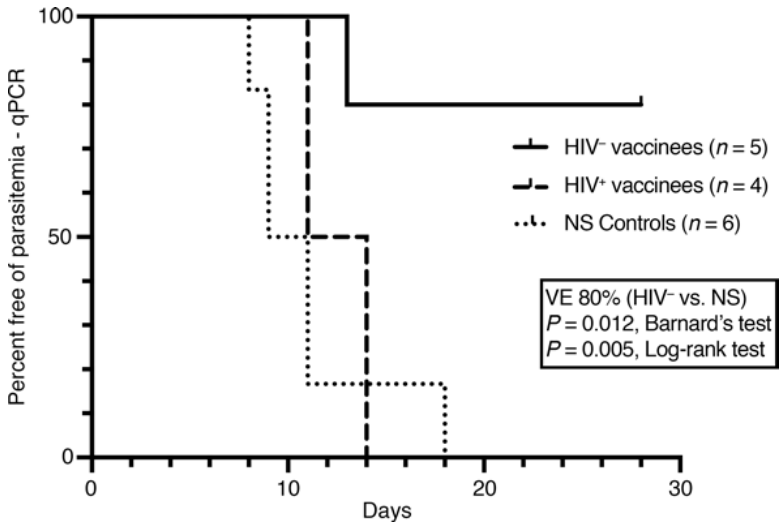

Figure 4. Time to parasitemia in vaccinees and controls participating in CHMI as assessed by qPCR. All 5 HIV⁻ vaccinees received 9.0 × 10^5 PfSPZ on days 1, 3, 5, 7, and 29. Three HIV⁺ vaccinees received 9.0 × 10^5 PfSPZ on days 1, 3, 5, 7, and 29; 1 HIV⁺ vaccinee received 9.0 × 10^5 PfSPZ on days 1, 3, 7, and 29. Three weeks after the last vaccination, participants underwent homologous CHMI.

antigen 1 (PfAMA1), exported protein 1 (PfEXP1), liver-stage antigen 1 (PfLSA1), cell-traversal protein for ookinetes and SPZ (PfCelTOS), and erythrocyte binding antigen 175 (PfEBA-175) (Supplemental Figure 3). HIV⁻ vaccinees did not develop significantly higher IgA, IgM, IgG1, IgG2, IgG3, or IgG4 antibodies to any of the malaria antigens compared with HIV⁺ vaccinees (Figure 6A), supporting the ELISA data showing that differences in the levels of antibodies between HIV⁻ and HIV⁺ vaccinees could not explain the differences in VE.

To further investigate how humoral profiles differed between the 2 groups, we performed a multivariate analysis. Using elastic-net to identify the core antibody features among the high dimensional humoral antibody profiles, our principal component analysis (PCA) separated HIV⁻ and HIV⁺ vaccinees primarily based on principal component 1 (PC1) (Figure 6B). Separation seen in PC1 was primarily driven by PfCSP and PfCelTOS functional antibody enrichment in HIV⁻ vaccinees, specifically antibody-dependent complement deposition (ADCD) activity and Fcγ3B binding of PfCSP antibodies and ADCD activity of PfCelTOS antibodies (Figure 6C). A network consisted of strongly correlated features of PfCSP functional attributes, including PfCSP Fcγ3B, Fcγ3A, and antibody-dependent neutrophil phagocytosis (ADNP), that were enriched in HIV⁻ vaccinees (Figure 6D). PfCSP Fcγ3B, Fcγ3A, and ADCD were each significantly enriched in HIV⁻ as compared with HIV⁺ vaccinees, while PfCSP ADNP trended toward enrichment (Figure 6E), demonstrating that the functional anti-PfCSP antibody response was diminished in HIV⁺ vaccinees. These findings suggest that lower functional anti-PfCSP antibody responses may have contributed to the decrease in VE found in HIV⁺ vaccinees.

On the other hand, PfCelTOS Fcγ2A, PfLSA1 IgG1, and PfSSP2/TRAP Fcγ2B features were enriched in HIV⁺ vaccinees (Figure 6C). Cocorrelation analysis revealed a network of the PfCelTOS, PfLSA1, and PfSSP2/TRAP features that were enriched in HIV⁺ vaccinees without additional close correlations observed (Figure 6D). Though PfCelTOS ADCD, PfCelTOS Fcγ2A, PfLSA1 IgG1, and PfSSP2 Fcγ2B had significant differences between HIV⁺ and HIV⁻ vaccinees, the prevaccination time points were similar or higher than the postvaccination time points (Figure 6F). This suggested that the differences observed for these features, largely enriched in HIV⁺ vaccinees, were less likely to be due to a response to vaccination. Taken together, these findings suggest that PfSPZ vaccination in HIV⁻ individuals resulted in higher functional anti-PfCSP antibody responses that potentially contributed to the greater VE in HIV⁻ vaccinees.

T cell responses to PfSPZ

T cell responses to PfSPZ were assessed by polychromatic flow cytometry on cryopreserved PBMCs acquired prior to immunization, 2 weeks after the fourth scheduled dose of PfSPZ Vaccine and 2 weeks after the fifth scheduled dose of vaccine in participants who received 9.0 × 10^5 PfSPZ of PfSPZ Vaccine per dose. The percentages of memory CD4⁺ T cells producing IFN-γ, IL-2, or TNF-α or expressing CD154 in response to PfSPZ stimulation (Figure 7 and Supplemental Figure 6) 2 weeks after the initial priming immunizations were significantly higher than before immunization (HIV⁻, P = 0.012; HIV⁺, P = 0.020; Kruskal-Wallis test). Cytokine expression 2 weeks after the final immunization in vaccinees, however, declined and was not significantly different from before immunization (HIV⁻, P = 0.54; HIV⁺, P = 0.70). There was no significant difference in the increase in median frequency of cytokine-producing memory CD4⁺ T cells between the HIV⁻ vaccinees and the HIV⁺ vaccinees (P = 0.423, Kruskal-Wallis test), although the median percentage of expression was higher in HIV⁻ (0.92) than in HIV⁺ (0.48) vaccinees. PfSPZ-specific CD8⁺ T cell responses 2 weeks after the fourth and fifth immunizations were undetectable for all but 2 vaccinees, 1 HIV⁻ and 1 HIV⁺ (Supplemental Figures 5 and 6).

Prior studies of PfSPZ Vaccine have shown an association between the levels of Vδ2⁺ gδ T cells prior to immunization and protection (5, 7, 19, 28). Although no statistically significant differences were identified, the following observations were made. In the HIV⁻ vaccinees, the median percentage of CD3⁺ T cells that were Vδ2⁺ (Figure 8A) was higher 2 weeks after the fourth

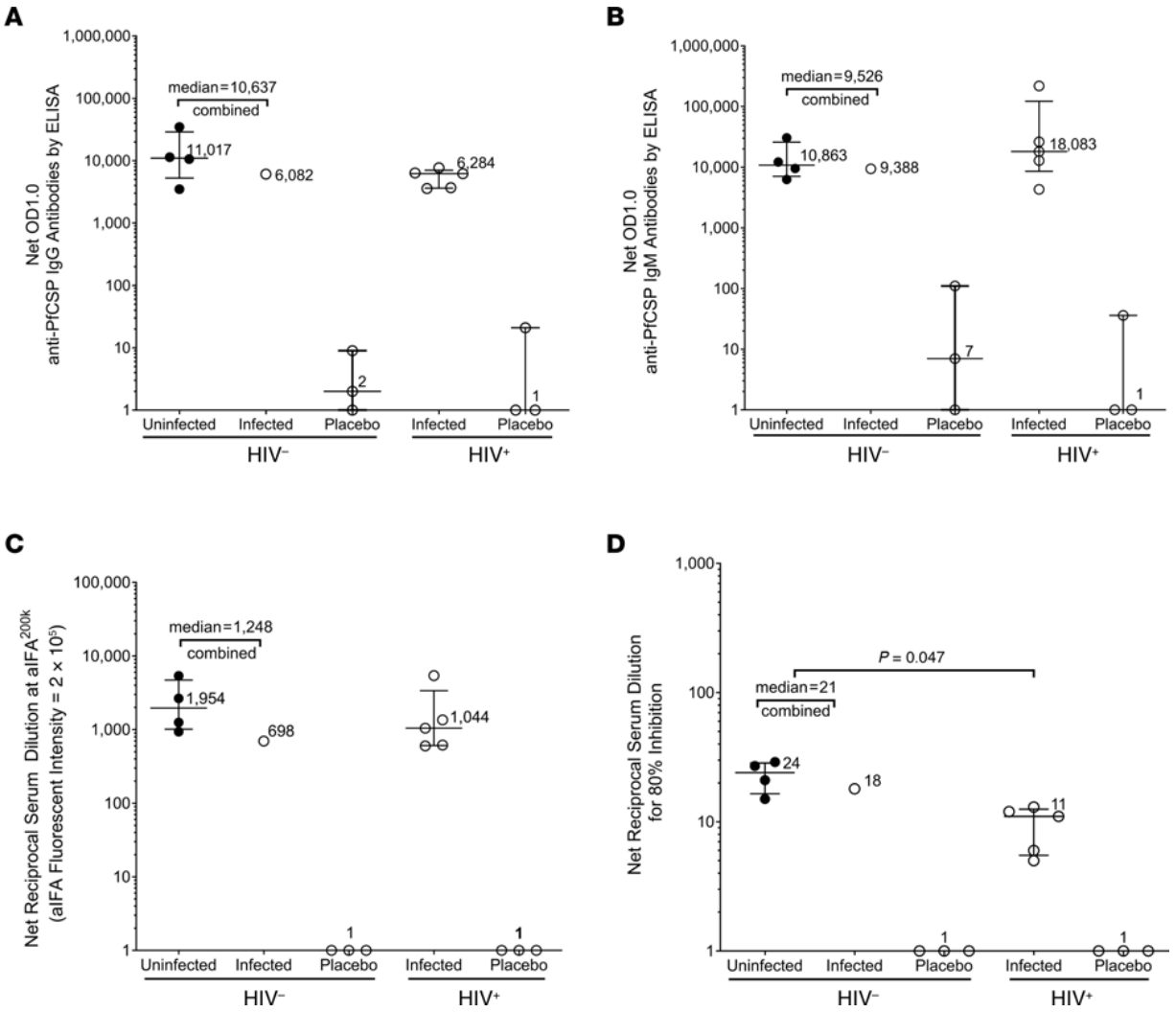

Figure 5. Antibodies to PfCSP and PfSPZ and functional activity of sera against PfSPZ. Antibodies and functional activity assessed in sera taken 2 weeks after the final dose of PfSPZ Vaccine in participants who were uninfected (protected) (black circles) and infected (white circles) during homologous CHMI with PfSPZ Challenge (NF54) administered 3 weeks after the final dose. Median and interquartile range of net OD 1.0 for IgG (**A**) and IgM (**B**) antibodies to PfCSP by ELISA. Median and interquartile range of net IgG antibodies to PfSPZ by aIFA (**C**). Net serum dilution for 80% inhibition of PfSPZ invasion of hepatocytes by aISI (**D**). There were no significant differences between HIV⁻ and HIV⁺ vaccinees for antibodies to PfCSP by ELISA or PfSPZ by aIFA. However, the serum dilution at which there was 80% inhibition of PfSPZ invasion of hepatocytes (aISI) was significantly higher in HIV⁻ vaccinees than in HIV⁺ vaccinees (P = 0.047, Wilcoxon-Mann-Whitney test adjusted for multiple comparisons).

dose compared with before immunization (4.41% versus 2.82%, P = 0.54) and higher than in the HIV⁻ controls (4.41% versus 1.35%, P = 0.25). In HIV⁺ vaccinees, the median percentage of CD3⁺ T cells that were Vδ2⁺ (Figure 8B) was higher 2 weeks after the fourth dose compared with before immunization (1.51% versus 0.75%, P = 0.70), but was not higher in vaccinees compared with the HIV⁺ controls (1.51% versus 1.90%, P = 0.88). The median percentage of CD3⁺ T cells that were Vδ2⁺ (Figure 8C) was higher in the HIV⁻ vaccinees compared with the HIV⁺ vaccinees before immunization (2.82% versus 0.75%) and after immunization (4.41% versus 1.51%, P = 0.59, Kruskal-Wallis).

Discussion

The primary objective of this clinical trial was to determine whether PfSPZ Vaccine was safe and well tolerated in persons living with HIV. No solicited AEs were reported in either HIV⁻ participants or HIV⁺ participants with full viral suppression and CD4 counts of more than 500 cells/μL after 9.0 × 10⁵ PfSPZ of PfSPZ Vaccine administered on days 1, 3, 5, 7, and 29. Unsolicited AEs were infrequent in both HIV⁺ and HIV⁻ participants, with no differences between groups. Leukopenia and neutropenia were seen more often in the HIV⁺ vaccinees and controls in comparison with the HIV⁻ groups, but did not appear to be vaccine related. Our long-term goal of using PfSPZ Vaccine in MVPs to halt transmission and eliminate malaria in geographically focused areas (1, 29, 30) without having to screen for HIV status was supported by this trial.

A concern with live attenuated vaccines is breakthrough infection by the vaccine strain, which can occur if the live infectious agent is not completely attenuated and/or if the vaccinee's immune system is required for complete attenuation and is functionally compromised. Disseminated varicella infection with vaccine strain has

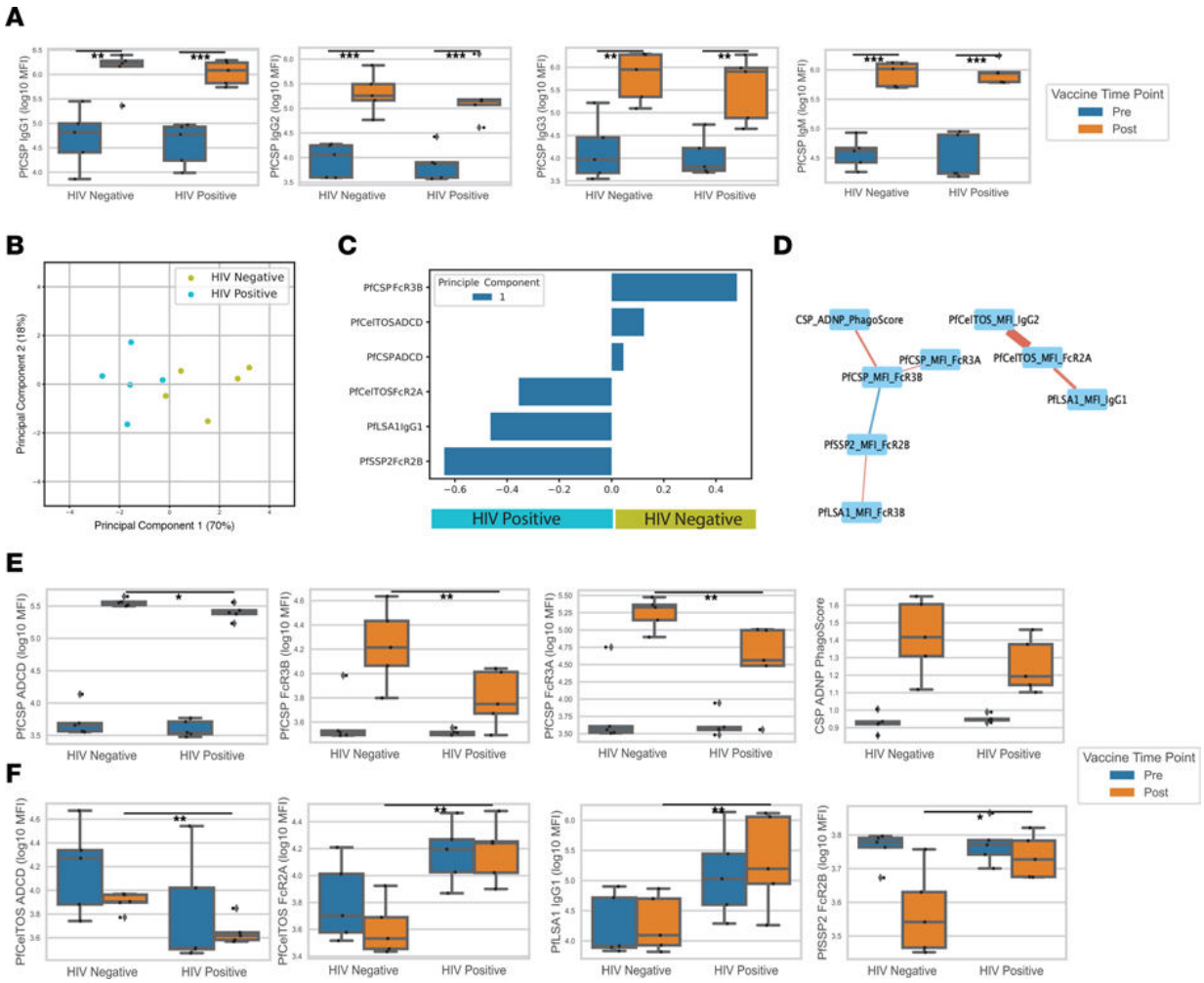

Figure 6. Systems serology analysis. (**A**) Box plots of PfCSP isotype and levels of IgG1, IgG2, IgG3, and IgM in prevaccination (blue) and postvaccination (orange) samples for HIV⁻ and HIV⁺ vaccinees. (**B**) PCA plot of postvaccination HIV⁺ (cyan) and HIV⁻ (green) participants based on the elastic-net selected features ($\alpha = 0.75$). Principle component 1 explained 70% of the variation observed. (**C**) Bar plot of the loadings for PC1. Features with a positive value were enriched in HIV⁻ individuals, and features with a negative value were enriched in HIV⁺ individuals. (**D**) Elastic-net selected features cocorrelation network. All measured features that had a Spearman's $r > 0.5$ and $P < 0.2$ with 1 of the selected features was included in this network plot. The color of an edge connotes the direction of correlation (red edge = +; blue edge = –), and the width of an edge connotes the significance of the relationship (wide edge = more significant; thin edge = less significant). (**E**) Box plots of CSP elastic-net selected and cocorrelated features from **C** and **D** of prevaccination (blue) and postvaccination (orange) samples for HIV⁻ and HIV⁺ vaccinees. (**F**) Box plots of elastic-net of non-CSP antibody features of prevaccination (blue) and postvaccination (orange) samples for HIV⁻ and HIV⁺ vaccinees. Mann-Whitney *U* method was used for statistical testing (**A**, **E**, and **F**). $^{*}P < 0.1$; $^{**}P < 0.05$; $^{***}P < 0.01$.

been reported in severely immunocompromised HIV⁺ individuals (31, 32). Bacille Calmette-Guérin (BCG) vaccination studies in HIV⁺ infants residing in Argentina and South Africa estimated disseminated infections occurred in approximately 1% (33–36) with up to 75% mortality. PfSPZ Vaccine has been administered to more than 2,000 vaccinees in previous trials with no vaccine breakthrough infections, and breakthrough infections were not expected in this trial because the attenuation of PfSPZ Vaccine is intrinsic to the parasite and not dependent on the recipient's immune system (37).

Although safe, PfSPZ Vaccine was not protective in HIV⁺ participants. VE against homologous CHMI at 3 weeks after the last dose of vaccine was high, 80% (4 of 5), in HIV⁻ participants, but all 4 HIV⁺ participants developed Pf parasitemia. Reduced VE in HIV⁺ participants has been seen after immunization in other challenge or natural infection models (38–42), and the findings of our trial are consistent. Persistent, subtle immunologic deficits in HIV⁺ individuals despite long-term virologic suppression with antiretroviral agents are the most likely explanation. HIV⁺ individuals on fully suppressive antiretroviral therapy (ART) and with normal CD4⁺ T cell counts exhibit altered function and dysregulation of CD4⁺ T cells (43), Vγ9Vd2 T cells (44), and monocytes (45), and depressed protective T cell–dependent immune responses (43, 44). Infection of CD4⁺ T follicular helper cells (T_{FH}), the major HIV reservoir in chronic asymptomatic infection, impairs B cell function in the germinal center, including maturation into plasma cells and long-lived memory B cells (46).

In this study, percentages of CD4⁺ T cells expressing IFN-γ, IL-2, TNF-α, or CD154 in response to in vitro PfSPZ stimulation were increased in both HIV⁻ and HIV⁺ vaccinees after the fourth immunization compared with the NS controls, with larger increases in HIV⁻ than HIV⁺ participants, although the differences in T cell responses between the HIV⁻ vaccinees (4 of 5 protected after

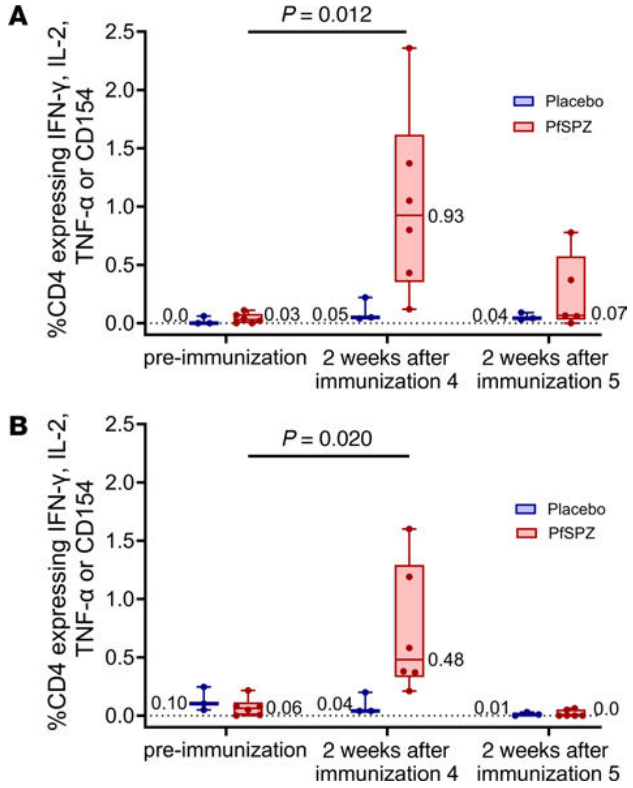

Figure 7. PfSPZ-specific memory CD4⁺ T cell responses before and after immunization. Percentages of memory CD4⁺ T cells in the blood expressing IFN-γ, IL-2, TNF-α, or CD154 at preimmunization or 2 weeks after the fourth and fifth doses of PfSPZ Vaccine (9.0×10^5) in HIV⁻ (**A**) and HIV⁺ (**B**) participants. Results show the percentages of cytokine-producing cells after incubation with PfSPZ minus the percentages of cytokine-producing cells after incubation with vaccine diluent (medium with 1% human serum albumin). Bars indicate median values within each group, and circles indicate individual participant data. The number of CD4⁺ T cells expressing IFN-γ, IL-2, TNF-α, or CD154 2 weeks after the initial series of 4 immunizations was significantly higher than before immunization ($P = 0.012$, HIV⁻; $P = 0.020$, HIV⁺, Kruskal-Wallis test), but not significantly different between HIV⁻ and HIV⁺ participants ($P = 0.59$). Cytokine expression 2 weeks after the final immunization in vaccinees was not significantly different from before immunization ($P = 0.54$, HIV⁻; $P = 0.70$, HIV⁺, Wilcoxon-Mann-Whitney test adjusted for multiple comparisons).

CHMI) and the HIV⁺ vaccinees (0 of 4 protected after CHMI) were not significant. CD8⁺ T cell responses to PfSPZ were not detected 2 weeks after the fourth or fifth immunizations. We believe that the dramatic reduction in numbers of detectable PfSPZ-specific CD4⁺ T cells in PBMCs after the fifth dose and the inability to detect PfSPZ-specific CD8⁺ T cells in PBMCS were due to trafficking of the protective T cells to the liver (2), consistent with the observation that levels of PfSPZ-specific T cells in PBMCs are highest after the first dose (or priming doses) of PfSPZ Vaccine (7), and with data from mice immunized with radiation-attenuated rodent malaria SPZ, which show that the movement of specific CD8⁺ T cells, such as effector memory cells, to the liver occurs rapidly after challenge (47).

A role for Vγ9Vδ2 T cells in the induction of PfSPZ VE has been previously described (5, 7, 19, 28, 48). Early in HIV infection, peripheral blood Vδ1 T cell frequency is increased and Vδ2 T cell frequency is decreased, with a reversal of the Vδ1/Vδ2 ratio (which normally favors Vδ2) (44, 49), and the remaining Vδ2 T cells exhibit decreased antigen-induced expression of IFN-γ and TNF-α and decreased proliferative/cytotoxic capacity. These changes are incompletely reversed despite years of fully suppressive ART (44, 49). Although we did not find statistically significant differences, our results are consistent in that the percentages of CD3⁺ γδ T cells that were Vδ2⁺ were higher in the HIV⁻ vaccinees than HIV⁺ vaccinees before immunization and 2 weeks after the fourth immunization and that percentages increased after immunization in the HIV⁻ vaccinees while percentages in the HIV⁺ vaccinees remained indistinguishable from those of HIV⁺ controls after the fourth immunization. The T cell data generated from this trial suggest that Vγ9Vδ2 T cell function is an important component of the protective immune response. We are further exploring this using RNA-Seq, cellular indexing of transcriptomes and epitopes (CITE-Seq), and plasma proteomics.

Seroconversion to protective antibody responses and peak antibody responses with most licensed vaccines is depressed and not as long lived as in healthy HIV⁻ children and adults (50). Total (51) and neutralizing (51) antibody responses to PfCSP are similarly reduced in HIV⁺ children after receiving the RTS,S/AS01 vaccine. All vaccinees in this study had IgG and IgM antibody responses to PfCSP by ELISA (Figure 5, A and B) and IgG antibodies to PfSPZ by automated immunofluorescence assay (aIFA) (Figure 5C), which were nonsignificantly higher in HIV⁻ as compared with HIV⁺ vaccinees. However, the median serum dilution at which there was 80% inhibition in the functional aISI was significantly higher in the HIV⁻ vaccinees (21) as compared with the HIV⁺ vaccinees (11) (Figure 5D). It is possible that this functional antibody activity played a role in the VE in the HIV⁻ participants and the lack of VE in the HIV⁺ participants.

By using a systems immunology approach to characterize the binding and functional antibody responses to a panel of relevant Pf antigens, we found additional evidence that HIV⁺ individuals had weakened functional antibody responses. A striking functional antibody difference between HIV⁺ and HIV⁻ vaccinees was PfCSP antibody binding to FcR3B, an FcR highly expressed on the surface of neutrophils (52), and in vitro PfCSP ADNP activity. It has been suggested that ADNP activity is important for antisporozoite immunity (53), and the results of these 2 assays point to a defect in anti-PfCSP ADNP responses in HIV⁺ individuals. Further, our findings also point to decreased complement activation of anti-PfCSP antibodies in HIV⁺ vaccinees. The importance of complement-fixing antibodies in antisporozoite immunity has been demonstrated in RTS,S vaccinees (54) and in immunity to natural infection (55). Taken together, our findings suggest that deficits in functional anti-PfCSP humoral immunity distinguish HIV⁺ from HIV⁻ individuals. HIV⁺ individuals have a less responsive B cell compartment with compromised B cell memory and increased markers of B cell exhaustion (56, 57). This results in impaired responses to infections — in terms of both antibody level and quality (58). More work, such as antibody avidity and dissociation assessments, is needed to clarify the underlying mechanism driving this difference in vaccine response and potentially efficacy in order to find vaccination strategies that will protect both HIV⁺ and HIV⁻ individuals.

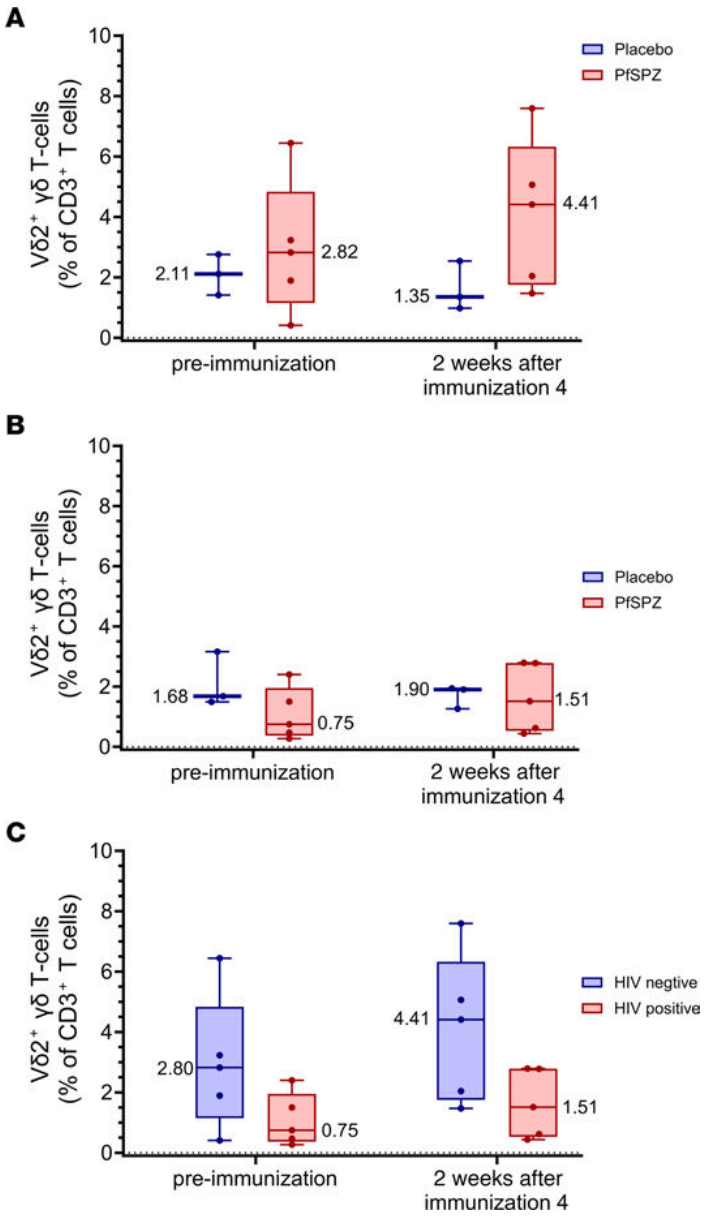

Figure 8. Vδ2⁺ γδ T cells before immunization and after fourth immunization. The percentages of CD3⁺ T cells that were Vδ2⁺ before immunization or 2 weeks after the fourth dose of PfSPZ Vaccine (9.0×10^5) or NS in HIV⁻ (**A**) and HIV⁺ (**B**) participants. Results show the percentages of unstimulated CD3⁺ T cells staining positive for Vδ2. Bars indicate median values within each group, and circles indicate individual participant data. In HIV⁻ vaccinees, the Vδ2⁺ percentages increased after immunization and were higher than the Vδ2⁺ percentages in NS controls, although the difference did not reach statistical significance ($P = 0.25$, Wilcoxon-Mann-Whitney test adjusted for multiple comparisons). In HIV⁺ vaccinees, there was a small increase in the Vδ2⁺ percentages after immunization, which was not different from that of NS controls ($P = 0.88$). The percentages of CD3⁺ T cells staining Vδ2⁺ was higher in HIV⁻ vaccinees compared with HIV⁺ vaccinees before and after immunization (**C**).

An interesting observation in this study was the number of Pf infections (7) identified in the HIV⁺ participants not attributable to PfNF54 — 2 during immunization (one of which either recrudesced during the 28-day period following CHMI or was replaced by a new infection) and 5 between days 28 and 56 after CHMI, following AL therapy (Figure 2). Six of the 7 infections were asymptomatic. In the HIV⁻ participants, parasitemia was only detected during the expected interval after CHMI (Figure 3), a finding consistent with 2 previous trials in HIV⁻ adults from the same study site (9, 11). Observational studies suggest both clinical malaria (59) and asymptomatic Pf parasitemia (60) may be more frequent or more severe in persons living with HIV, with higher levels of parasitemia (61) and correlated with lower CD4 counts (60, 62). Incidence of asymptomatic infection has not been shown to differ significantly, however, between HIV⁺ individuals with CD4 counts of more than 400 cells/μL and HIV⁻ individuals residing in the same community. Although the frequency of asymptomatic parasitemia in this study may have alternate explanations, such as different living conditions or areas of residence within Bagamoyo town that could increase exposure, these findings suggest increased susceptibility to infection in persons living with HIV, even with a fully suppressed HIV viral load and CD4 counts above 500 cells/μL.

Limitations of the trial. This trial provides evidence for the safety of PfSPZ Vaccine and PfSPZ Challenge in HIV$^+$ individuals, with the caveat that all enrolled HIV$^+$ participants were on antiretroviral treatment and had CD4$^+$ T cell counts higher than 500/μL. Safety has yet to be established in those with depressed CD4$^+$ T cell counts or suffering from opportunistic infections. Because this was a first trial that we know of in an immunocompromised study population, the number of participants was small, limiting the power to detect safety or immunology differences between groups. In addition, we have learned since performing this trial that clearance of parasitemia in all prospective vaccinees prior to the first dose is required for maximal vaccine-induced protection, even if the density of parasitemia is below the limit of detection by TBS, due to the immunosuppressive effects of parasitemia (63). Given the small sample size of the trial and the fact that all WT infections documented during immunization were in HIV$^+$ participants, it is possible that an imbalance in subpatent (nondetectable by qPCR) parasitemias between the 2 groups contributed to the absence of protection seen in the HIV$^+$ group. We also considered that the difference in age between the HIV$^+$ (older) and HIV$^-$ (younger) groups may have affected protection. However, a pooled analysis of CHMI outcomes in 80 HIV$^-$ vaccinees 18 to 48 years of age from 4 trials receiving 9.0×10^5 PfSPZ of PfSPZ Vaccine on days 1, 8, and 29 showed no significant difference in age between participants who did or did not develop parasitemia by qPCR by day 28 (our unpublished observations).

We achieved the primary goal of this clinical trial, establishing that PfSPZ Vaccine was well tolerated and safe in a small number of HIV$^+$ individuals. Expanded testing of PfSPZ Vaccine in larger numbers of HIV$^+$ participants, including those with lower CD4$^+$ T cell counts or viremia or others with immunosuppressed status, is merited. However, to make PfSPZ vaccines most useful, we must determine how to overcome the lack of VE of PfSPZ Vaccine in the HIV$^+$ participants. One approach will be to assess different doses or dosing regimens of PfSPZ Vaccine, including our downselected regimen of 9.0×10^5 PfSPZ on days 1, 8, and 29. Another will be to include the institution of presumptive treatment for malaria prior to the first vaccine dose to reduce the immunosuppressive effects of latent parasitemia. A third will be to move to more potent PfSPZ vaccines, such as PfSPZ-CVac (CQ) (64, 65), or genetically attenuated late arresting replication competent (LARC) PfSPZ vaccines, which have been manufactured and will enter clinical trials in 2024 (66).

Methods

Study design and population

This single-center, randomized, double-blind, placebo-controlled trial was conducted in the Bagamoyo Clinical Trials Unit, IHI, between February 2018 and August 2018. Healthy HIV$^-$ and HIV$^+$ males and females ages 18 to 45 years were enrolled after they provided informed consent, successfully completed a test of study understanding, and met prespecified inclusion and exclusion criteria (ClinicalTrials.gov NCT03420053) (Supplemental Table 11 and Supplemental Table 12). For HIV$^+$ participants, this included undetectable HIV viral load by PCR, CD4 of more than 500 cells/μL, and a stable antiretroviral regimen for at least 3 months.

Investigational products

Sanaria PfSPZ Vaccine comprises radiation-attenuated, aseptic, purified, vialed, cryopreserved PfSPZ stored in liquid nitrogen vapor phase (LNVP) at –150 to –196°C. Preparation of investigational productions (IP) in 0.5 mL of diluent (human serum albumen in PBS) was done under the supervision of the study pharmacist. PfSPZ Vaccine or NS (0.5 mL) was administered by DVI through a 25-gauge needle. Sanaria PfSPZ Challenge (NF54) was used for CHMI. PfSPZ Challenge is identical to PfSPZ Vaccine, except it is not radiation attenuated and is infectious. Handling and administration were similar to that of PfSPZ Vaccine. NS was the placebo, as it is indistinguishable from the vaccine, can be administered intravenously, and is virtually free of adverse effects, providing a strict comparator for the assessments of safety.

Care for HIV$^+$ participants

Maintaining the standard of care for HIV$^+$ adults provided by national Care and Treatment Centers (CTC) was prioritized to maximize safety for the HIV$^+$ study participants. The study team coordinated care with CTC staff throughout the course of the study. All HIV$^+$ participants remained on ART during the trial. Assessments for safety included clinical evaluation with WHO staging, assessments of medication adherence, and safety laboratory assessments, including CD4 counts and HIV viral loads (Supplemental Table 13). Clinical and laboratory evaluation at the CTCs were maintained and encouraged; participants could be referred for care to the CTC providing their HIV-related care, should there be indications of clinical failure (a new or recurrent clinical event indicating severe immunodeficiency after 6 months of effective treatment), immunological failure (CD4 count <250 cells/mm^3 following clinical failure or persistent CD4 levels <100 cells/mm^3), or virological failure (HIV viral load above 1,000 copies/ml based on 2 consecutive viral load measurements within 3 to 6 months, with medication adherence support following the first viral load test) (67).

Randomization and intervention

Twenty-one adult participants were allocated into 3 groups based upon HIV status. Group 1 consisted of 9 non–HIV-infected participants randomized to receive 9.0×10^5 PfSPZ of PfSPZ Vaccine (n = 6) or NS control (n = 3) on days 1, 3, 5, 7, and 29 by DVI. Following a safety assessment 7 days after the fourth dose in group 1, a sentinel safety group (group 2a) of HIV$^+$ participants (n = 3) received 4.5×10^5 PfSPZ of PfSPZ Vaccine on days 1, 3, 5, 7, and 29 by DVI. Following a safety assessment 7 days after the fourth dose in group 2a, 9 HIV$^+$ participants allocated to group 2b were randomized to receive 9.0×10^5 PfSPZ of PfSPZ Vaccine (n = 6) or NS (n = 3) on days 1, 3, 5, 7, and 29 by DVI. Randomization was by a computer-generated list without restriction, with each participant entered sequentially to the list as they were enrolled. Only the lead pharmacist had access to this list. For groups 1 and 2b, the participants and investigators were blinded to treatment assignment.

AE assessment

Solicited AEs following immunization were recorded utilizing prespecified criteria (Supplemental Table 4) beginning after the first immunization until 2 days (local) or 7 days (systemic) after the fourth immunization and for 2 (local) or 7 (systemic) days after the fifth immunization. Solicited AEs following administration of PfSPZ

Challenge were recorded utilizing prespecified criteria for 2 (local) or 5 (systemic) days after CHMI (Supplemental Table 4). Subsequent assessment of an expanded set of solicited systemic AEs representative of symptomatic Pf infection continued until a participant was diagnosed and completed treatment for malaria or until day 28 after CHMI (Supplemental Table 4). Unsolicited AEs were assessed by an open-ended questioning and recorded from day 1 to day 14 after each immunization or series of immunizations and to day 28 after administration of PfSPZ Challenge for CHMI. All AEs were assessed for severity and relatedness to PfSPZ Vaccine or PfSPZ Challenge administration; these assessments were blinded for groups 1 and 2b. Laboratory abnormalities, including those involving hematology, ALT, creatinine, CD4, and quantitative HIV nucleic acid testing (Cepheid Xpert HIV-1), were assessed for changes from baseline, severity, and relatedness (Supplemental Table 13).

VE

VE was assessed by homologous CHMI with 3.2×10^3 PfSPZ of PfSPZ Challenge administered by DVI 3 weeks after the last immunization for participants in groups 1 and 2b. Participants were admitted to the facility 7 days after receiving PfSPZ Challenge (day 8), and inpatient observations were performed continuously until either (a) patients were diagnosed with malaria, treated, and confirmed to be malaria free (documented 2 negative daily blood smears) or (b) at 21 days (day 22) after receiving PfSPZ Challenge, if no parasitemia had been detected. TBSs were collected and read twice daily from day 8 to day 15 and then once daily until discharge on day 22. All participants who received PfSPZ Challenge were seen for follow-up 28 days after CHMI for safety assessment and TBS/qPCR and presumptive treatment with antimalarials (see below). Participants who were treated for documented parasitemia through day 29 and who were taking efavirenz or nevirapine for ART had an additional sample drawn for blood smear and PCR on day 43. The final clinical review was scheduled at a study visit on day 57.

Detection of Pf parasites

Participants were assessed for parasitemia by TBS and qPCR at screening, prior to the first immunization and prior to CHMI. AFter CHMI, TBSs were prepared and read on days 6 and 7, twice daily on days 8 to 14, and daily on days 15 to 20 or until positive, with daily qPCR samples obtained over the same interval. Follow-up TBSs and qPCR were assessed on day 28 after CHMI in all participants and again on day 42 after CHMI in any HIV^+ participant taking efavirenz or nevirapine who was treated for parasitemia in the 28 days after CHMI.

Slide preparation and reading for TBSs were performed as described (68). In brief, 10 μL of blood collected in EDTA was placed on a 10 mm by 20 mm rectangle on a glass slide, dried, and stained. For asymptomatic individuals, approximately 0.5 μL of blood was assessed. For symptomatic individuals, approximately 2.0 μL of blood was assessed. Two asexual erythrocytic stage Pf parasites had to be identified for a slide to be considered positive. All positive TBSs were confirmed by qPCR prior to the initiation of treatment. TBSs were continued after initiation of treatment until 2 consecutive smears were negative.

Parasites were quantified by the PlasQ assay, a multiplex qPCR assay targeting the pan-*Plasmodium* spp. 18S rDNA (69) and the high copy number Pf-specific varATS region (70), which has an overall lower limit of detection of 50 parasites/mL (71), using a sample volume of 180 μL. A single positive result was considered positive for infection with Pf. After the start of CHMI, the time of first blood sample positivity by qPCR was used to determine infection status and calculation of prepatent period. All samples were analyzed by qPCR in real time as they were continuously collected from the participants.

Parasite genotyping was applied to selected samples when necessary to distinguish NF54 from naturally acquired Pf infection. Genotyping was performed by the Swiss Tropical Public Health Institute using published methods (72), targeting the sequences for merozoite surface protein (MSP) 1 and 2, glutamate-rich protein (GLURP), and microsatellites PfPK2, TA40, TA60, and TA81 to establish identification.

Treatment of Pf infections

Symptomatic and asymptomatic *Plasmodium* sp. infections diagnosed or confirmed by qPCR in participants prior to the CHMI were treated according to national guidelines with AL (80 mg artemether/480 mg lumefantrine twice a day for 3 days) under directly observed therapy (DOT). All participants with positive TBSs in the 28-day interval following CHMI were treated with AL within 24 hours of first positive TBS confirmed by qPCR. Participants remaining TBS negative were presumptively treated with AL on day 28, regardless of qPCR findings.

Immunology assays

Samples for analysis of humoral and cellular immune responses and transcriptomics were obtained prior to each immunization and prior to CHMI and at specific times after immunizations and after CHMI.

Standard antibody assays. Sera were separated and frozen at –80°C within 4 hours of collection. IgG and IgM antibodies to PfCSP and IgG antibodies to MSP5 were assessed by ELISA and IgG antibodies to PfSPZ by aIFA, as described (64, 65). Functional activity was assessed by the ISI assay (64, 65). Definitions for a positive response were taken relative to the pre–dose 1 measurement. For ELISA, samples were considered positive if the difference between the postimmunization OD 1.0 and the preimmunization OD 1.0 (net OD 1.0) was 50 or more and the ratio of the postimmunization OD 1.0 to preimmunization OD 1.0 (ratio) was 3.0 or more. For aIFAs, participants with a net reciprocal serum dilution for 2 Å~ 105 arbitrary fluorescence units (AFUs) of 150 or more and a ratio reciprocal serum dilution of 3.0 or more were considered positive. For aISI, participants with a net reciprocal serum dilution for 80% inhibition of 10 or more in the ISI assay and a ratio reciprocal serum dilution for 80% inhibition of 3.0 or more in the ISI were considered positive.

Systems serology assays. Recombinant PfCSP, PfSSP2/PfTRAP, PfAMA1, PfEXP1, PfLSA1, and PfEBA175 were used. Antigen-specific isotype level and FcR binding were measured by a multiplex Luminex assay, as previously described (73). The ADNP assay was performed with neutrophils isolated from 2 separate human donors, and the antibody-dependent cell phagocytosis (ADCP) assay was performed with THP-1 cells in technical duplicate, both as previously described (73). ADCD was performed with a Luminex bead array assay measuring C3 deposition triggered by antigen-specific polyclonal sera adapted from previously published work (74).

T cell analyses. T cell responses in cryopreserved PBMCs were assessed by multiparameter flow cytometry as described previously for $CD4^+$ and $CD8^+$ (5). After thawing, PBMCs were rested for 8 hours, then stimulated for 17 hours with PfSPZ Vaccine diluent (PBS with 1% human serum albumin; CSL Behring) or 1.5×10^5 viable,

irradiated, aseptic, purified, cryopreserved PfSPZ from a single production lot. For the last 5 hours of the stimulation, 10 μg/ml brefeldin A (BD Biosciences) was added to the culture. After stimulation, cells were stained as previously described (75). Briefly, cells were washed and stained with viability dye for 20 minutes at room temperature, followed by a 20-minute surface staining at room temperature for the markers CD4, CD8, CD14, CD20, CD38, CD45RA, CD56, TCR Vα-7.2, CD161, TCR-γδ, TCR-Vδ1, TCR-Vδ2, TCR-Vγ9, or CXCR6. Cells were washed, fixed, and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences) and were stained intracellularly for CD3, IFN-γ, IL-2, TNF-α, perforin, and Ki-67. On completion of staining, cells were collected on a BD FACSymphony Flow Cytometer. Samples were analyzed using FlowJo, version 10.6.1 (FlowJo LLC). Cytokine-positive cells were determined after gating on nonnaive T cells. All antigen-specific cytokine frequencies were reported after background subtraction of identical gates from the same sample incubated with negative control stimulation (human serum albumin).

Ex vivo measurements of $Vd2^+$ and $Vd2^-$ gδ T cells were performed using PBMCs. Briefly, cryopreserved PBMCs were thawed and stained with Aqua Viability Dye (Invitrogen) for 15 minutes. Cells were then washed and surface stained with a cocktail of antibodies for anti-CD3 Alexa Fluor 700, CD4-BV421, CD8 allophycocyanin-H7, gdTCR-PE, CD27–PE–Cy7, CD45RO-PECF594, and Vδ2-FITC and incubated for 20 minutes (Supplemental Figure 7). All antibodies were purchased from BD Biosciences. Cells were washed and acquired on an LSR II flow cytometer equipped with a blue, red, and violet laser. Subsequent analysis of flow cytometry data was done using FlowJo (version 10.5.3).

Statistics

Small group sizes were selected to balance the chance to detect any possible untoward reactions against the desire to limit the number of participants involved for safety purposes. All participants who received at least 1 dose of IP were included in the safety analysis; all participants who participated in CHMI were included in the efficacy analysis except 1 HIV^+ participant who was found to have a non-NF54 parasite infection. Categorical variables were summarized using absolute (*n*) and relative (%) frequencies. Continuous variables were summarized using mean and SD, median, and range. Comparisons of categorical variables between groups were analyzed using Barnard's 2-sided exact unconditional test; comparisons of continuous variables were analyzed using the Mann-Whitney 2-sided test. VE was estimated as 1– (attack rate in vaccine participants/attack rate in NS control participants) based upon parasitemia detected by qPCR. For purposes of VE calculations, the HIV^- and HIV^+ participants receiving placebo were to be combined, assuming infection rates would be similar in both.

The immune responses of study participants in each group were compared with the immune responses of the corresponding placebo groups. For standard antibody assays, differences between vaccinees and controls were analyzed using the Mann-Whitney test for net OD 1.0 and OD 1.0 ratios. The Holm-Bonferroni method was used to calculate adjusted *P* values. For systems serology analyses, univariate and multivariate statistical analyses were performed in R (version 4.0.0) or Python (version 3.9.1). Prior to analysis, Luminex and ADCD data were $\log_{10}$ transformed, and all data were centered and scaled. For univariate analysis, significance was determined by Mann-Whitney *U* test. For feature selection, the systemseRology R package (version 1.0) (https://github.com/LoosC/systemsseRology) was used. Elastic-net parameters were optimized using tuneLength of 10 and leave-one-out cross validation. PCA of the elastic-net selected features was performed with the sklearn package (version 1.1.2). Cocorrelation network analysis was performed in Cytoscape (version 3.8).

For T cell analyses, the differences in the percentages of $CD4^+$ T cells expressing cytokines or expressing markers of activation were analyzed using the Kruskal-Wallis test and adjusted for multiple comparisons using the Holm-Bonferroni method to calculate an adjusted *P* value. Any *P* value of less than 0.05 was considered significant.

Study approval

This clinical trial (BSPZV3a) is registered at ClinicalTrials.gov (NCT03420053). Following successful completion of a written test of understanding, all study participants provided written, informed consent prior to participation in the trial. This trial was approved by the IRBs of the IHI and the National Institute for Medical Research (NIMR), both in Tanzania, and was conducted in compliance with the Declaration of Helsinki and Good Clinical Practice guidelines. Results are presented following the CONSORT reporting guidelines (76).

Data availability

The study protocol and all data for this trial held by Sanaria are available through the ImmPort data repository (Study SDY1909; www.ImmPort.org). Values for all data points in graphs are reported in the Supporting Data Values file.

Author contributions

SJ was the principal investigator (PI) and FM the lead clinician of the clinical trial. SJ, TLR, SA, and SLH designed the trial. Conduct of the trial was supported by MQ, MR, AT, GN, BMB, LM, KK, BSS, ERJ, and PFB. BKLS, TM, YA, and ERJ provided the study product. TS, M Mpina, and CD managed the clinical laboratory, including performance or TBS and diagnostic PCR. TAM, PR, and LWPC were responsible for data management and analysis. NK, JDH, GA, YZ, and M Mendu performed and analyzed the humoral immunology studies. IZ, PED, PAS, and RS performed and analyzed cellular immunology. LWPC, SJ, and SLH wrote the manuscript with contributions from all authors in the writing or review stages. SJ and LWPC are co–first authors, with SJ listed first as the study PI.

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4.8 Paper VI: Published Article

Multi-Dose Priming Regimens of PfSPZ Vaccine: Safety and Efficacy against Controlled Human Malaria Infection in Equatoguinean Adults: Randomized controlled trial

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Multi-Dose Priming Regimens of PfSPZ Vaccine: Safety and Efficacy against Controlled Human Malaria Infection in Equatoguinean Adults

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Abstract. *Plasmodium falciparum* sporozoite (PfSPZ) Vaccine is composed of radiation-attenuated, aseptic, purified cryopreserved PfSPZ. Multiple clinical trials empirically assessing two to six doses have shown multi-dose priming (two to four doses the first week) to be optimal for protection in both 4- and 16-week regimens. In this randomized, double-blind, normal saline (NS) placebo-controlled trial, four groups (G) of 18- to 32-year-old Equatoguineans received multi-dose priming regimens with or without a delayed final dose at 4 or 16 weeks. The regimens were G1: days 1, 3, 5, 7, and 113; G2: days 1, 3, 5, and 7; G3: days 1, 3, 5, 7, and 29; and G4: days 1, 8, and 29. All doses were 9×10^5 PfSPZ. Tolerability, safety, immunogenicity, and vaccine efficacy (VE) against homologous controlled human malaria infection (CHMI) 6–7 weeks after vaccination were assessed to down-select the best regimen. All four regimens were safe and well tolerated, with no significant differences in adverse events (AEs) between vaccinees ($N = 84$) and NS controls ($N = 20$) or between regimens. Out of 19 controls, 13 developed Pf parasitemia by quantitative polymerase chain reaction (qPCR) after CHMI. Only the vaccine regimen administered on study days 1, 8, and 29 gave significant protection (7/21 vaccinees versus 13/19 controls infected, VE 51.3%, $P = 0.03$, Barnard's test, two-tailed). There were no significant differences in antibodies against Pf circumsporozoite protein (PfCSP), a major SPZ antigen, between protected and nonprotected vaccinees or controls pre-CHMI. The six controls not developing Pf parasitemia had significantly higher antibodies to blood stage antigens Pf exported protein 1 (PfEXP1) and Pf merozoite surface protein 1 (PfMSP1) than the controls who developed parasitemia, suggesting naturally acquired immunity against Pf limited infections in controls. This study identified a safe, protective, 4-week, multi-dose prime vaccination regimen for assessment in future trials of PfSPZ Vaccine.

INTRODUCTION

Malaria continues to be a major global health problem, with the WHO African Region reporting 215 million cases (94% of the global burden) in 2019. Malaria incidence rates worldwide have remained static at ~57 cases per 1,000 population at risk since 2015.[1] The increasing prevalence of molecular markers of artemisinin resistance and detection of resistance to all main insecticide classes throughout sub-Saharan Africa threaten control measures currently deployed. If the goal of malaria elimination is to be achieved, additional tools are needed, including vaccines that can prevent infection and thereby block transmission.

Sanaria® PfSPZ Vaccine (Sanaria Inc., Rockville, MD), composed of radiation-attenuated, aseptic, purified, cryopreserved, whole *Plasmodium falciparum* (Pf) sporozoites (SPZ), is designed to achieve these objectives. PfSPZ Vaccine has been assessed in 21 completed or ongoing trials in the United States, European Union, and Africa, and shown to be safe and well tolerated,[2–17] with almost no differences in adverse event (AE) profiles between vaccinees and normal saline (NS) placebo recipients in 12 of the 13 trials among the 21 that used a randomized, double-blind, placebo-controlled design (in one trial conducted in Burkina Faso, there was an increased frequency myalgia in vaccinees—Sirima and Laurens, unpublished). Vaccine efficacy (VE) > 90% against homologous (same parasite strain in vaccine and challenge) controlled human malaria infection (CHMI) at 3–11 weeks after last dose has been shown in the United States,[3,5] Tanzania,[11] and Mali,[17] and can last for at least 14 months.[4] In field studies, during 24 weeks of follow-up post-vaccination in three trials in Mali and one in Burkina Faso, VE against first episode of parasitemia ranged from 48% to 57% by time-to-event analysis (one minus the hazard ratio)[7,17] (Sirima and Laurens, unpublished; Diawara and Healy, unpublished), and in both Burkina Faso and the most recent Mali trial, VE against infection was sustained during a second malaria transmission season, as was VE against clinical malaria in Mali (Sirima and Laurens, unpublished; Diawara and Healy, unpublished). Vaccine efficacy against clinical malaria has also been demonstrated in Kenyan infants.[16]

The most promising VE against CHMI has been seen with vaccine doses of 9.0×10^5, with protection reduced when

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higher doses were tested in malaria-exposed Tanzanian[11] and Equatoguinean[14] adults. However, optimal timing and number of doses have not been defined. Previous studies tested longer vaccination schedules (three to five doses administered over 16–20 weeks), with relatively even spacing.[2–11,13–15,17] Recent data indicate that accelerated vaccination schedules, particularly for the initial priming immunizations (multi-dose priming), allow shortening of the vaccination period and may provide better VE. For example, in a study in the United States (Warfighter 2 trial), a five-dose regimen consisting of four priming doses over the first 6 days (days 1, 3, 5, and 7) with a boost at 16 weeks using 4.5×10^5 PfSPZ per dose gave significant VE (40%) against heterologous CHMI (different Pf strains in the vaccine and challenge), whereas three regimens of three evenly spaced administrations over 16 weeks using higher doses (0.9, 1.8, and 2.7×10^6 PfSPZ) did not provide significant VE (20–23%).[15] A subsequent study in Germany (MAVACHE trial) showed that the VE was 77% and 79% against homologous and heterologous CHMI, respectively, when the boost was administered on day 29; however, both a multi-dose prime (two doses on days 1 and 8) and the boost were required, as just two administrations of the same (9.0×10^5) or higher (1.35 or 2.7×10^6) doses of PfSPZ protected only 67% and 50%, respectively (Mordmüller, unpublished). A condensed administration schedule of PfSPZ Vaccine maintaining high VE such as that tested in Germany would facilitate its deployment in the field.

Here, we report the results of a randomized, double-blind, NS placebo-controlled clinical trial (EGSPZV3) assessing the safety, immunogenicity, and VE of four different multi-dose priming regimens with or without a delayed booster dose, each dose containing 9.0×10^5 PfSPZ of PfSPZ Vaccine administered by direct venous inoculation (DVI). Two of the regimens tested, both described above, had been the best among several evaluated in two prior regimen-optimization trials. To better compare these two leading regimens, two bridging groups were added in this study to assess the effect of the interval prior to booster dose (15 weeks in the first leading regimen, 3 weeks in the second) and to examine the need for a booster dose.

The trial enrolled healthy, malaria-exposed adults living on Bioko Island in Equatorial Guinea. We selected this population rather than malaria-naive individuals because the most important application planned for PfSPZ-based vaccines is their use in mass vaccination programs (MVPs) in endemic areas to regionally eliminate malaria transmission. It was therefore appropriate to conduct this regimen-comparison study in the target population, although the ethical concerns associated with conducting CHMI in pediatric age-groups limited participation to adults.[18] Vaccine efficacy was assessed 6–7 weeks following final immunization by homologous CHMI. The trial was designed to identify an optimal immunization regimen for further testing in Phase 2 and 3 clinical trials.

MATERIALS AND METHODS

Study location and population. This single-center trial was conducted at the Baney Clinical Research Center located near the capital city Malabo on Bioko Island between August 2018 and March 2019. Healthy male and female adults of age 18–45 years were recruited from the Baney and Rebola districts and Malabo. After successfully completing a test of study understanding and signing informed consent forms (ICFs), prospective participants were screened and enrolled according to inclusion and exclusion criteria (Supplemental Tables 4 and 5; https://clinicaltrials.gov/show/NCT03590340). Individuals testing positive for HIV, hepatitis C, or hepatitis B were excluded from participation. Women of childbearing potential were required to use injectable depot hormonal contraception.

Investigational products. The investigational product (IP), Sanaria® PfSPZ Vaccine, consists of radiation-attenuated (metabolically active but nonreplicating), aseptic, purified, vialed, cryopreserved PfSPZ and is stored in liquid nitrogen vapor phase at −150 to −196°C.[19] After thaw, vialed PfSPZ were diluted in phosphate-buffered saline and human serum albumin in a biological safety cabinet, and injected within 30 minutes. Normal saline was used as a neutral comparator for assessment of safety as it is visibly indistinguishable from the vaccine, can be administered by DVI, and is not associated with AEs. PfSPZ Vaccine or NS in 0.5 mL was administered by DVI through a 25-gauge needle over several seconds. Sanaria® PfSPZ Challenge (NF54), used for CHMI, was manufactured, stored, and administered identically to PfSPZ Vaccine apart from the attenuation (irradiation) step. When administered by DVI at the standard dose of 3.2×10^3 PfSPZ, PfSPZ Challenge has led to patent parasitemia in 100% of 78 malaria-naive adults[20–29] and 6/7 Equatoguineans[14] on first CHMI.

Randomization and intervention. The 104 participants were allocated into four groups of 26 participants (Figure 1). In all groups, participants were randomized to receive PfSPZ Vaccine ($N = 21$ per group, 9.0×10^5 PfSPZ per dose) or NS ($N = 5$ per group). In Groups 1–3, 9.0×10^5 PfSPZ were administered four times on days 1, 3, 5, and 7 followed by 1) a final dose on day 113/week 16 (regimen 1 as previously studied in the Warfighter 2 trial, NCT02601716[15]), 2) no final dose (regimen 2, a bridging group to examine the need for a final dose), or 3) a final dose on day 29/week 4 (regimen 3, a bridging group with a shorter interval to the final dose to match regimen 4). In regimen 4, 9.0×10^5 PfSPZ were administered on days 1 and 8 with a final dose on day 29 (best regimen from MAVACHE trial, NCT02704533). Although group assignment was unblinded, participants, clinical staff, and study outcome assessors were blinded to treatment assignment within each group. Because parasitemia is immunosuppressive[30–32] and appears to inhibit the ability of PfSPZ to induce a protective immune response,[28,33,34] all participants received a full treatment course of AL (artemether 80 mg/lumefantrine 480 mg) twice a day for 3 days under directly observed treatment (DOT) prior to first immunization, allowing at least 14 full days between the last drug dose and the first immunization. The same regimen was used to treat any malaria infections acquired through natural transmission during the study.

Vaccine efficacy. Vaccine efficacy was assessed by homologous CHMI of 3.2×10^3 PfSPZ of PfSPZ Challenge (NF54) administered by DVI. Controlled human malaria infection was targeted for 8 weeks after the last immunization for all participants. To prevent the confounding of VE assessment by naturally acquired Pf infection, given that the study was located in an area of active malaria transmission, participants completed a second full treatment course of AL under

A

Study Weeks	-2	-1	0	1	2	3	4	5	6	8	10	12	14	16	18	20	22	24	28	32
Group 1	AL		V1	V2	V3	V4								V5		AL		CHMI		LSV
Group 2	AL		V1	V2	V3	V4		AL		CHMI				LSV						
Group 3	AL		V1	V2	V3	V4	V5		AL		CHMI				LSV					
Group 4	AL		V1		V2		V3		AL		CHMI				LSV					

Multi-dose prime

B

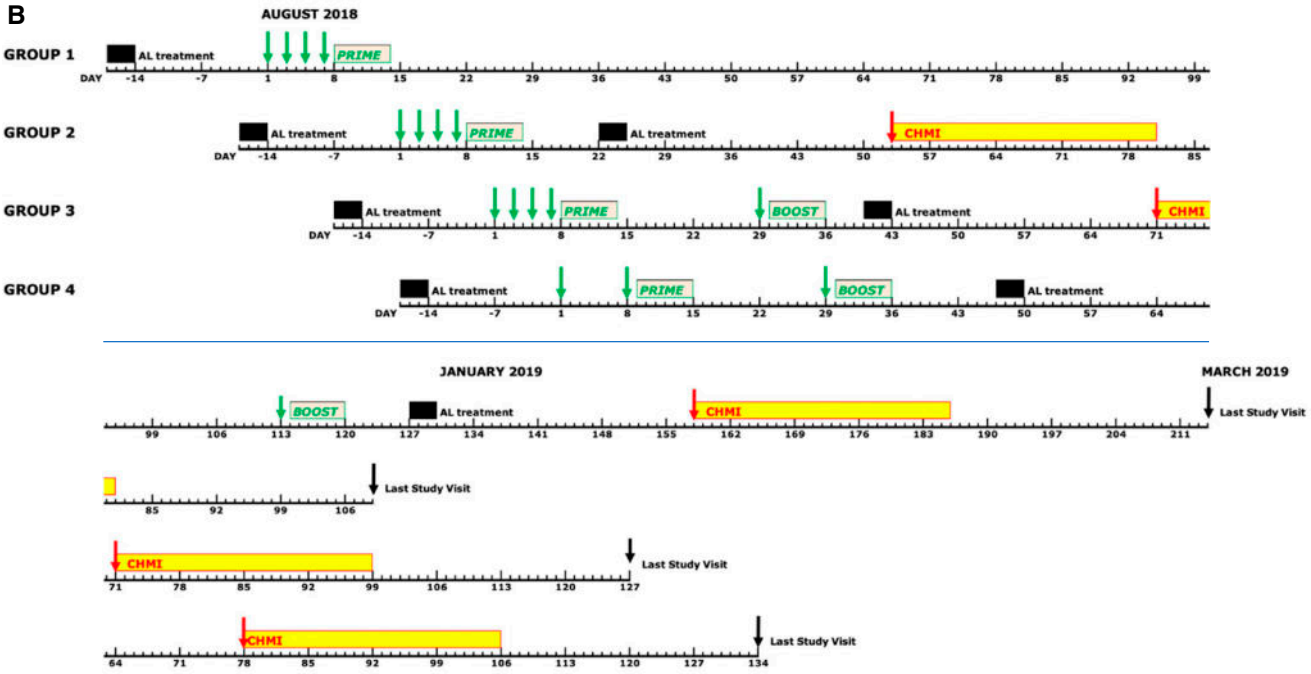

FIGURE 1. Schema and execution plan for EGSPZV3 clinical trial. (**A**) Shows the design of the four study groups. Group 1 was the best regimen from the Warfighter 2 clinical trial,[15] Group 4 was the best regimen from the MAVACHE trial (Mordmüller, unpublished), and Groups 2 and 3 were bridging groups to explore the importance of the boost and the length of the prime-boost interval. AL = artemether/lumefantrine; V1–V5 = vaccinations 1–5; CHMI = controlled human malaria infection; LSV = last study visit. Minimum days between AL and V1: 14 days. Minimum days between AL and CHMI: 23 days. (**B**) Shows the execution plan for the study. Green arrows: immunizations; red arrows: CHMI; black arrows: last study visits. This figure appears in color at www.ajtmh.org.

DOT approximately 3 weeks after final immunization and at least 23 days prior to CHMI to minimize the possibility of residual drug effects at the time of CHMI. Twenty-three days is four to eight times the 3- to 6-day half-life of the longer acting drug partner, lumefantrine. After PfSPZ Challenge injection, participants were observed for 30 minutes and then discharged from the clinic. Follow-up for parasitemia and for signs and symptoms of malaria began on day 6 after CHMI, performed on an outpatient basis (days 6 and 7) and then inpatient basis (day 8 onward) using thick blood smear (TBS), quantitative polymerase chain reaction (qPCR), and clinical assessment until day 21 or until Pf parasitemia was diagnosed and treatment of malaria with AL completed, whichever came first. Samples for TBS and qPCR were obtained daily on days 6 and 7, every 12 hours beginning on day 8 through day 14, then daily from day 15 through day 21. Any positive TBS was confirmed by qPCR by an independent technician prior to treatment. All other qPCR testing was performed retrospectively. Participants remaining TBS negative on day 21 continued with alternate-day outpatient monitoring until day 28. Thick blood smear sampling could be performed more frequently at the discretion of study investigators if volunteers had symptoms or signs consistent with malaria. After initiation of treatment, TBS was assessed daily until two consecutive samples were negative by TBS. Individuals who dropped out of the study prior to day 28 without a malaria diagnosis and those who remained negative on day 28 were pre-emptively treated with AL and confirmed negative by qPCR. A sample for qPCR was obtained at the final scheduled study visit (42 days after CHMI) to confirm that no study participants were left with a residual malaria infection at the end of the study.

Adverse events assessment. Solicited AEs following inoculation were assessed utilizing a prespecified list of signs and symptoms (Supplemental Table 6) for 2 days (local AEs) or 7 days (systemic AEs) after each injection using in-person clinic or telephone visits and were rated for severity. All solicited AEs were considered related to IP administration and attributed

to the preceding injection. Solicited AEs following administration of PfSPZ Challenge for CHMI were recorded utilizing the same prespecified criteria as for PfSPZ Vaccine or NS injection, except that the list of AEs was expanded on day 6 post CHMI to include additional symptoms that might indicate clinical malaria and collection of systemic AEs continued until the diagnosis and treatment of Pf parasitemia, or until day 28, whichever came first. Solicited AEs recorded through 5 days after CHMI were attributed to PfSPZ Challenge, and from day 6 onward to parasitemia (Supplemental Table 6).

Unsolicited AEs were assessed by open-ended questioning and recorded from day 1 to day 14 after each injection or series of multi-dose prime injections and from day 1 to day 28 after CHMI and were rated for severity. During the first 5 days after CHMI start, unsolicited AEs were considered potentially related to PfSPZ Challenge, and thereafter through to day 28 to asexual blood stage parasitemia. A relatedness attribution to parasitemia was kept only if the subject turned out to be positive for Pf by TBS or qPCR.

Hematology and biochemistry testing was performed 14 days after the priming regimen and the final dose; abnormal results were rated for severity and clinical significance (Supplemental Table 7).

Detection of Pf parasites and parasite DNA. Subjects had scheduled assessments for parasitemia by TBS at four time points outside of CHMI: at screening, and prior to the first dose, the final dose and CHMI (for Group 2, which did not receive a delayed final dose, the third sample was taken 2 weeks after the final dose of the priming regimen). Except at screening, all positive TBS were confirmed retrospectively by qPCR after the conclusion of the study. Additional TBS were to be made for any symptomatic individual.

Slide preparation and reading for TBSs were performed as described.[35] In brief, 10 μL of blood collected in ethylenediaminetetraacetic acid were evenly spread over a 10 mm × 20 mm rectangle on a glass slide, dried, and stained with Giemsa. For asymptomatic individuals, ~0.5 μL of blood were assessed. For symptomatic individuals, ~2.0 μL of blood were assessed. Two asexual erythrocytic stage Pf parasites had to be identified for a sample to be considered positive; thus, the theoretical lower limit of detection for a positive TBS was 4 parasites/μL blood in an asymptomatic subject and 1 parasite/μL in a symptomatic subject. Subjects were monitored by qPCR until day 28 after CHMI or until malaria treatment based on TBS positivity.

Parasites were quantified by qPCR using the PlasQ qPCR assay that has a lower limit of dection of 50 parasites/mL, as described.[36] A single positive result was considered positive for infection with Pf. After the start of CHMI, the time of first blood sample positivity by qPCR was used to determine infection status and calculation of prepatent period.

Antibody assays. Blood for antibody testing was drawn at baseline prior to the first immunization, 2 weeks after the final immunization and prior to CHMI. Serum was separated and frozen at −80°C within 4 hours of collection. IgG antibodies to Pf circumsporozoite protein (PfCSP), Pf merozoite surface protein 1 (PfMSP1), and Pf exported protein 1 (PfEXP1) were assessed by ELISA as described.[10] Samples for PfCSP were considered positive (seroconversion) if the difference between the postimmunization optical density (OD) 1.0 and the preimmunization OD 1.0 (net OD 1.0) was > 50 and the ratio of postimmunization OD 1.0 to preimmunization OD 1.0 (ratio) was > 3.

Statistics. The sample size of 21 subjects in each vaccine group with a pooled total of 20 subjects receiving NS was selected to have a 90% power to detect a VE of 35–50% for each vaccine group compared with the controls, allowing for a 20% dropout rate from each group and assuming that no more than one control subject would fail to develop detectable parasitemia. Categorical variables were summarized using absolute (*n*) and relative (%) frequencies. Continuous variables were summarized using mean and SD, median, and range. Comparisons of categorical variables between groups were analyzed using Barnard's two-sided exact unconditional test; for comparisons of continuous variables including differences between vaccinees and controls in the results of antibody assays were analyzed using the Mann–Whitney two-sided test. Vaccine efficacy was estimated as 1—(attack rate in vaccine subjects/attack rate in NS control subjects) based upon parasitemia detected by qPCR. The immune responses of study subjects in each group were compared with the immune responses of the pooled placebo group. Within each group, immune responses in subjects protected were compared with the immune responses in subjects not protected against CHMI. For antibody assays, differences between vaccinees and controls were analyzed using the Mann–Whitney test for net OD 1.0 and OD 1.0 ratios. No adjustments were made for multiple comparisons.

Study approval. Ethical approval was obtained from the National Ethics Committee of Equatorial Guinea and the Ifakara Health Institute Institutional Review Board. The volunteers were briefed on the specifics of the planned study, had to pass a written assessment of understanding of the study, and signed informed consent before any study procedures were done.

RESULTS

A total of 104 subjects (91 male, 13 female) aged 18–32 years were enrolled and allocated to one of the four study groups. Twenty-six subjects in each group were randomized to receive vaccine ($N = 21$) or NS ($N = 5$) (Figure 2). Vaccinees in each group were well balanced with respect to age and body mass index (BMI) when compared with each of the other groups or the pooled control groups (Supplemental Table 1). Few women were enrolled in the study—a consequence of the requirement for stringent pregnancy prevention that many women refused. All 13 female subjects were randomized by chance to receive the vaccine. Five subjects withdrew or were lost to follow-up prior to CHMI—four from Group 1, which had the longest dosing period (113 days) and one from Group 3; all five had been randomized to receive PfSPZ Vaccine.

Parasitemia in subjects prior to CHMI. No volunteer was symptomatic for malaria during the preimmunization and immunization phases of the study and thus no blood smears were performed other than at protocol-specified time points of screening, prior to first vaccination, prior to final dose (or 2 weeks after the fourth priming dose in the case of Group 2), and prior to CHMI. TBS and qPCR results from these timepoints are presented in Supplemental Table 2.

Five participants were positive by TBS at screening among those later enrolled in the trial—one in Group 1 and two each in Groups 3 and 4. All subjects were treated presumptively with AL after screening and prior to V1 as stipulated by the protocol. At the next time point, prior to V1, one subject

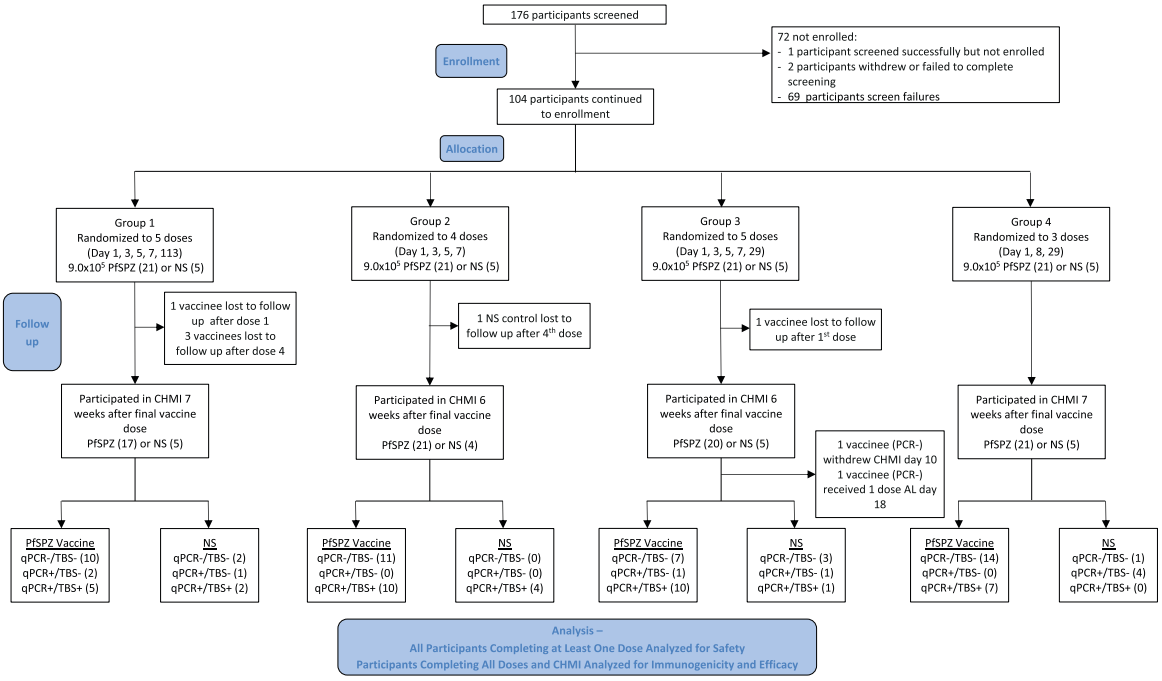

FIGURE 2. CONSORT flow diagram. This figure appears in color at www.ajtmh.org.

(Group 3, NS control) was TBS positive (confirmed by qPCR, density of 1.84 genome copies/μL) 14 days after the last dose of AL and was treated again with AL, with immunization deferred 3 weeks. A second participant (Group 1, PfSPZ Vaccine) was retrospectively qPCR positive 16 days after the last dose of AL (1.31 genome copies/μL) and may have been parasitemic at V1. Both subjects likely acquired malaria by natural transmission.

At the time of the last vaccine dose, 38–126 days after being treated with AL, one participant was positive by TBS and this subject plus four more were found positive by qPCR, indicating that they had probably acquired Pf infections by natural transmission during the immunization phase. Among these five subjects, two were vaccinees from Group 4 (densities of 0.39 genome copies/μL blood and 0.70 genome copies/μL blood), two were controls from Group 3 (16.31 genome copies/μL blood and 227.4 genome copies/μL blood), and one, with a density of 6,740 genome copies/μL blood, was a NS control from Group 4. This last subject, although asymptomatic, had a positive TBS, was treated with AL and received the final injection 41 days after treatment, this constituting a 20-day delay in the final dose. The other four may therefore have been parasitemic at the time they received their final dose. All subjects were subsequently presumptively treated with AL 24–29 days before CHMI as stipulated by the protocol and no subjects were TBS or PCR positive prior to CHMI.

All three vaccinees and three of the four controls identified as qPCR positive during the immunization period remained negative during CHMI follow-up. Only the control from Group 4 whose TBS had been positive prior to V3 developed parasitemia after CHMI (day 20).

Vaccine efficacy. *Normal saline controls.* Out of 20 controls, 19 participated in one of the four CHMIs conducted 6–7 weeks after the final dose of NS, which was within the protocol-defined window. One control subject was lost to follow-up after last injection and did not participate. Out of 19 controls, 13 were positive by qPCR after CHMI (seven of these positive by TBS) and six controls were negative by qPCR and TBS. Results for the controls were pooled for comparison to each of the vaccine groups (Table 1). All subjects who tested negative throughout the 28 days of observation were presumptively treated on day 28 with AL and confirmed qPCR negative on the last visit day 42.

PfSPZ Vaccine, Group 1. Out of 21 subjects, 17 were immunized with five doses of 9.0×10^5 PfSPZ of PfSPZ Vaccine on days 1, 3, 5, 7, and 113, and underwent CHMI 7 weeks after the last vaccine dose. One subject was lost to follow-up after the first immunization and three subjects traveled outside the study area after the fourth immunization. Seven of 17 subjects were positive by qPCR (5/7 positive by TBS) and 10/17 were negative by both qPCR and TBS (Table 1). Vaccine efficacy at 7 weeks after last dose of vaccine was 39.8% ($P = 0.13$, Barnard's test, two-tailed) (Figure 3).

PfSPZ Vaccine, Group 2. A total of 21 subjects were immunized with four doses of 9.0×10^5 PfSPZ of PfSPZ Vaccine on days 1, 3, 5, and 7, and underwent CHMI 6 weeks after last vaccine dose. Out of 21 subjects, 10 were positive by qPCR (10/10 positive by TBS) and 11/21 were negative by both qPCR and TBS (Table 1). Vaccine efficacy at 6 weeks after last dose of vaccine was 30.4% ($P = 0.22$) (Figure 3).

TABLE 1
VE against homologous CHMI

		# Completing CHMI per protocol	Median time from last vaccine dose to CHMI (range)	# Without parasitemia at 28 days by		
				TBS	qPCR	VE by qPCR*
PfSPZ Vaccine	Group 1 (1, 3, 5, 7, 113)	17	52 days	12	10 (58.8%)	39.8% ($P = 0.13$)
	Group 2 (1, 3, 5, 7)	21	46 days (42–46 days)	11	11 (52.4%)	30.4% ($P = 0.22$)
	Group 3 (1, 3, 5, 7, 29)	18†‡	42 days	8	7 (38.9%)	10.7% ($P = 0.74$)
	Group 4 (1, 8, 29)	21	48 days	14	14 (66.7%)	51.3% ($P = 0.03$)
Controls	Pooled	19	–	12	6 (31.6%)	–
Total		96		57	48	

CHMI = controlled human malaria infection; qPCR = quantitative polymerase chain reaction; TBS = thick blood smear; VE = vaccine efficacy.
* VE calculated as VE = one-risk ratio. *P* values calculated using Barnard's test, two-tailed.
† One subject withdrew from inpatient observation on day CHMI+10 for personal reasons, was treated and was not included in VE calculations or counted in the 18 completing CHMI.
‡ One subject was unintentionally treated with single dose of AL on day CHMI+18. This subject remained negative throughout the duration of CHMI follow-up but was not included in VE calculations or counted in the 18 completing CHMI.

PfSPZ Vaccine, Group 3. A total of 20 subjects were immunized with five doses of 9.0×10^5 PfSPZ of PfSPZ Vaccine on days 1, 3, 5, 7, and 29, and underwent CHMI 6 weeks after last vaccine dose. Two subjects, both recipients of PfSPZ Vaccine, were excluded from per protocol analysis. One subject, persistently TBS and qPCR negative to day 18 after CHMI, was unintentionally given a single dose of AL on day 18; this subject remained negative for the duration of the study. The other subject declined continued participation in the inpatient phase of the study on day 10 after CHMI. This subject, who was qPCR and TBS negative on day 10, was treated preemptively with AL and discharged with safety visits on post CHMI days 28 and 42. Out of 18 remaining subjects, 11 were positive by qPCR (10/11 positive by TBS) and seven were negative by both qPCR and TBS (Table 1). Vaccine efficacy at 6 weeks after last dose of vaccine was 10.7% ($P = 0.74$) (Figure 3).

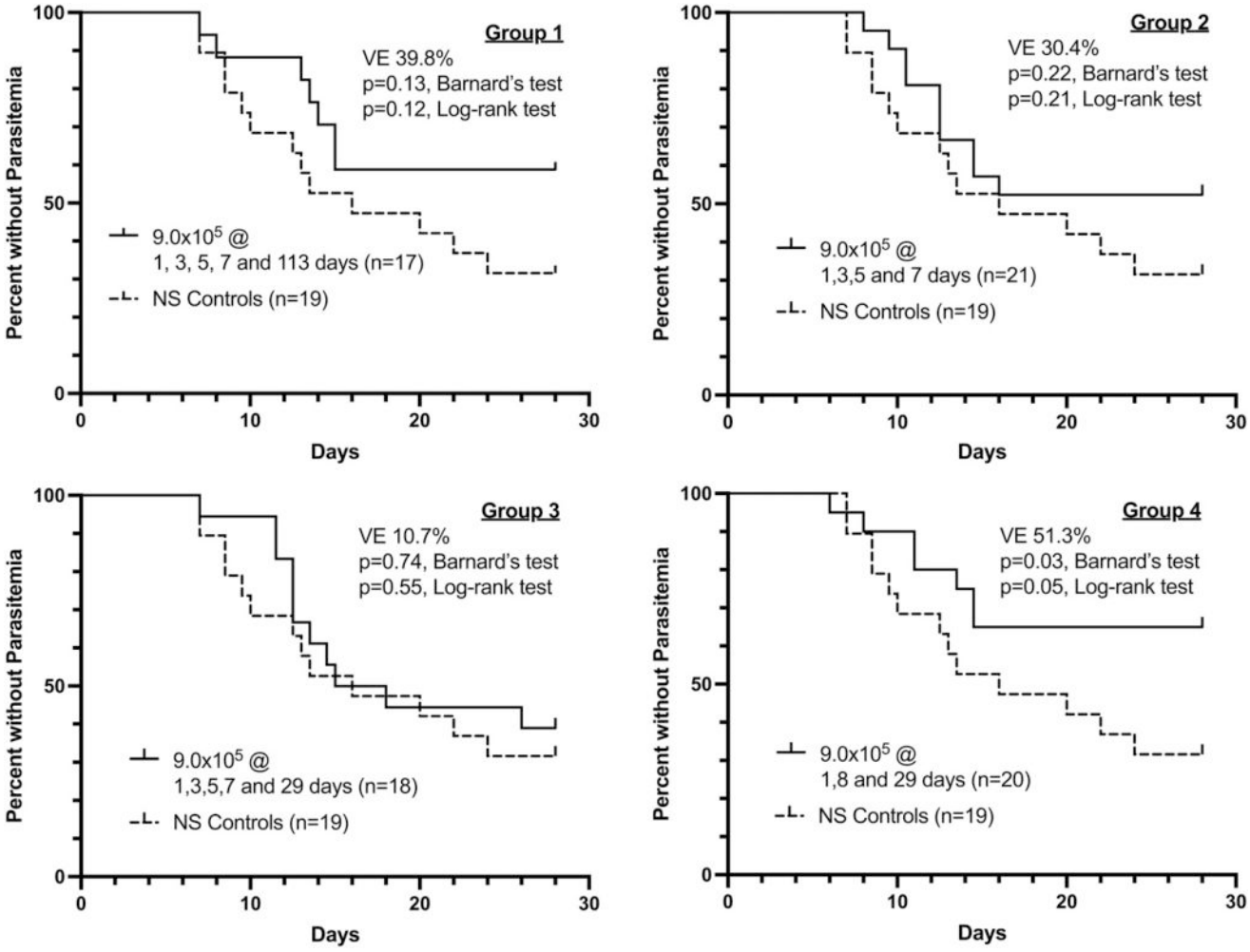

FIGURE 3. Kaplan–Meier survival curves in vaccinees and controls as assessed by quantitative polymerase chain reaction (qPCR).

PfSPZ Vaccine, Group 4. A total of 21 subjects were immunized with three doses of 9.0×10^5 PfSPZ of PfSPZ Vaccine on days 1, 8, and 29, and underwent CHMI 7 weeks after last vaccine dose. Seven were positive by qPCR (7/7 positive by TBS) and 14 were negative by both qPCR and TBS (Table 1). Vaccine efficacy at 7 weeks after last dose of vaccine was 51.3% ($P = 0.03$) (Figure 3).

Male versus female. There were no differences in VE among the men and women undergoing CHMI (data not shown).

Antibody responses. *Antibodies to PfCSP.* IgG antibodies against PfCSP measured by ELISA were assessed in participants from all groups prior to the first immunization, 2 weeks after the final immunization, and prior to CHMI. For each vaccine group, the median net OD 1.0 at 2 weeks after the final dose and 6 weeks after the final dose (prior to CHMI) were significantly higher than the median net OD 1.0 for the control subjects (Figure 4, $P < 0.0001$ for all comparisons, Wilcoxon–Mann–Whitney test). In the three groups who received vaccine doses at days 1, 3, 5, and 7, the median net OD 14 days later was similar for all three groups (Supplemental Figure 1) and was slightly lower 14 days after the fifth (final) dose in Groups 1 and 3. For the subjects within each vaccine group who received PfSPZ Vaccine, and separately for the pooled control group, there was no significant difference in median net OD 1.0 between the infected and uninfected subjects at either the post-immunization or pre-CHMI timepoint ($P > 0.25$ for all comparisons).

Antibodies to PfEXP1 and PfMSP1. IgG antibodies against PfMSP1 and PfEXP1 were assessed in participants from all groups prior to CHMI (Figure 5). In vaccine Groups 1, 2, and 4, and in the control group, the median antibody level to PfEXP1 was higher in subjects who were uninfected after CHMI; this difference was statistically significant for Group 2 and for the NS control group. The median antibody level to PfMSP1 was also higher in uninfected subjects for each group; the difference was statistically significant only for the NS control group. All antibody responses from the trial are provided in Supplemental Table 3.

Safety. *Solicited AEs following immunization.* No SAEs or grade 3 AEs, solicited or unsolicited, were reported by any subject participating in this trial prior to CHMI. Out of 84 vaccinees, 12 (14%) experienced 14 grade 1 local AEs compared with two (10%) NS controls who experienced three grade 1 local AEs ($P = 0.69$, Barnard's test, two-tailed) (Table 2). Nine of 84 (11%) vaccinees experienced 14 systemic AEs (12 grade 1, 2 grade 2) compared with no NS controls ($P = 0.13$). The most common solicited systemic AEs were headache (5) and arthralgia (5). No increase in AEs with successive doses was observed and only one subject reported a solicited systemic AE after the final dose. No subject in any group experienced an unsolicited AE that was considered related to immunization. There were no differences between any of the four groups with respect to the number or severity of AEs associated with vaccination.

Laboratory abnormalities following immunization. There were no significant differences (Barnard's test, two-tailed) in the number of subjects experiencing any laboratory abnormalities grade 2 or higher between vaccinees and controls. Grade 2 or higher laboratory abnormalities were reported in 18 vaccinees (21%) and seven controls (35%) ($P = 0.22$). The most commonly reported laboratory abnormality was increased eosinophils (15 vaccinees, six controls, $P = 0.25$), followed by increased AST (two vaccinees, one control, $P = 0.69$) and neutropenia (two vaccinees, zero controls, $P = 0.60$).

AEs after CHMI. For solicited AEs reported on days 1–5 after CHMI, three subjects reported tenderness at the site of injection, one subject reported mild headache, and one

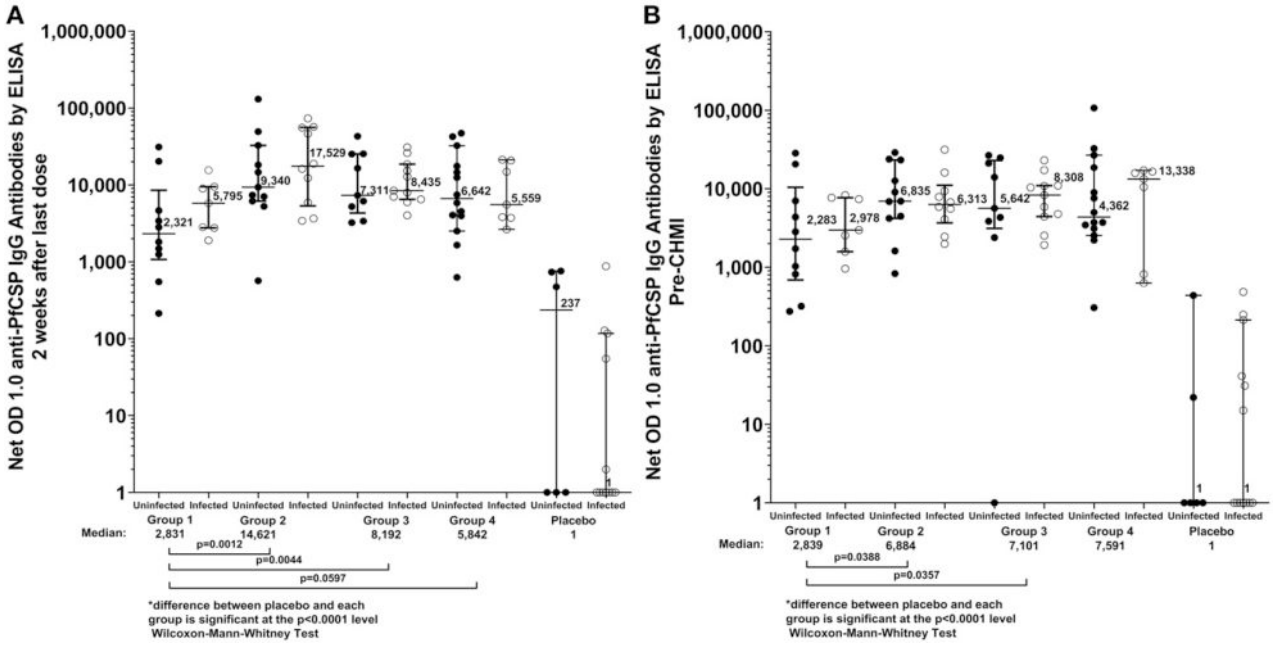

FIGURE 4. Antibodies to *Plasmodium falciparum* circumporozoite protein (PfCSP) by ELISA 2 weeks after the final vaccine dose (**A**) and immediately prior to controlled human malaria infection (CHMI) (6–7 weeks after the final vaccine dose) (**B**). For each group results are paired by subjects who were not infected (•) or infected (○) after CHMI.

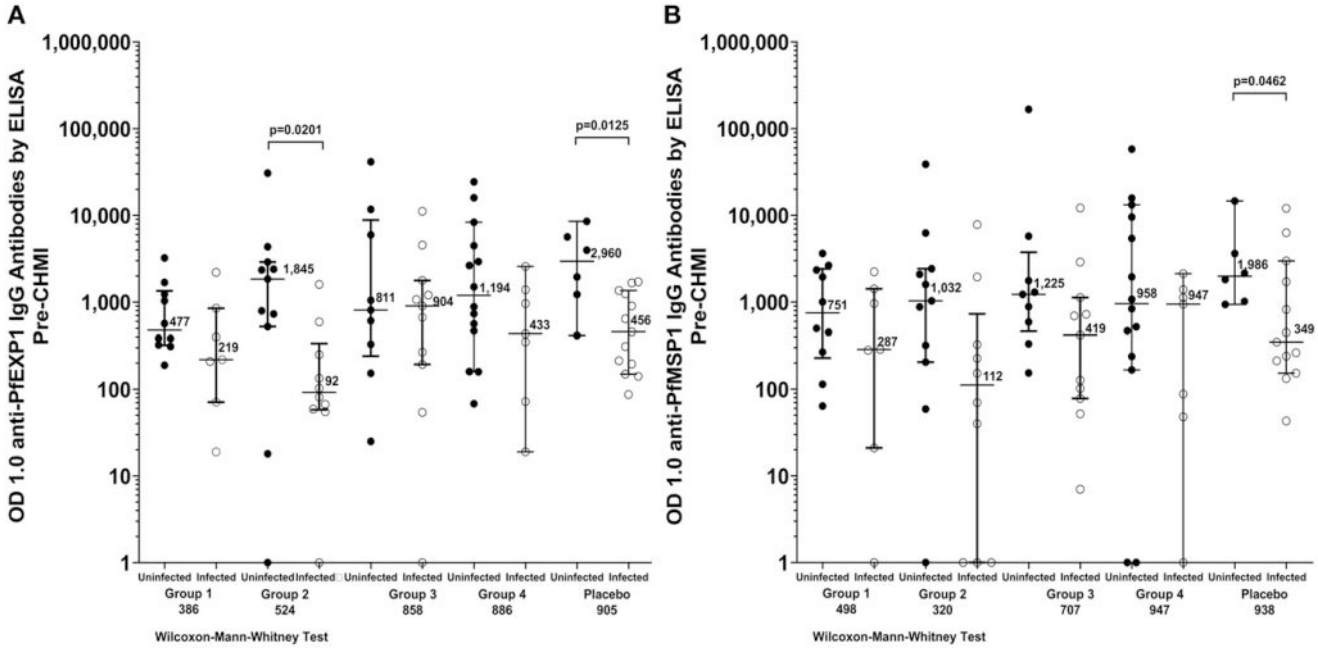

FIGURE 5. Antibodies to Pf exported protein 1 (PfEXP1) (**A**) and Pf merozoite surface protein 1 (PfMSP1) (**B**) by ELISA prior to controlled human malaria infection (CHMI). For each group, results are paired by subjects who were not infected (●) or infected (○) after CHMI.

reported diarrhea. For solicited AEs reported on days 6–28 after CHMI, those rated as grade 2 or 3 (11 AEs in six subjects) were all considered related to malaria as they correlated with the presence of parasitemia by TBS (all 11 AEs) and were highly correlated by time of initial presentation with the prepatent period by TBS ($r = 0.87$, $P = 0.0019$, Spearman correlation, two-tailed). Two subjects had grade 3 fever considered related to Pf infection—one vaccinee (fever on day 13, qPCR positive day 12.5, and TBS positive day 19) and one control (fever on day 18, qPCR positive day 13, and TBS positive day 19). In contrast, a grade 1 solicited AE on days 6–28 after CHMI corresponded to parasitemia by TBS in only 37 of 78 episodes (47%) and to parasitemia by qPCR in only 45 of 78 episodes, 58%).

DISCUSSION

The EGSPZV3 study was designed to assess and compare vaccination regimens with a goal to identify the regimen with an acceptable safety profile and the highest vaccine-induced protection for future clinical development. We first selected the best regimen from the Warfighter 2 trial in the United States,[15] which consisted of four priming doses on days 1, 3, 5, and 7 and a delayed final dose at 16 weeks (Group 1 in the EGSPZV3 trial, Figure 1). This multi-dose prime regimen had provided approximately twice the protection compared with three other regimens studied in the same study, each of which consisted of three widely-spaced single injections also administered over 16 weeks. Vaccine efficacy was 40% against a stringent heterologous CHMI at

TABLE 2
Solicited adverse events postvaccination (number of subjects experiencing AE)

	PfSPZ vaccine					Normal saline
	Group 1 ($N = 21$)	Group 2 ($N = 21$)	Group 3 ($N = 21$)	Group 4 ($N = 21$)	Total AE ($N = 84$)	Groups 1–4 ($N = 20$)
Any local solicited adverse event*	1 (4.8%)	3 (14%)	6 (29%)	2 (9.5%)	12 (14%)	2 (10%)
Tenderness	1 (4.8%)	3 (14%)	6 (29%)	1 (4.8%)	11 (13%)	1 (5%)†
Bruising	0	0	1 (4.8%)	0	1 (1.2%)	0
Swelling	0	0	0	1 (4.8%)	1 (1.2%)	0
Pruritus	0	0	0	0	0	1 (5%)‡
Any solicited systemic adverse event*	2 (9.5%)	3 (14%)	3 (14%)	1 (4.8%)	9 (11%)§	0§
Headache	0	1 (4.8%)	3 (14%)	1 (4.8%)	5 (6.0%)	0
Fatigue	0	1 (4.8%)	0	0	1 (1.2%)	0
Myalgia	1 (4.8%)	0	0	0	1 (1.2%)	0
Arthralgia	0	1 (4.8%)	2 (9.5%)	0	3 (3.6%)	0
Chills	0	0	1 (4.8%)	0	1 (1.2%)	0
Generalized pruritis	1 (4.8%)	0	0	0	1 (1.2%)	0

AE = adverse event; PfSPZ = *Plasmodium falciparum* sporozoites.
* Subjects may have more than one of the listed individual adverse events.
† Group 4.
‡ Group 1.
§ Comparison between vaccine and placebo for total AEs: $P = 0.13$, Barnard's test, two-tailed.

12 weeks ($P = 0.04$ using Barnard's test) and higher than the 20%, 21%, and 23% VE at 12 or 24 weeks shown by the other regimens, none of which achieved statistical significance.[15] However, the regimen required five doses and took 16 weeks to complete, a complex approach that would be sub-optimal to implement in the field. A second regimen demonstrating the best protection in a regimen-comparison trial used a two-dose prime (days 1 and 8) and a 4-week final dose, an approach offering distinct advantages in speed and simplicity (Group 4 in the EGSPZV3 trial, Figure 1). The regimen provided 83% VE against heterologous CHMI at 9.5 weeks in the MAVACHE trial in Germany (NCT02704533) (Mordmüller, unpublished).

To best compare these two leading regimens, we included two additional groups: the four-dose prime from Warfighter 2 without a final dose (Group 2), to determine whether a final dose was needed, and the four-dose prime from Warfighter 2 with a 4-week rather than 16-week final dose (Group 3) to match the 4-week final dose in Group 4, based on the premise that a final booster dose might be needed but did not depend on a 16-week delay. We further decided to conduct the study in a malaria-exposed population, as this is the most important target population for field application of PfSPZ-based vaccines.

In the current study, of the four regimens evaluated, only the 1-, 8-, and 29-day regimen gave statistically significant VE (51.3%) against homologous CHMI at 6–7 weeks. Based on these results, this dose regimen has been down-selected for future Phases 2 and 3 testing in malaria naïve and malaria preexposed populations. An appealing aspect of this regimen is that it is completed in 4 weeks' time, compared with earlier regimens that extended over 16–20 weeks.[2,3,6–8,10,11,14,15,17] This will increase the feasibility of MVPs where, to halt malaria transmission, entire populations need to be immunized as quickly and efficiently as possible.

It was unclear in this study why Group 4 outperformed Group 3, as the only difference between the two groups was a four-dose (Group 3) rather than a two-dose (Group 4) multi-dose prime. Recent data suggest that innate immune activation and specific myeloid signatures prevaccination diminish protection afforded by PfSPZ Vaccine in malaria-exposed African infants (Senkpeil, unpublished), and examination of whether four sequential doses might enhance these features is a subject for future study.

Although the EGSPZV3 trial met its overall objective, the analysis of VE in the four groups lost expected power because six of the 19 controls did not develop detectable Pf parasitemia after CHMI. In previous trials with PfSPZ Challenge (NF54) administered by DVI at a dose of 3,200 PfSPZ, parasitemia developed in 79/79 (100%) of malaria-naive control subjects[20–29] and in 35/35 limited malaria preexposed Tanzanian control subjects undergoing first CHMI[8,10] (Jongo, unpublished). Data from more heavily malaria-exposed populations provide a somewhat different picture, however. In our previous trial in Equatorial Guinea,[14] where malaria exposure is heavier than in Bagamoyo, Tanzania, 6/7 (86%) controls developed parasitemia by qPCR and 4/6 (67%) by TBS after CHMI. In a study in neighboring Gabon, among adults with normal hemoglobin (lacking sickle cell trait), 9/11 (82%) developed parasitemia by qPCR and 7/11 (64%) by TBS, and those with sickle cell trait were similar, with 7/9 (78%) developing parasitemia by qPCR, and 5/9 (56%) by TBS.[24] In a CHMI trial in Mali, where lifelong malaria exposure is particularly intense, using the same PfNF54 strain and 3,200 PfSPZ dose for CHMI as in the above studies, 8/15 (53%) control subjects developed Pf parasitemia by qPCR and only 1/15 (7%) had a positive TBS.[17] Thus, the control infection rate of 13/19 (68%) in our current study in Equatorial Guinea is not surprising.

We assessed antibodies to PfEXP1 and PfMSP1 prior to CHMI in order to determine why parasitemia did not develop in some controls. The uninfected control subjects had 6.4-fold higher antibody levels to PfEXP1 ($P = 0.013$) and 5.4-fold higher levels of antibodies to PfMSP1 ($P = 0.046$) than did the infected controls (Figure 5). This indicated that these uninfected subjects had naturally acquired immunity to Pf blood stage parasites that prevented them from developing parasitemia after administration of PfSPZ Challenge; three of the six controls who did not develop parasitemia had parasitemia detected by qPCR during the period of immunization, suggesting that infection acquired post artemether-lumefantrine pretreatment may have contributed to this naturally acquired immunity. The findings were similar in uninfected versus infected vaccinees following CHMI in the various vaccine groups, with uninfected vaccinees showing higher levels of antibodies to both PfEXP1 and PfMSP1 in all cases except for PfEXP1 in Group 3, and this difference achieved statistical significance for PfEXP1 in Group 2 (Figure 5). Another factor that might have affected results was sickle cell trait or other hemoglobinopathies, although these were not assessed based on the data provided above that the proportion developing parasitemia in Gabon following administration of PfSPZ Challenge (NF54) did not appear to be significantly influenced[24] by sickle cell trait. In Tanzania, alpha-thalassemia heterozygosity had no apparent effect on infectivity.[35]

The VE seen against homologous CHMI in this trial was moderate. In the same study population in Equatorial Guinea, three doses of 2.7×10^6 PfSPZ administered at 8-week intervals of PfSPZ Vaccine did not give significant VE (27%) against homologous CHMI at 15 weeks after last dose.[14] In the same earlier study, three doses of 1×10^5 nonattenuated (fully infectious) PfSPZ administered at 4-week intervals under cover of chloroquine (PfSPZ-CVac approach) conferred significant 55% VE against homologous CHMI at 14 weeks after the last dose despite using 9-fold fewer PfSPZ (3×10^5 versus 2.7×10^6 in Group 4). These data are consistent with the greater potency of PfSPZ-CVac in malaria-naive populations.[22,37]

In the current trial, there was no correlation between antibodies to PfCSP 2 weeks after the last dose of vaccine or just prior to CHMI and protection status (Figure 4). Antibody levels 2 weeks after the day 7 (fourth) dose were similar in Groups 1–3 (Supplemental Figure 1) and did not increase further post final dose in Groups 1 or 3. Furthermore, at the time of CHMI, Group 1 had significantly lower PfCSP antibody levels than Groups 2 and 3, a finding for which we have no explanation (Figure 4 and Supplemental Figure 1). Correlations between anti-PfCSP antibody levels (post-vaccination or pre-CHMI) and protection have been identified in some but not all prior CHMI studies in malaria-exposed African adults immunized with PfSPZ Vaccine,[8,11,14] and this inconsistency may be the product of the small sample sizes used in these studies. In contrast, when PfSPZ Vaccine has been evaluated for protection against naturally transmitted Pf malaria in the field, larger sample sizes have been studied, and have consistently shown statistically significant differences in

the anti-PfCSP antibody responses (or the fold rise in antibody responses) between those infected and those uninfected during the follow-up period (Sirima and Laurens, unpublished[7,17]). Because liver resident CD8 T cells are thought to underlie the protection induced by PfSPZ Vaccine but cannot be measured in the periphery,[3] the anti-PfCSP antibody results in field protection studies have been interpreted to be a marker for underlying cellular responses rather than a protective mechanism *per se*. It is possible that stronger associations between anti-PfCSP antibodies and protection would have been found in the current study had larger sample sizes been evaluated. At this point, it is not known how multi-dose priming may affect antibody or cellular responses to PfSPZ Vaccine.

PfSPZ Vaccine administered by DVI was safe and very well tolerated in all four regimens. Mild headache and mild arthralgia were the most commonly reported systemic AEs, but the frequency was not statistically different between vaccinees and controls or between study groups 1–4. There were no grade 3 AEs, no grade 3 laboratory abnormalities, and no SAEs attributed to administration of PfSPZ Vaccine.

Administration of PfSPZ Challenge for CHMI in this malaria-exposed adult population, using TBS positivity to initiate treatment of parasitemia resulting from CHMI, was also safe and well tolerated. There were very few AEs in the 5 days after inoculation, and these were all mild in severity. As in other studies in Africa and our previous experience in Equatorial Guinea, symptoms and signs consistent with malaria (those occurring on or after day 6, the time of first emergence of parasites from the liver into the bloodstream) were minimal.[8,11,14,17,24,35,38–40] Six of 47 subjects developing parasitemia experienced grade 2 AEs, two of whom concurrently developed grade 3 fevers. All grade 2 and 3 AEs were associated with Pf parasitemia detected by TBS and the onset of grade 2 and 3 AEs was highly correlated with the time of positive TBS. In contrast, grade 1 AEs appeared to reflect background rates in the community as they occurred with equal frequency in those who did not develop parasitemia.

In summary, we were encouraged that significant VE was demonstrated with an immunization regimen of 9.0×10^5 PfSPZ on days 1, 8, and 29. Achieving optimal VE is challenged by the effects of current and previous infections with Pf and possibly other *Plasmodium* species circulating on Bioko Island.[36] To better understand and define VE and these confounders, further studies with this regimen are planned or underway in the United States, Equatorial Guinea, Germany, and Mali in populations with the degree of prior Pf exposure varying according to the area of residence and the participant's age.

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CHAPTER 5: Optimization of recruitment and enrolment process through application of local population parameters

CHAPTER 5: Optimization of recruitment and enrolment process through application of local population parameters

5.1 General overview

This section provides details substantiating the description provided for thesis aim 2 in section 2.2. The need to involve communities where interventions are needed, in the early stages of product's clinical development plans, has revolutionized the process for improving access to interventions. Initial phase 1a studies are usually conducted in areas where the product has originated. More recently, even both phase 1a and phase 1b have been implemented using single protocols (Steiner-Monard et al., 2019). Thus, synchronization of procedures between protocols assessing similar products in Africa may necessitate extrapolation of recruitment and enrolment criteria with minor modifications. We have identified several issues that are fundamental and require more than minimal attention to ensure that, modifications that are made, do actually reflect the implementation of GCP compliant clinical trials in Africa. These include; availability of reliable means to identify volunteers (eg. National identification cards); availability of reliable means to maintain constant communication with the participants (eg limited network coverage and access to mobile phones); means of proper documentation of guardianship; local perception on testing for pregnancy and use of highly effective means of contraception as a requirement in clinical trials settings; community involvement; availability of routine medical check-up practices and medical records; risk of exposures to diseases; and hereditary disorders. Hence, to ensure GCP compliance and human subject protection, criteria for recruitment and enrolment of participants in clinical trials involving local population in Africa need to be optimized.

5.1.1 The process for criteria optimization

The process to optimize such criteria has two major elements; the first element defines how each criterion is linked to the protocol and the second element defines, how each criterion is linked to the population where the protocol will be implemented.

5.1.1.1 Defining the link between the criteria and the protocol

This first step is performed under the key assumption that, the primary source of recruitment and enrolment criteria are the key protocol elements including the design, objectives, methodology, target population and product characteristics as reported in pre-clinical or existing clinical data. Hence, each criterion in the existing or newly developed list of recruitment and enrolment criteria must be adequately justified in reference to such protocol elements. Additional criteria referenced to GCP and regulatory requirements that are relevant to the protocol elements must similarly be justified prior to their application.

During implementation of this work, each criterion was assessed by investigators and placed under the respective technical category as demonstrated in Figure *5*; (1) Criteria addressing GCP or regulatory compliance (2) Criteria assessing compliance to specific IP requirements (3) Clinical criteria requiring minimum clinical setup (4) Clinical criteria requiring specialized setup. This categorization may require technical input from team members that are competent in each respective category, sponsor or even staff from the ethical or regulatory bodies. This categorization, provides the potential alert mechanism to identify redundant and unnecessary criteria or gaps due to missing criteria in reference to each of the four technical categories. Furthermore, proper allocation of resources can be achieved depending on the technical category that the criteria is placed, including the opportunity to decide on where and how such criteria can be assessed. At this point, an outline of resources and logistics requirements can be developed and registered on the risk log for further quality process. This process of criteria categorization serves to generate an ideal but objective set of criteria for the protocol in either of the two scenarios; (1) during the development of new set of criteria or (2) during extrapolation of criteria to be applied for the different population. Next to this step, is the second element which defines, how each criterion is linked to the population where the protocol will be implemented, as detailed in section 5.1.1.2.

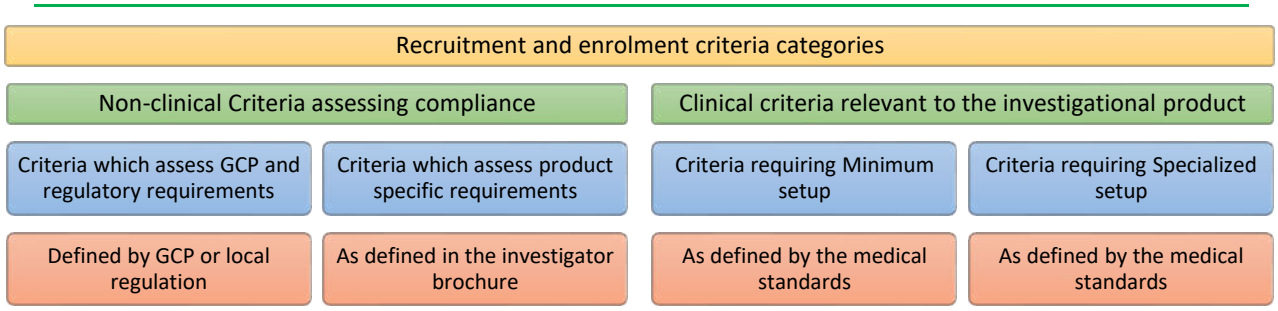

Figure 5: Criteria categories showing the two functional categories; each has two technical categories.

5.1.1.2 Defining the link between the criteria and the local target population

This second step is performed for the ideal criteria that have been adequately linked to the protocol elements as described in the previous section. Investigators and sponsor should work together to assess each criterion in reference to what is already known about the target population to which the criteria will be applied. This assessment may necessitate the revision of; existing data, lessons learned from previous experience or any other reliable source of information that will provide an indication of how best each of the criterion may be applied to the target population.

Situations such as, when a relatively newer intervention needs to be assessed, or newer clinical investigation site needs to be involved, may require additional active community engagement and / or piloting of procedures that can be used to assess such criteria during the implementation. Furthermore, capacity strengthening and / or capacity building for the team of investigators may be required. During the implementation of activities which are in the scope of this thesis, investigators and sponsor were faced by both situations; (i) to generate initial safety data set on the safety of PfSPZ based malaria vaccine candidates and related CHMI application in African population, (ii) to establish new clinical investigation site in Equatorial Guinea, that would complement and support early assessment in different African sites. The strategy to accomplish the linking of criteria for recruitment and enrolment included, (i) capacity-strengthening for the team with experience in conducting clinical trials in Tanzania (ii) capacity-building for the newer site established in Bioko Island, through active community engagement activities and community-based criteria assessment through pilot studies.

The rationale for the strategy outlined above, is that, (i) the experienced team in Tanzania would be tasked with the leadership role in a program of work that was anchored to the strong North-South and South-South collaboration of partner organizations from three continents (Africa, Europe and America) (ii) significant capacity building was required on the training and infrastructure development for the new clinical investigation site established in Equatorial Guinea (iii) both sites, needed prior preparatory community engagement plans to address several issues that are outlined in section 5.1.

During earlier studies conducted in Bagamoyo, pilot protocols were developed and obtained relevant IRB approvals. Each pilot assessment was specifically intended to address key issues linked to the clinical trial protocol that was subsequently implemented. Paper I, reported tolerability and safety results for the first trial in Africa to assess malaria infectivity by using PfSPZ-CHMI among healthy adults following immunization with PfSPZ Vaccine in Tanzania. Prior to implementation of this trial, the sponsor and investigators determined that, as part of the risk mitigation, potential volunteers should be recruited from the higher learning institutions in Dar es Salaam, to ensure that, participants to be enrolled would best address consenting and compliance matters related to this trial. Hence, the pilot process was implemented for testing of procedures that were associated with recruitment and screening of these volunteers in order to identify challenges that needed to be addressed. Pilot testing of recruitment and enrolment procedures was also performed prior to the implementation of the first ever assessment of feasibility, tolerability and safety of PfSPZ Vaccine in pediatric population in Tanzania (Paper II and Paper III). Furthermore, pilot testing of recruitment and enrolment producers was indicated and performed prior to the implementation of the first ever assessment of tolerability and safety of PfSPZ Vaccine in individuals living with HIV in Tanzania (Paper II and Paper III). In Equatorial Guinea, we conducted a pilot study to optimize recruitment and screening procedures for future clinical trials and to create a registry of (~2500) potential research participants on Bioko Island. Further details for the pilot study conducted in Equatorial Guinea are provided in section 5.2

5.1.1.3 Impact of recruitment and enrolment criteria optimization

The process to optimize recruitment and enrolment criteria that we described in section 5.1.1, bring together, the two components, one describing the process used to establish the link between the criteria and the protocol and other describing the process used to establish the link between the criteria to the population where the protocol will be implemented. The benefits of using such an approach, are beyond achieving the required sample size evident and can be seen right from the process itself. The timelines of how the trials were advanced is demonstrated in Table *2* indicating, a relatively smooth transition from one trial to the other, almost parallel with timely completion within the planned time frame, among those clinical trials conducted within the scope of this thesis work. For clinical trials that we conducted in the scope of this work, we used CONSORT diagrams to report disposition data of volunteers, with lost to follow-up rate of less than 10% despite the longer follow-up schedules during these initial trials. Beyond the individual trials, this process of optimization was the key to identify the specific needs and priorities for capacity strengthening and capacity building beyond the two centers where primary activities were conducted. Furthermore, clinical and laboratory data collected during the pilot protocol implementation, contributed significantly to optimizations needed for the development of local population-based tools for standardizing safety assessment, details of which are provided in section 6.2.

5.2 Paper VII: Submitted for Publication

Assessment of Health Status and Creation of a Registry of Potential Research Participants Age 1.5 to 50 Years on Bioko Island, Equatorial Guinea

Journal: *American Journal of Tropical Medicine & Hygiene*

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LRH: LÓPEZ MIKUE AND OTHERS

RRH: EQUATORIAL GUINEA MALARIA VACCINE PILOT STUDY

Assessment of Health Status and Creation of a Registry of Potential Research Participants Age 1.5 to 50 Years on Bioko Island, Equatorial Guinea

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Abstract

A program to control malaria established in 2004 on Bioko Island, Equatorial Guinea achieved significant reductions in malaria prevalence but thereafter progress stalled, leading to a proposal to develop and implement a highly effective malaria vaccine to increase impact. After conducting several clinical trials of Sanaria® PfSPZ vaccines against *Plasmodium falciparum* malaria demonstrating safety and efficacy, the Bioko Island Malaria Elimination Project (BIMEP) planned a larger Phase 3 trial to support vaccine licensure for specific target populations and eventual island-wide malaria elimination. The Equatorial Guinea Pilot Study for Recruitment, Screening and Participant Registry (EGRESPAR) assessed health status of the target population and generated a registry of eligible children and adults interested in participating in the trial. Households in areas with malaria prevalence ≥15% were randomly selected aiming to register 2100 healthy Equatoguineans divided equally into 1.5-9-, 10-17-, and 18-50-year age groups. 6493 persons from 1807 households completed questionnaires, 2021 were screened using Phase 3 enrollment criteria, 643 were excluded and 1378 were entered into the registry. Among those screened, 13.6% had *Plasmodium* and 1.8% *Loa loa* on thick blood smears (TBSs), 4.8% hepatitis B, 0.5% hepatitis C, and 2.1% HIV, with hepatitis and HIV leading to exclusion. Adults were 2-fold more likely to have clinically relevant medical conditions meeting exclusion criteria than children. In conclusion, there was a significant prevalence of infections and chronic medical conditions in the Bioko Island population, particularly in adults. EGRESPAR successfully generated a registry to support initiation of a large-scale Phase 3 vaccine trial.

Background

Malaria is a major public health problem in Equatorial Guinea with *Plasmodium falciparum* the most prevalent malaria species in the country and responsible for most malaria-related illness and death worldwide.[1] The prevalence of *P. falciparum* exceeds 50% in rural areas of the Equatorial Guinea mainland,[2,3] which constitutes more than 92% of the land area. Bioko Island, located 23 miles off the coast, is the site of the capital city Malabo and a high prevalence area like the mainland[4,5] until implementation of the Bioko Island Malaria Control Project (BIMCP) in 2004 by Medical Care Development International (MCDI), now MCD Global Health, under the supervision of the National Malaria Control Program (NMCP) of the Ministry of Health and Social Welfare of Equatorial Guinea (Ministerio de Sanidad y Bienestar Social de Guinea Ecuatorial (MINSABS)).[6-8] The program has focused on indoor residual spraying, distribution of long-lasting insecticide-impregnated bednets, larval source management, entomological monitoring and human case detection and treatment, these interventions tailored to local transmission characteristics determined by detailed yearly malaria indicator surveys. Following BIMCP implementation, the prevalence of *P. falciparum* parasitemia detected by rapid diagnostic tests (RDT) in 2-14-year-old children living on Bioko Island was reduced from 45% in 2004 to 14% in 2012.[9,10] However, progress stalled thereafter, despite continued intensive control efforts, and in 2023 prevalence was 13.8% (BIMEP Malaria Indicator Survey, unpublished data).

A consortium consisting of MINSABS, the Equatorial Guinea Ministry of Mines and several US-based energy companies (Marathon EG Production Limited, Noble Energy, Atlantic Methanol Production Company [AMPCO], and EG LNG) initiated funding of the Bioko Island Malaria Elimination Project (BIMEP) to support the conduct of clinical trials of Sanaria's aseptic, purified, cryopreserved, *P. falciparum* (Pf) sporozoite (SPZ) vaccines.[11] The BIMEP consortium

consisted of the funders, Sanaria Inc., MCD Global Health, the Ifakara Health Institute (Tanzania), Swiss Tropical and Public Health Institute (which provided additional funds) and the Malaria Research and Training Center (Mali) with technical support from the WHO. Four studies were conducted on Bioko Island and two in Bagamoyo, Tanzania to support vaccine development, licensure, and implementation. The four studies on Bioko Island were EGSPZV1, a randomized, double-blind, placebo-controlled trial (RCT) performed between March and September 2015 in 33 Equatoguinean men to assess the safety and immunogenicity of Sanaria® PfSPZ Vaccine (radiation-attenuated PfSPZ);[12] EGSPZV2, a RCT performed between November 2016 and January 2018 in 119 Equatoguinean adults, children and infants, to assess the safety and immunogenicity of PfSPZ Vaccine in younger and older age groups and additionally to compare the efficacy of PfSPZ Vaccine and a second vaccination approach, Sanaria® PfSPZ-CVac (CQ) (chemoprophylaxis vaccination: non-attenuated PfSPZ combined with administration of the antimalarial chloroquine [CQ] to attenuate the parasites *in vivo*) in adults, using controlled human malaria infection (CHMI) to assess efficacy;[13, 14] EGSPZV3, a RCT performed between August 2018 and April 2019 in 104 Equatoguinean adults to compare the efficacy against CHMI of four different PfSPZ Vaccine regimens with a goal to down-select the best regimen for ongoing development;[15] and EGMALEP, a cleared cohort study performed between January and September 2019 in 240 adults, children and infants to measure malaria incidence in preparation for a planned Phase 3 trial.[16] The two trials in Tanzania were BSPZV2, a RCT performed between December 2015 and August 2016 in 93 adults, children and infants to assess safety and immunogenicity in younger age groups (similar to EGSPZV2) and also to compare the efficacy against CHMI of different immunization regimens of PfSPZ Vaccine in adults;[17, 18] and BSPZV3a, a RCT performed between February and July 2018 to assess safety and efficacy of PfSPZ Vaccine against CHMI in 9 HIV- and 12 HIV+ Tanzanian adults.[19]

These clinical studies added to the findings from trials in several other African countries demonstrating that PfSPZ Vaccine was safe in African infants, children and adults,[20-25] could provide 78% to 100% protection against CHMI when administered by optimal routes and schedules;[25-28] and could provide 41-86% efficacy against naturally transmitted Pf infection in adults in the field[20, 24, 25](Diawara et al, submitted). The studies also demonstrated that the potency of PfSPZ-CVac (CQ) was higher than that of PfSPZ Vaccine[14] providing up to 100% protection against both homologous and heterologous strain CHMI at less than a quarter of the dose,[29, 30] although development of PfSPZ Vaccine remained prioritized over that of PfSPZ-CVac (CQ) for safety reasons.

Based on these results, the BIMEP initiated plans to conduct a randomized, double-blind, placebo-controlled Phase 3 trial of PfSPZ Vaccine on Bioko Island to provide pivotal data on field efficacy to support regulatory submissions to African regulatory agencies, the United States Food and Drug Administration (US FDA) and the European Medicines Agency (EMA), with a goal to licensure and subsequent program integration. The ultimate objective was to initiate mass vaccination programs (MVPs) to eliminate *P. falciparum* from Bioko Island and to immunize frequent travelers to the mainland to prevent re-importation.

The anticipated number of participants in the trial (2100) was several-fold higher than the trials previously conducted on Bioko Island, raising questions regarding the feasibility of recruiting and retaining such a large study population. To address such questions, the present study was designed, called the Equatorial Guinea Pilot Study for Recruitment, Screening and Participant Registry (EGRESPAR). The objectives were to optimize procedures and thereby minimize costs of significantly expanding recruitment, screening, and enrollment, to assess the health status of potential research participants, and to generate a Clinical Trials Registry of eligible individuals to be tapped for the Phase 3 trial. It was intended that EGRESPAR would sensitize

potential research participants and their communities to local PfSPZ vaccine development efforts and to promote the potential benefits of an island-wide vaccination program, thereby improving rates of recruitment, screening, and enrollment as well as cost profiles, study efficiency, participant compliance and resulting quality of data.

This study recruited participants from selected areas of Bioko Island in and around the capital city Malabo. Enrollment criteria reproduced those anticipated for the Phase 3 trial (**Supplementary Table 1**). Potential research participants were identified using the BIMEP's mapping system to identify households[31] and conduct home visits, and were then consented and screened at recruitment venues (tier 1 screening) and the clinical research center (CRC) (tier 2 screening). This paper reports on the findings in the 2021 participants screened before changing development priorities and the SARS-CoV-2 pandemic interrupted the study and halted preparations for the Phase 3 trial.

Methods

Study area and population

The study enrolled healthy Equatoguinean male and female children, adolescents and adults who were 18 months to 50 years of age at the time of screening. The study population came from localities within 12 administrative areas with relatively high malaria prevalence and low likelihood that the malaria present in the community was travel associated[32] (**Figure 1**). These communities were Fishtown III Ballares, Hacienda la Natividad I, Cipriano Tómo, San Luis II, Vigatana, Eulogio Oyo Riquesa, Vivienda Sociales detrás de GePetrol, Buena Esperanza II, Magdalena Mora, Sacriba Fang, Potao and Basupú. The population is composed of several ethnic groups, including Fang, Bubi, Annobones, Ndowe, Bisio, and Fernandino. Permission was obtained from local government officials and community representatives to use the

BIMEP/NMCP household database to randomly identify individuals to be invited to participate in the study [31]. Naturally acquired immunity (which manifests as reduced susceptibility to clinical disease) increases progressively with age in the study population, and could potentially affect vaccine trial outcomes, including safety, tolerability, immunogenicity, and efficacy.Therefore, these individuals were stratified into three balanced age groups (1.5-9, 10-17 and 18-50 years) to mirror the plan for similar age stratification in the Phase 3 trial.

Study sites

The EGRESPAR study was headquartered at the BIMEP Clinical Research Center (CRC) located in the Rotonda Arab area, Malabo, Bioko Island, but was conducted primarily through household visits and 5 satellite clinics located strategically in San Luis II, Eulogio Oyo Riquesa, Buena Esperanza II, Potao and Basupú (**Figure 1**). Serious illnesses in these areas are generally cared for at the government-owned Malabo Regional Hospital. There are also several private hospitals in close proximity to these communities. All five clinical sites were modified to serve as recruitment venues (RV) with sufficient office space, a registration desk, a waiting area, clinical examination rooms, a nursing area, space for supplies and storage, and internet access. The CRC site was upgraded to have a pharmacy and document archive. The clinical hematology and chemistry laboratories were located at the newly constructed Baney Research Center (Centro de Investigaciones de Baney) launched in February 2019.

Study design

EGRESPAR was a cross-sectional, non-interventional study lasting from September 2019 to March 2020. This single center study expanded the recruitment and screening approach previously approved by community leaders and used to enroll participants into the prior studies.[12-16]

Ethics review and approval

EGRESPAR received approval from the Equatorial Guinea National Ethics Committee (CENGE).[33] The study was also approved by two institutional review boards (IRBs) used by Tanzanian and Swiss collaborators, respectively: the Ifakara Health Institute (IHI) IRB and the Ethics Committee of Northwest and Central Switzerland (ENKZ).

Community engagement and sensitization

Prior to initiating recruitment, the clinical team met with MINSABS and local community leadership to explain the study, and launched information campaigns through radio, television, posters, and flyers. Permission to recruit was obtained from local officials. Subsequently, community meetings were held to sensitize the population to the study. Recruitment was done by door-to-door household visits and limited, IRB-approved pre-consent screening (**Supplementary Form 1**) during household visits to avoid scheduling individuals for consenting at RV visits who were clearly ineligible due to age or general health status. The number of individuals per age category per household which could be entered into the EGRESPAR registry was restricted to two to reduce the potential influence of household-related factors affecting trial endpoints in a future vaccine trial, including reduced transmission rates should a high proportion of household members receive the test vaccine.

Selection of study population and rationale

Study areas were selected in locations on Bioko Island where malaria prevalence was $\geq$15%, and the parasites appeared to locally acquired (as opposed to imported by travelers to the mainland) as determined by yearly malaria indicator surveys (MIS) conducted by the BIMEP that included travel history[32] and by the results of the prior incidence study.[16] The total local population residing within the study areas was 16,557 persons in 3,955 households. The 12

sectors with a malaria prevalence ≥15% included 11,890 persons in 2,786 households (2018 malaria indicator survey). Microsoft Visual Foxpro Version 9.0 package was used to randomly select and order households and then to select and order individuals within each age group within each household. Household selection was constrained to assure a balanced geographic distribution within each community.

Participant eligibility and screening

The goal of the initial home visit was to invite potentially eligible individuals to RVs for informed consent and first tier screening. Suitability for screening was determined during the home visit by the IRB-approved pre-consent screening criteria (**Supplementary Form 1** and **Supplementary Table 2**) which, if met, resulted in an invitation to the local RV in the next days. RV visits began with presentations about the study followed by informed consent. Adults were asked to provide written consent for themselves or their children, 9-17-year-olds were asked to provide written assent, and 6-8-year-olds to provide verbal assent. The clinical team then collected socio-demographic and medical history data and measured vital signs (respiratory and pulse rate, systolic and diastolic blood pressure, axillary temperature) and anthropometric indices including weight, height, and body mass index (BMI) and, for children age 11-17, Z-score BMI for age and for children below 11 years, Z-score weight for age (**Supplementary Tables 4a** and **4b**), to evaluate nutritional status. Individuals were not asked about gender identity. The individual being screened (or the parent or guardian) was notified at the end of the screening visit or later by telephone if he or she (or the dependent child) was excluded from further procedures (**Supplementary Table 3a**) or invited to the CRC for second tier screening (**Supplementary Table 3b**). At the CRC, ECGs and general physical examinations were performed (the latter without pelvic or rectal examinations unless indicated by medical history or other findings). Blood samples were collected for thick blood smear (TBS) for malaria

parasitemia (also used to screen for *Loa loa* or *Mansonella perstans* microfilaremia), hepatitis B (Hexagon HBsAG rapid test for hepatitis B surface antigen, Human Diagnostics Worldwide), hepatitis C (Hexagon HCV rapid test for hepatitis C antibodies, Human Diagnostics Worldwide) and HIV (Determine HIV1-2 Combo Test, Alere; Uni-Gold HIV-1/2 Test, Trinity; and SD Bioline HIV 1/2 Test, Standard Diagnostic Inc. for antibodies to HIV-1 and -2). Complete blood count with differential (neutrophils, lymphocytes, eosinophils) (Horiba ABX Pentra 60 C+ hematology analyzer), and biochemistry including alanine aminotransferase (ALT), creatinine, and random blood glucose (Roche Cobas Integra 400 plus chemistry analyzer) (Supplementary Tables 5a and 5b). Positive rapid tests for hepatitis B, hepatitis C and HIV were confirmed using Roche Cobas E411 ElectroChemiLuminescence (ECL) automated analyzer (**Supplementary Tables 5a** and **5b**). A urine sample was collected for human chorionic gonadotropin pregnancy testing in females aged 9-45 years. Pre-test (and later post) test counseling was provided for hepatitis and HIV assessments.

Those with a history or clinical manifestations of serious or chronic disease (**Supplementary Tables 1, 3a** and **3b**) including hypertension, cardiac disease, diabetes, renal disease, hepatitis B or C or HIV infection were excluded and appropriate healthcare referrals made for further care. Also excluded were those perceived to have increased risk of non-adherence to study procedures, including the intention to move from the study area during the anticipated Phase 3 study period. Participants with minor illnesses or found to be malaria, *L. loa* or *M. perstans* positive were treated by the study team clinician according to local treatment guidelines and could be included[34]. Those passing all eligibility criteria were asked if they were willing to have their names and contact information entered into the Clinical Trial Registry.

Compliance with study visits and procedures

Longitudinal compliance with study visits and procedures was encouraged by using multiple contact methods (phone calls to the participant, home visits by community mobilizers, and phone calls to close contacts as needed), transportation to the RV or CRC if needed, and compensation for attending each visit. A participant received 3,000 Central African Francs (just over 5 U.S. dollars) in cash for visits to RVs and the CRC. In addition, light snacks/drinks were provided at the RV and CRC. Payment was made to a parent or guardian in the case of minors and was specified that the payment should be used to support the child's well-being or advancement. In addition, participants were reimbursed for the cost of transport to the RV or CRC (for scheduled but not unscheduled visits) in case the participant was unable to use transport provided by the study. Study doctors were available 24 hours a day, 7 days a week to attend study participants who had signed consent forms and not yet reached study end, which was defined as the point where the individual was excluded or, if enrolled, their name was or was not entered into the registry.

Data management

At the household level, information was collected using the ODK software system (https://getodk.org) using tablets. At the RV and CRC information was collected on paper-based case reports forms and these were entered into a customized electronic database, Castor EDC^{R}. Data were entered and verified by independent teams.

Sample size

This was a descriptive study with no hypotheses testing and hence the number of participants to be included in the registry was driven by the anticipated sample size for the Phase 3 trial, which would require 2100 research participants. The study originally targeted approximately

3000 individuals equally divided into three age categories, but this goal was not met due to study interruption (see below).

Statistical analysis

Descriptive statistical assessment was performed. The number of persons screened, the number of screen failures, and the reason for screen failure and enrollment were tabulated overall and by age group and sex. Demographic, laboratory as well as clinical characteristics were summarized in tables and figures. Frequency tables were used to summarize distributions of categorical data while continuous data were summarized using mean, standard deviation and range. STATA (version 15; Stata Corp, College Station, TX, USA) and R Statistical Software (version 3.4.3, https://www.r-project.org/) were used for summary statistics and graphical analysis, respectively.

Results

Door to door canvasing of households

Following the randomized, hierarchical sampling frame used to select households and individuals from the BIMEP/NMCP household database (see Methods), door-to-door canvasing of the pre-selected households and household members was conducted from 09 September 2019 to 18 February 2020. 6493 individuals living in 1807 (77.7%) of the 2325 pre-selected households that were visited during this period expressed an interest in participating (or in their dependents participating) and responded to the IRB-approved pre-consent screening questionnaire, while in 36 (1.5%) households there was no interest in participating. 251 (10.8%) additional households had no eligible volunteer, 92 (4.0%) were uninhabited, 79 (3.4%) had no phone to maintain contact with the study team, 56 (2.4%) were closed, and 4 (0.2%) could not be located (**Figure 2**).

Disposition of screened participants at recruitment venue and CRC

The questionnaires from 6493 interested individuals were reviewed. 5110 (78.7%) were invited to the RV for screening and of these, 2021 (39.5%) attended the RV and 3089 (60.5%) were on the waiting list to attend the RV when the study was ended. An additional 715 (11.0%) were invited but were lost to follow up prior to the RV visit, 29 (10.3%) were excluded because they would have exceeded their age category limit per household, and 639 were kept in reserve as back-ups (**Figure 2**). At the RV visit, all 2021 attendees, which included 599, 464 and 958 individuals in the 1.5-9 years, 10-17 years, and 18-50 years respectively, signed consent or provided assent to proceed with screening (**Table 1**). 418 (20.7%) of 2021 failed the screening criteria applicable at the first tier RV level, accounting for 10.7%, 12.7% and 30.8% of total screened in age categories 1.5-9 years, 10-17 years, and 18-50 years respectively (**Table 1** and **Supplementary Table 3A**). Major reasons included unwillingness to meet contraception requirements (n=110, with 94 in the 18-50 year age category and 16 in the 10-17 year age category), anthropometric parameters outside the specified range (n=107, with 93 cases [87%] in adults) and medical history, symptoms, signs and/or laboratory values suggestive of a systemic disorder or chronic illness (n=104, with 78 cases [75%] in adults). An additional 225 (11.1%) failed the second-tier screening criteria at the CRC accounting for additional 3.8%, 7.8% and 17.3% of total screened in the three age categories (**Table 1** and **Supplementary Table 3B**). The main reasons for exclusion overall were medical history, symptoms, signs and/or laboratory values suggestive of a systemic disorder or chronic illness (n=180, with 138 cases [77%] in adults), positivity for HIV or hepatitis (n=118, with 93 cases [79%] in adults), history of arrhythmias, prolonged QT interval or other cardiac disease (n=30, with 26 cases [87%] in adults). Overall, non-clinical reasons for exclusion such as not residing in the selected community, not willing or unable to attend the required appointments at the CRC for the next

two years and not agreeing to be contacted by telephone and by home visit were equally common in all three age groups (**Supplementary Table 3A)**. Clinical reasons such as anthropometric parameters outside reference interval, medical history, symptoms, signs and/or laboratory values suggestive of a systemic disorder or chronic illness and not sufficiently healthy as per the clinical judgement based on medical history, examination and investigations done in this study including positive HIV, hepatitis B virus or hepatitis C virus tests were most common in adults (**Supplementary Table 3A, Supplementary table 3B).** 1378 (68.2%) met all eligibility criteria and agreed to be entered into the Clinical Trial Registry, including 512, 369 and 497 individuals in the 1.5-9 years, 10-17 years, and 18-50 years age categories, respectively. These were 85.5%, 79.5% and 51.9% of total screened in these categories (**Figure 2** and **Table 1**).

Health data from RV screening results

Any abnormalities in vital signs (HR, RR, BP, T) and anthropometric measurements (BMI and BMI or weight for age) from the 2021 participants undergoing tier 1 screening were assessed for clinical significance by study clinicians and reviewed against age-relevant reference intervals (**Supplementary Tables 4a** and **4b**) and protocol eligibility criteria (**Supplementary Table 1**). The distributions of clinical parameters (pulse rate, respiratory rate, axillary temperature, systolic and diastolic blood pressure, QTcF) are presented in **Figure 3**. The numbers of persons with clinical parameters sufficiently outside the reference interval to be deemed clinically relevant for exclusion were: elevated pulse rate 16 (0.8%), elevated respiratory rate 11 (0.5%), elevated axillary temperature 8 (0.4%), elevated systolic blood pressure 3 (0.2%), elevated diastolic blood pressure 5 (0.3%), elevated systolic and diastolic pressures 30 (1.5%) and abnormal ECG 22 (1.4%). Findings for each age category are provided in **Table 2A**. The numbers of participants outside the reference interval and deemed

clinically relevant for exclusion for each anthropometric parameter are provided in **Table 2B**. For BMI among the adult group, 93 (9.7%) had BMI outside the reference interval. Four (0.4%) had values below the lower limit (18 kg/m^2) and 89 (9.3%) had values above the upper limit (30 kg/m^2). Among the children, 14 (1.3%) children had their Z-score [BMI for age (children aged 11 years to 17 years) or weight for age (children below 11 years)] outside the reference interval. Two (0.2%) had values below the lower limit (< (-2SD)) and 12 (1.1%) had values above the upper limit (> (+2SD)) (see **Supplementary Table 4a** and **4b**).

Health data from CRC screening results

Among the 1603 participants who attended the CRC visit: 217 (13.6%) were TBS positive for malaria accounting for 11.7%, 18.9% and 12% of the total tested in age categories 1.5-9 years, 10-17 years and 18-50 years respectively; 28 (1.8%) were *L. loa* positive accounting for 0.4%, 1.2% and 3.2% of total tested in the same age categories; 34 (2.1%) were HIV positive accounting for 0%, 0.3% and 5% of total tested in the same age categories; 77 (4.8%) were hepatitis B surface antigen positive accounting for 0.6%, 4.5% and 8.5% of total tested in the same age categories; and 8 (0.5%) were hepatitis C antibody positive accounting for 0.4%, 0.3% and 0.8% of total tested in the same age categories (**Table 2C**). All participants found positive for malaria and *L. loa* were treated and post treatment tests were performed to confirm negative status and eligibility for inclusion. Individuals who tested positive for HIV and hepatitis were excluded and provided with post-test counselling and referred to relevant treatment centers for further management. Safety laboratory parameters most frequently found to be outside reference interval were eosinophils, elevated in 365 (22.8%) [upper limit of normal 1.6*10^3/µl (1.5-9 years), 2.1*10^3/µl (10-17 years), 0.78*10^3/µl (18- 50 years)] and random blood glucose, elevated in 303 (19%) [upper limit of normal 97.3 mg/dL (1.5-9 years), 95.5 mg/dL (10-17 years), 109.8 mg/dL (18- 50 years)]. Hemoglobin was lower than the reference interval

[8.6-13.2 g/dL (1.5-9 years), 9.6-14.1 g/dL (10-17 years) and 12-17.4 g/dL (18-50 years males), 9.60-14.10 g/dL (18-50 years females)] in 179 (11.2%) participants and this occurred with the greatest frequency in 10-17-year-olds (22%) (**Table 2D**). The distribution of laboratory parameters is presented in **Figure 4**.

Characterization of participants entered into EGRESPAR registry

A total of 1378 participants were eligible and agreed to be entered into the Clinical Trials Registry [by age category: 512 (1.5-9 years), 369 (10-17 years) and 497 (18- 50 years)]. The overall mean age ±SD in years of those entered was 15.9±10.9 (**Table 3**). The mean age +/- SD in years for those in the three age categories were 5.9±2.5, 13.4±2.2 and 28.1±8.1, respectively. Overall, similar numbers of males and females were enrolled. The percentages of females in age categories 1.5-9 years, 10-17 years, and 18-50 years were 53.1%, 51.2% and 42.7% respectively. The number of participants from the selected 12 administrative areas of the Bioko Island were: Fishtown III Ballares 66, Hacienda la Natividad I 27, Cipriano Tómo 59, San Luis II 57, Vigatana 80, Eulogio Oyo Riquesa 23, Vivienda Sociales detrás de GePetrol 412, Buena Esperanza II 131, Magdalena Mora 35, Sacriba Fang 24, Potao 52 and Basupú 412.

Early termination of EGRESPAR

While the study was underway, development priorities shifted based on the creation of a new genetically-attenuated PfSPZ parasite demonstrating a replication-competent, late-liver stage arresting (LARC) phenotype developed by Sanaria and its collaborators at the Seattle Children's Research Institute (Goswami submitted). This new vaccine, PfSPZ-LARC2 Vaccine, held the potential for safety and tolerability equal to PfSPZ Vaccine combined with superior efficacy achieved at less than one fourth the dose, improving the chances of successful malaria

elimination and reducing the cost of goods. However, as the vaccine had not yet been clinically tested, this pivot was not compatible with the timelines specified by the BIMEP funders. Also, in mid-February 2020, the SARS-CoV-2 pandemic reached Africa, leading to cessation of household visits and a month later to the closure of the study when the clinical team, clinical laboratory and funding were re-assigned by MINSABS to confronting the pandemic.

Discussion

P. falciparum malaria is a persistent health concern on Bioko Island [3], mirroring the situation in much of sub-Saharan Africa.[1] The BIMEP, one of Africa's most comprehensive and well-funded malaria control programs[33] achieved progressive reductions in malaria prevalence during the first eight years of implementation, but impact plateaued, leading to an ambitious plan to develop, license and field a malaria vaccine chosen for its potential to block infection. Sanaria's PfSPZ vaccines were selected for development, and a consortium of funders and collaborators initiated the BIMEP with a long-term goal of supplementing traditional malaria control measures with mass vaccination programs to eliminate *P. falciparum* transmission on Bioko Island. Six clinical trials were completed toward this objective including five assessing radiation-attenuated PfSPZ Vaccine and one assessing PfSPZ-CVac (CQ). The demonstrations of safety, good tolerability and protection against CHMI from these trials[12-15, 17-19], combined with the results of other studies of PfSPZ vaccines in the US, EU and Africa[20-28, 35-39], led to the planning of a Phase 3 trial for licensure. The Equatorial Guinea Pilot Study for Recruitment, Screening and Participant Registry (EGRESPAR) was conducted to optimize recruitment practices, assess the health status of the target population and generate a registry of eligible individuals in three age groups. The study used the eligibility criteria planned for the Phase 3 trial.

The participants in this study were from areas of higher malaria prevalence in and around Malabo. They considered themselves (or were considered by their parents) to be healthy. Tier

1 screening of 2021 individuals at the recruitment venues focused on medical history, vital signs and anthropometric measurements and resulted in 418 (20.7%) exclusions. Clinically relevant abnormalities sufficient for exclusion were evident on medical history or physical examination including chronic illnesses and obesity in 187 cases (**Tables 2A**, **2B** and **2C**). A similar number of women were unwilling to follow the strict pregnancy prevention measures required. The 1603 (79.3%) persons who passed Tier 1 assessment were further screened by ECG, physical examination and laboratory tests at the CRC, including tests for malaria, *L. loa*, HIV, hepatitis B and C, and an additional 225 were excluded, with 118 of these because of chronic infections. As expected, there were variations in the prevalence of chronic infections among participants in different age categories (**Table 3B)**. Except for malaria prevalence, which was highest in adolescents, the prevalence of other conditions was at least two-fold higher among adults compared to children.

The screening parameters used at recruitment venues and for laboratory assessment at the CRC for the present study resulted in higher exclusion rates among adult volunteers than children. These exclusions reflected the concern that chronic medical conditions could potentially interfere with interpretation of results and jeopardize the safety of participants selected for the Phase 3 vaccine trial.

Overall, there was a balanced sex ratio achieved for the registry, with slightly higher percentage of females among children and higher percentage of males in adults, the latter explained by the unwillingness of many women to meet requirements for avoidance of pregnancy and breast feeding. There was variation in the number of participants from the different administrative areas. The highest number of participants were registered from the densely populated semi-rural (Basupú) and urban (Vivienda Sociales de Gepetrol and Buena Esperanza) locations. Other areas were less densely populated with households dispersed over larger areas. The

majority of participants had age-appropriate levels of education (**Table 3**). The majority of adults indicated either secondary school education (71.6%) or university level (20.8%), with less than 8 percent indicating primary school only or illiterate.

EGRESPAR was halted by changing development priorities and the COVID-19 pandemic prior to reaching the target numbers for the registry but still largely achieved its major objectives, including establishment of the stepwise screening approach beginning at the community level (recruitment venues) and moving to the clinic level (CRC), enabled the study team to optimize the use of limited resources, a key consideration for reducing Phase 3 costs and accelerating timelines. The selection, upgrade and practical use of 5 satellite sites and the BIMEP CRC created the infrastructure needed for administration of investigational products and clinical follow-up during the Phase 3 program. Furthermore, conduct of EGRESPAR improved knowledge of the disease dynamics of the study areas, supported capacity building, and gave confidence to the implementation teams that the conduct of a 2100-person trial was feasible. Despite its interruption and the postponement of further Phase 3 planning, EGRESPAR provided a foundation for the success of any future clinical trials research on Bioko Island.

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Disclosures

Laurence Lemiale, B. Kim Lee Sim, L. W. Preston Church, Peter F. Billingsley, Stephen L. Hoffman, and Thomas L. Richie were fulltime employees of Sanaria Inc. at the time the study was conducted. The other authors declare that they have no competing interests.

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Figure legends

Figure 1: Map of the EGRESPAR study area showing the locations and (number) of sectors (small squares) containing households that were visited, color-coded by malaria prevalence in 6-10-year-olds (see key). All sectors were in 12 administrative areas of Bioko Island (black borders).

Figure 2: Consort diagram for the household and participant disposition.

Figure 3. Distributions of clinical parameters of volunteers participated in EGRESPAR study, stratified by age and sex). Panels show the distribution of (a) pulse rate (beats/min), (b) respiratory rate (breaths/min), (c) axillary temperature (°C), (d) systolic blood pressure (mmHg), (e) diastolic blood pressure (mmHg), and (f) iQTcF intervals (ms). The QT intervals were corrected for the effect of heart rate using the Fridericia formula. Boxes show the median and the upper and lower quartiles, and the whiskers define maximum and minimum values excluding outliers.

Figure 4. Distributions of laboratory parameters of volunteers participated in EGRESPAR study, stratified by age and sex. Panels a to g are showing the hematological parameters and h to j are showing the biochemistry parameters. Panels show the distribution of (a) white blood cells (10^3/µl), (b) red blood cells (10^6/µl), (c) hemoglobin (g/dL), (d) platelets (10^3/ µl), (e) lymphocytes (10^3/µl), (f) neutrophils (10^3/µl), (g) eosinophils (10^3/µl), (h) alanine aminotransferase (U/L), (i) blood glucose (mg/dL), and (j) creatinine (mg/dL). Boxes show the median and the upper and lower quartiles, and the whiskers define maximum and minimum values excluding outliers.

Table 1: Disposition of 2021 participants who provided consent for screening

Description	All	Age groups in years		
		1.5-9	10-17	18-50
Provided consent	2021	599	464	958
Eligible at recruitment venue (RV)	1603 (79.3)	535 (89.3)	405 (87.3)	663 (69.2)
Eligible at clinical research center (CRC)	1395 (87.0)	518 (96.8)	373 (92.1)	504 (76.0)
Entered in EGRESPAR registry	1378 (98.8)	512 (98.8)	369 (98.9)	497 (98.6)

Values are n (%); denominators for calculating % in each row are the n's in the row above.

Table 2A: Volunteer numbers (%) with clinical parameters outside reference interval at RV screening and with ECG parameters outside reference interval (with or without clinically significant abnormalities) at CRC screening

Parameter	Group	Total =2021 (936 male, 1085 female)		Age group					
				1.5 - 9 years n=598 (284 male, 314 female)		10 - 17 years n=465 (214 male, 251 female)		18 - 50 years n=958 (438 male, 520 female)	
		NCS N (%)	CS N (%)	NCS N (%)	CS N (%)	NCS N (%)	CS N (%)	NCS N (%)	CS N (%)
Pulse rate	All	335 (16.6)	16 (0.8)	191 (31.9)	9 (1.5)	51 (11.0)	1 (0.2)	93 (9.7)	6 (0.6)
	Male	160 (17.1)	10 (1.1)	87 (30.6)	7 (2.5)	19 (8.9)	1 (0.5)	54 (12.3)	2 (0.5)
	Female	175 (16.1)	6 (0.6)	104 (33.1)	2 (0.6)	32 (12.7)	0	39 (7.5)	4 (0.8)
Respiratory rate	All	649 (32.1)	11 (0.5)	256 (42.8)	8 (1.3)	159 (34.2)	2 (0.4)	234 (24.4)	1 (0.1)
	Male	263 (28.1)	7 (0.7)	123 (43.3)	5 (1.8)	62 (29.0)	2 (0.9)	78 (17.8)	0
	Female	386 (35.6)	4 (0.4)	133 (42.4)	3 (1.0)	97 (38.6)	0	156 (30.0)	1 (0.2)
Axillary Temperature	All	367 (18.2)	8 (0.4)	137 (22.9)	4 (0.7)	92 (19.8)	3 (0.6)	138 (14.4)	1 (0.1)
	Male	161 (17.2)	4 (0.4)	57 (20.1)	4 (1.4)	39 (18.2)	0	65 (14.8)	0
	Female	206 (19.0)	4 (0.4)	80 (25.5)	0	53 (21.1)	3 (1.2)	73 (14.0)	1 (0.2)
Systolic blood pressure only	All	372 (18.4)	3 (0.1)	130 (21.7)	0	100 (21.5)	0	142 (14.8)	3 (0.3)
	Male	122 (13.0)	3 (0.3)	54 (19.0)	0	36 (16.8)	0	32 (7.3)	3 (0.7)
	Female	250 (23.0)	0	76 (24.2)	0	64 (25.5)	0	110 (21.2)	0
Diastolic blood pressure only	All	218 (10.8)	5 (0.2)	76 (12.7)	1 (0.2)	33 (7.1)	1 (0.2)	109 (11.4)	3 (0.3)
	Male	97 (10.4)	3 (0.3)	33 (11.6)	1 (0.4)	17 (7.9)	1 (0.5)	47 (10.7)	1 (0.2)
	Female	121 (11.2)	2 (0.2)	43 (13.7)	0	16 (6.4)	0	62 (11.9)	2 (0.4)
Both systolic and diastolic	All	178 (8.8)	30 (1.5)	101 (16.9)	1 (0.2)	30 (6.5)	0	47 (4.9)	29 (3.0)
	Male	103 (11.0)	13 (1.4)	55 (19.4)	1 (0.4)	19 (8.9)	0	29 (6.6)	12 (2.7)
	Female	75 (6.9)	17 (1.6)	46 (14.6)	0	11 (4.4)	0	18 (3.5)	17 (3.3)
		n = 1602 (806 male, 796 female)		n=534 (251 male, 283 female)		n=406 (197 male, 209 female)		n=662 (358 male, 304 female)	
ECG	All	444 (27.7)	22 (1.4)	165 (30.9)	2 (0.4)	100 (24.6)	2 (0.5)	179 (27.0)	18 (2.7)
	Male	274 (34.0)	14 (1.7)	82 (32.7)	1 (0.4)	58 (29.4)	2 (1.0)	134 (37.4)	11 (3.1)
	Female	170 (21.4)	8 (1.0)	83 (29.3)	1 (0.4)	42 (20.1)	0	45 (14.8)	7 (2.3)

Values are n (%); CS, clinically significant ; NCS, not clinically significant

Reference ranges are available in Supplementary Table 5.

Table 2B: Numbers (%) of adults (BMI) and children (BMI/Weight for age) with anthropometric parameters outside reference interval at screening

Parameter (reference interval)		Total		Age group					
				1.5 - 9 years		10 - 17 years		18 - 50 years	
BMI (18 to 30 kg/m^2) (n=958)	Total	958		NA		NA		958	
	Male	438		NA		NA		438	
	Female	520		NA		NA		520	
		<LLRi N (%)	>ULRi N (%)	<LLRi N (%)	>ULRi N (%)	<LLRi N (%)	>ULRi N (%)	<LLRi N (%)	>ULRi N (%)
	All	4 (0.4)	89 (9.3)	NA	NA	NA	NA	4 (0.4)	89 (9.3)
	Male	1 (0.2)	16 (3.7)	NA	NA	NA	NA	1 (0.2)	16 (3.7)
	Female	3 (0.6)	73 (14.0)	NA	NA	NA	NA	3 (0.6)	73 (14.0)
BMI/Weight for age (Z score ± 2 SDs) (n=1063)	Total	1063		598		465		NA	
	Male	498		284		214		NA	
	Female	565		314		251		NA	
		<LLRi N (%)	>ULRi N (%)	<LLRi N (%)	>ULRi N (%)	<LLRi N (%)	>ULRi N (%)	<LLRi N (%)	>ULRi N (%)
	All	2 (0.2)	12 (1.1)	1 (0.2)	3 (0.5)	1 (0.2)	9 (1.9)	NA	NA
	Male	1 (0.2)	4 (0.8)	1 (0.4)	2 (0.7)	0	2 (0.9)	NA	NA
	Female	1 (0.2)	8 (1.4)	0	1 (0.3)	1 (0.4)	7 (2.8)	NA	NA

LLRi, lower limit of reference interval; ULRi, upper limit of reference interval; BMI, body mass index

Table 2C: Number (percent) of individuals with infections among participants screened at CRC

category		Total	Age groups		
			1.5 - 9 y	10 - 17 y	18 - 50 y
	Total	1599	533	405	661
	Male	806	251	197	358
	Female	793	282	208	303
Plasmodium total (% of total screened)	All	217 (13.6)	62 (11.6)	76 (18.8)	79 (12.0)
	Male	115 (14.3)	30 (12.0)	36 (18.3)	49 (13.7)
	Female	102 (12.9)	32 (11.3)	40 (19.2)	30 (9.9)
P. falciparum only (% of total positive)	All	185 (85.3)	50 (80.6)	64 (84.2)	71 (89.9)
	Male	95 (82.6)	23 (76.7)	31 (86.1)	41 (83.7)
	Female	90 (88.2)	27 (84.4)	33 (82.5)	30 (100)
P. falciparum + P. malariae (% of total positive)	All	30 (13.8)	11 (17.7)	11 (14.5)	8 (10.1)
	Male	18 (15.7)	6 (20.0)	4 (11.1)	8 (16.3)
	Female	12 (11.8)	5 (15.6)	7 (17.5)	0
P. falciparum + P. ovale (% of total positive)	All	1 (0.5)	0	1 (1.3)	0
	Male	1 (0.9)	0	1 (2.8)	0
	Female	0	0	0	0
P. falciparum + P. malariae + P. ovale (% of total positive)	All	1 (0.5)	1 (1.6)	0	0
	Male	1 (0.9)	1 (3.3)	0	0
	Female	0	0	0	0
Loa loa	All	28 (1.8)	2 (0.4)	5 (1.2)	21 (3.2)
	Male	18 (2.2)	0	2 (1.0)	16 (4.5)
	Female	10 (1.3)	2 (0.7)	3 (1.4)	5 (1.7)
HIV	All	34 (2.1)	0	1 (0.2)	33 (5)
	Male	3 (0.4)	0	1 (0.5)	2 (0.6)
	Female	31 (3.9)	0	0	31 (10.2)
Hepatitis B	All	77 (4.8)	3 (0.6)	18 (4.4)	56 (8.5)
	Male	49 (6.1)	2 (0.8)	11 (5.6)	36 (10.1)
	Female	28 (3.5)	1 (0.4)	7 (3.4)	20 (6.6)
Hepatitis C	All	8 (0.5)	2 (0.4)	1 (0.2)	5 (0.8)
	Male	4 (0.5)	0	1 (0.5)	3 (0.8)
	Female	4 (0.5)	2 (0.7)	0	2 (0.7)

Values are n (%)

Table 2D: Volunteer numbers (%) with hematological and blood biochemistry parameters outside reference interval at CRC screening*

Parameter**			Age groups		
			1.5 - 9 years	10 - 17 years	18 - 50 years
Total tested		1599	533	405	661
Male		806	251	197	358
Female		793	282	208	303
Hematology					
White blood cells (increased)	All	75 (4.7)	9 (1.7)	29 (7.2)	37 (5.6)
	Male	39 (4.8)	7 (2.8)	14 (7.1)	18 (5.0)
	Female	36 (4.5)	2 (0.7)	15 (7.2)	19 (6.3)
White blood cells (decreased)	All	61 (3.8)	26 (4.9)	11 (2.7)	24 (3.6)
	Male	35 (4.3)	15 (6.0)	8 (4.1)	12 (3.4)
	Female	26 (3.3)	11 (3.9)	3 (1.4)	12 (4.0)
Lymphocytes (increased)	All	152 (9.5)	6 (1.1)	74 (18.3)	72 (10.9)
	Male	72 (8.9)	3 (1.2)	38 (19.3)	31 (8.7)
	Female	80 (10.1)	3 (1.1)	36 (17.3)	41 (13.5)
Lymphocytes (decreased)	All	34 (2.1)	16 (3.0)	8 (2.0)	10 (1.5)
	Male	19 (2.4)	9 (3.6)	6 (3.0)	4 (1.1)
	Female	15 (1.9)	7 (2.5)	2 (1.0)	6 (2.0)
Neutrophils (increased)	All	33 (2.1)	14 (2.6)	7 (1.7)	12 (1.8)
	Male	17 (2.1)	8 (3.2)	4 (2.0)	5 (1.4)
	Female	16 (2.0)	6 (2.1)	3 (1.4)	7 (2.3)
Neutrophils (decreased)	All	215 (13.4)	21 (3.9)	58 (14.3)	136 (20.6)
	Male	139 (17.2)	12 (4.8)	38 (19.3)	89 (24.9)
	Female	76 (9.6)	9 (3.2)	20 (9.6)	47 (15.5)
Eosinophils (increased)	All	386 (24.1)	35 (6.6)	151 (37.4)	200 (30.3)
	Male	233 (28.9)	22 (8.8)	79 (40.3)	132 (36.9)
	Female	153 (19.3)	13 (4.6)	72 (34.6)	68 (22.4)
Eosinophils (decreased)	All	16 (1.0)	16 (3.0)	0	0
	Male	7 (0.9)	7 (2.8)	0	0
	Female	9 (1.1)	9 (3.2)	0	0
Red blood cells (increased)	All	25 (1.6)	1 (0.2)	0	24 (3.6)
	Male	25 (3.1)	1 (0.4)	0	24 (6.7)
	Female	0	0	0	0
Red blood cells (decreased)	All	59 (3.7)	23 (4.3)	17 (4.2)	19 (2.9)
	Male	24 (3.0)	13 (5.2)	8 (4.1)	3 (0.8)
	Female	35 (4.4)	10 (3.5)	9 (4.3)	16 (5.3)
Hemoglobin (decreased)	All	157 (9.8)	21 (3.9)	86 (21.2)	50 (7.6)
	Male	106 (13.2)	15 (6.0)	73 (37.1)	18 (5.0)
	Female	51 (6.4)	6 (2.1)	13 (6.3)	32 (10.6)

Platelets (increased)		201 (12.6)	43 (8.1)	97 (24.0)	61 (9.2)
		80 (9.9)	23 (9.2)	40 (20.3)	17 (4.7)
		121 (15.3)	20 (7.1)	57 (27.4)	44 (14.5)
Platelets (decreased)	All	44 (2.8)	6 (1.1)	3 (0.7)	35 (5.3)
	Male	25 (3.1)	1 (0.4)	1 (0.5)	23 (6.4)
	Female	19 (2.4)	5 (1.8)	2 (1.0)	12 (4.0)
Biochemistry					
Alanine aminotransferase (increased)	All	109 (6.8)	95 (17.8)	14 (3.5)	0
	Male	49 (6.1)	44 (17.5)	5 (2.5)	0
	Female	60 (7.6)	51 (18.1)	9 (4.3)	0
Alanine aminotransferase (decreased)	All	61 (3.8)	16 (3.0)	6 (1.5)	39 (5.9)
	Male	36 (4.5)	7 (2.8)	1 (0.5)	28 (7.8)
	Female	25 (3.2)	9 (3.2)	5 (2.4)	11 (3.6)
Creatinine (increased)	All	26 (1.6)	6 (1.1)	1 (0.2)	19 (2.9)
	Male	20 (2.5)	2 (0.8)	0	18 (5.0)
	Female	6 (0.8)	4 (1.4)	1 (0.5)	1 (0.3)
Creatinine (decreased)	All	221 (13.8)	26 (4.9)	158 (39.0)	37 (5.6)
	Male	90 (11.2)	14 (5.6)	73 (37.1)	3 (0.8)
	Female	131 (16.5)	12 (4.3)	85 (40.9)	34 (11.2)
Blood glucose (increased)	All	301 (18.8)	196 (36.8)	48 (11.9)	57 (8.6)
	Male	174 (21.6)	105 (41.8)	30 (15.2)	39 (10.9)
	Female	127 (16.0)	91 (32.3)	18 (8.7)	18 (5.9)
Blood glucose (decreased)	All	16 (1.0)	0	3 (0.7)	13 (2.0)
	Male	13 (1.6)	0	3 (1.5)	10 (2.8)
	Female	3 (0.4)	0	0	3 (1.0)

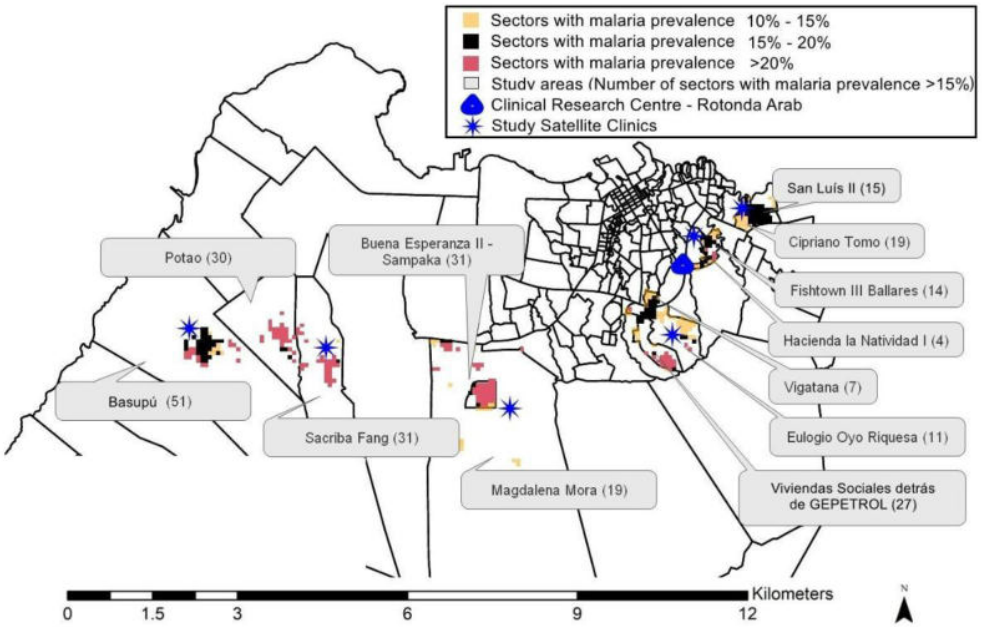
Values are n (%), with percent calculated using the figures in the column heading (total tested, male and female for the rows below labelled all, male and female, respectively)
**None of the abnormal values were clinically significant*
***Reference ranges available in **Supplemental Tables 5a** and **5b**.*

Table 3. Demographic characteristics of 1378 participants in EGRESPAR registry [number (percent)]

Parameter		Total	Age groups		
			1.5- 9 years	10 - 17 years	18 - 50 years
N		1378	512 (37.2)	371 (26.9)	495 (35.9)
Sex					
Male n (%)		705 (51.2)	240 (46.9)	181 (48.8)	284 (57.4)
Female n (%)		673 (48.8)	272 (53.1)	190 (51.2)	211 (42.6)
Age					
Mean ± SD (range) years	All	15.9±10.9 (1.6 - 50.4)	5.9±2.4 (1.6 - 10.0)	13.4±2.2 (10.1 - 17.9)	28.2±8.1 (18.0 - 50.4)
	Male	16.5±10.6 (1.9 - 49.7)	6.0±2.3 (1.9 - 10.0)	13.5±2.2 (10.1 - 17.9)	27.2±7.7 (18.0 - 49.7)
	Female	15.4±11.2 (1.6 - 50.4)	5.9±2.4 (1.6 - 10.0)	13.2±2.1 (10.0 - 17.9)	29.4±8.4 (18.0 - 50.4)
Education (127 lacking data)	All	1251	386 (30.9)	371 (29.7)	494 (39.5)
	Male	658	194 (29.5)	181 (27.5)	283 (43.0)
	Female	593	192 (32.4)	190 (32.0)	211 (35.6)
Illiterate n (%)	All	3 (0.2)	0	0	3 (0.6)
	Male	3 (0.5)	0	0	3 (1.1)
	Female	0	0	0	0
Primary only n (%)	All	682 (54.5	386 (100)	261 (70.4)	35 (7.1)
	Male	340 (51.7)	194 (100)	131 (72.4)	15 (5.3)
	Female	342 (57.7)	192 (100)	130 (68.4)	20 (9.5)
Secondary and primary n (%)	All	463 (37.0)	0	110 (29.6)	353 (71.5)
	Male	244 (37.1)	0	50 (27.6)	194 (68.6)
	Female	219 (36.9)	0	60 (31.6)	159 (75.4)
University or higher n (%)	All	103 (8.2)	0	0	103 (20.9)
	Male	71 (10.8)	0	0	71 (25.1)
	Female	32 (5.4)	0	0	32 (15.2)
Residence	All	1378	512 (37.2)	371 (26.9)	495 (35.9)
	Male	705 (51.2)	240 (46.9)	181 (48.8)	284 (57.4)
	Female	673 (48.8)	272 (53.1)	190 (51.2)	211 (42.6)
Fishtown III Ballares	All	65 (4.7)	21 (4.1)	22 (5.9)	22 (4.4)
	Male	28 (4.0)	7 (2.9)	7 (3.9)	14 (4.9)
	Female	37 (5.5)	14 (5.1)	15 (7.9)	8 (3.8)
Hacienda la Natividad I	All	27 (2.0)	9 (1.8)	4 (1.1)	14 (2.8)
	Male	15 (2.1)	6 (2.5)	1 (0.6)	8 (2.8)
	Female	12 (1.8)	3 (1.1)	3 (1.6)	6 (2.8)
Cipriano Tomo	All	59 (4.3)	22 (4.3)	13 (3.5)	24 (4.8)
	Male	28 (4.0)	7 (2.9)	3 (1.7)	18 (6.3)
	Female	31 (4.6)	15 (5.5)	10 (5.3)	6 (2.8)

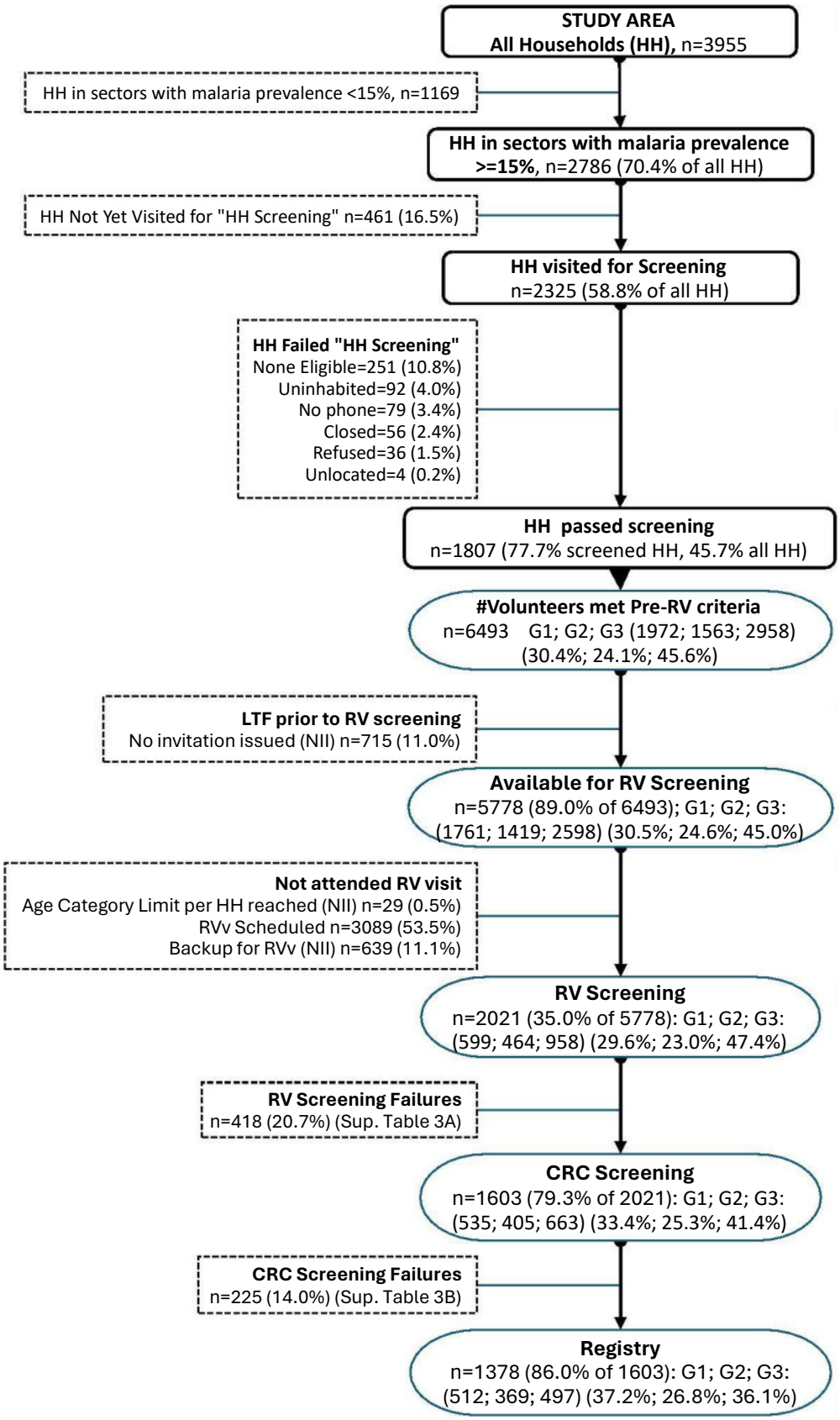
	All	57 (4.1)	20 (3.9)	13 (3.5)	24 (4.8)
San Luis II	Male	28 (4.0)	9 (3.8)	5 (2.8)	14 (4.9)
	Female	29 (4.3)	11 (4.0)	8 (4.2)	10 (4.7)
	All	80 (5.8)	26 (5.1)	17 (4.6)	37 (7.5)
Vigatana	Male	46 (6.5)	13 (5.4)	10 (5.5)	23 (8.1)
	Female	34 (5.1)	13 (4.8)	7 (3.7)	14 (6.6)
	All	23 (1.7)	5 (1.0)	10 (2.7)	8 (1.6)
Eulogio Oyo Riquesa	Male	13 (1.8)	2 (0.8)	5 (2.8)	6 (2.1)
	Female	10 (1.5)	3 (1.1)	5 (2.6)	2 (0.9)
	All	404 (29.3)	160 (31.3)	97 (26.1)	147 (29.7)
Vivienda Sociales detras de GePetrol	Male	189 (26.8)	78 (32.5)	45 (24.9)	66 (23.2)
	Female	215 (31.9)	82 (30.1)	52 (27.4)	81 (38.4)
	All	131 (9.5)	46 (9.0)	39 (10.5)	46 (9.3)
Buena Esperanza II	Male	69 (9.8)	19 (7.9)	22 (12.2)	28 (9.9)
	Female	62 (9.2)	27 (9.9)	17 (8.9)	18 (8.5)
	All	35 (2.5)	16 (3.1)	13 (3.5)	6 (1.2)
Magdalena Mora	Male	14 (2.0)	7 (2.9)	3 (1.7)	4 (1.4)
	Female	21 (3.1)	9 (3.3)	10 (5.3)	2 (0.9)
	All	24 (1.7)	11 (2.1)	8 (2.2)	5 (1.0)
Sacriba Fang	Male	10 (1.4)	4 (1.7)	3 (1.7)	3 (1.1)
	Female	14 (2.1)	7 (2.6)	5 (2.6)	2 (0.9)
	All	52 (3.8)	23 (4.5)	16 (4.3)	13 (2.6)
Potao	Male	31 (4.4)	15 (6.3)	11 (6.1)	5 (1.8)
	Female	21 (3.1)	8 (2.9)	5 (2.6)	8 (3.8)
	All	414 (30.0)	150 (29.3)	118 (31.8)	146 (29.5)
Basupu	Male	228 (32.3)	71 (29.6)	65 (35.9)	92 (32.4)
	Female	186 (27.6)	79 (29.0)	53 (27.9)	54 (25.6)
	All	7 (0.5)	3 (0.6)	1 (0.3)	3 (0.6)
Other places	Male	6 (0.9)	2 (0.8)	1 (0.6)	3 (1.1)
	Female	1 (0.1)	1 (0.4)	0	0

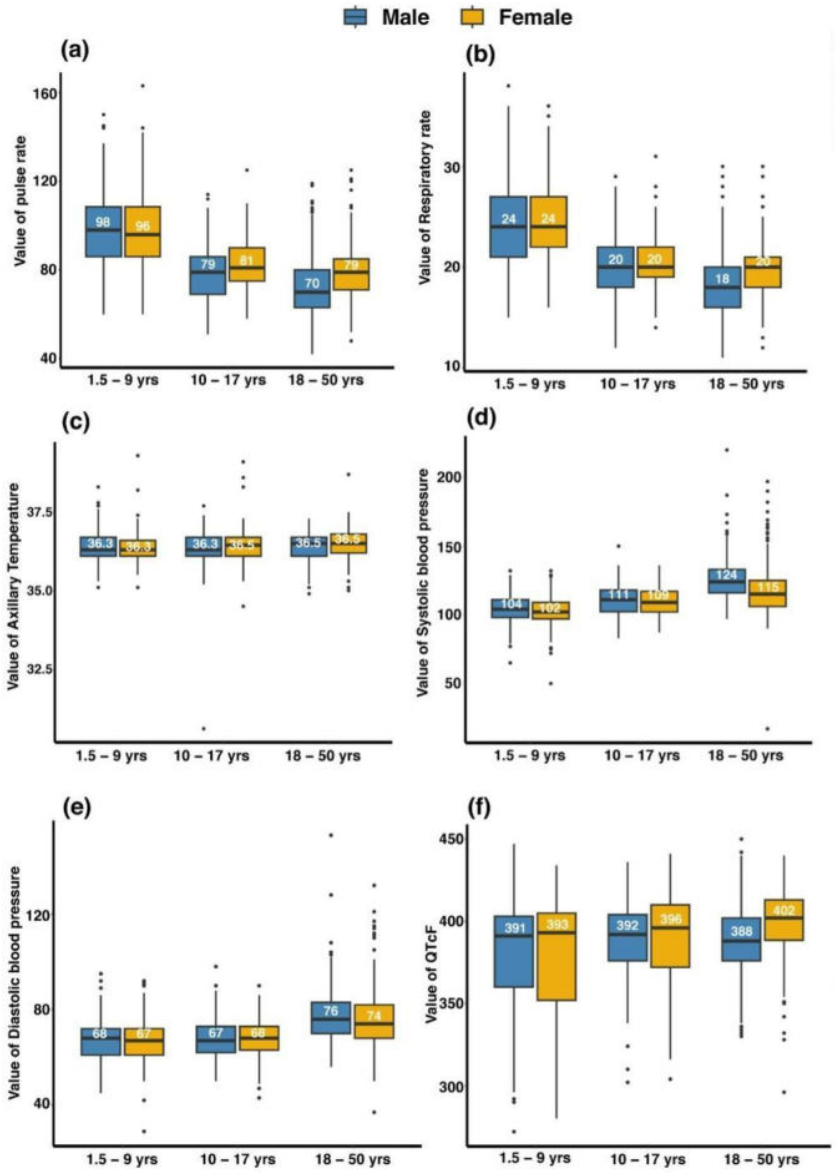
Values are n (%)



Map of the EGRESPAR study area showing the locations and (number) of sectors (small squares) containing households that were visited, color-coded by malaria prevalence in 6-10-year-olds (see key). All sectors were in 12 administrative areas of Bioko Island (black borders).

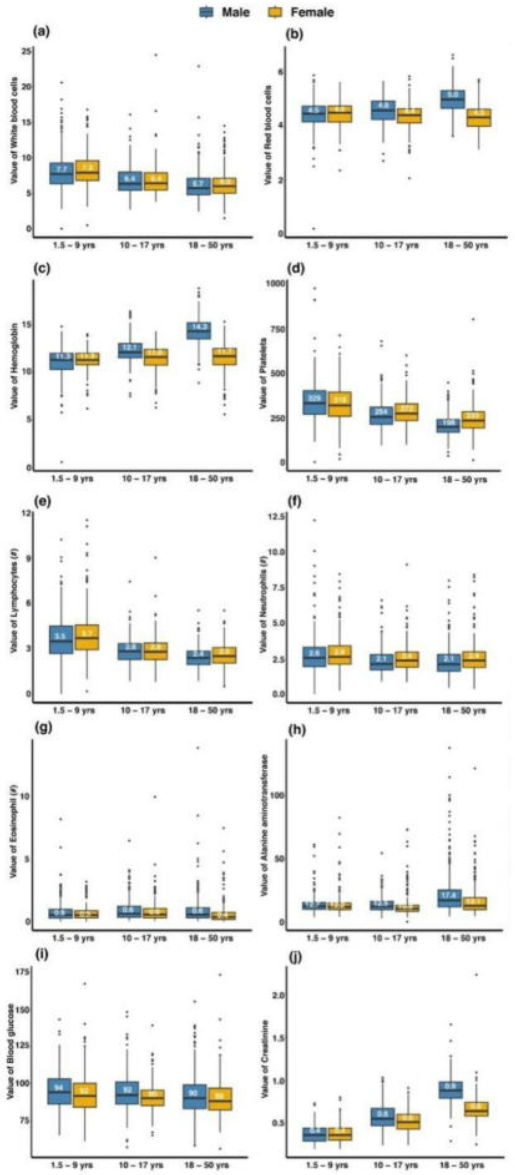
239x152mm (144 x 144 DPI)


STUDY AREA
All Households (HH), n=3955
HH in sectors with malaria prevalence <15%, n=1169
HH in sectors with malaria prevalence >=15%, n=2786 (70.4% of all HH)
HH Not Yet Visited for "HH Screening" n=461 (16.5%)
HH visited for Screening
n=2325 (58.8% of all HH)
HH Failed "HH Screening"
None Eligible=251 (10.8%)
Uninhabited=92 (4.0%)
No phone=79 (3.4%)
Closed=56 (2.4%)
Refused=36 (1.5%)
Unlocated=4 (0.2%)
HH passed screening
n=1807 (77.7% screened HH, 45.7% all HH)
#Volunteers met Pre-RV criteria
n=6493 G1; G2; G3 (1972; 1563; 2958)
(30.4%; 24.1%; 45.6%)
LTF prior to RV screening
No invitation issued (NII) n=715 (11.0%)
Available for RV Screening
n=5778 (89.0% of 6493); G1; G2; G3:
(1761; 1419; 2598) (30.5%; 24.6%; 45.0%)
Not attended RV visit
Age Category Limit per HH reached (NII) n=29 (0.5%)
RVv Scheduled n=3089 (53.5%)
Backup for RVv (NII) n=639 (11.1%)
RV Screening
n=2021 (35.0% of 5778): G1; G2; G3:
(599; 464; 958) (29.6%; 23.0%; 47.4%)
RV Screening Failures
n=418 (20.7%) (Sup. Table 3A)
CRC Screening
n=1603 (79.3% of 2021): G1; G2; G3:
(535; 405; 663) (33.4%; 25.3%; 41.4%)
CRC Screening Failures
n=225 (14.0%) (Sup. Table 3B)
Registry
n=1378 (86.0% of 1603): G1; G2; G3:
(512; 369; 497) (37.2%; 26.8%; 36.1%)



Distributions of clinical parameters of volunteers participated in EGRESPAR study, stratified by age and sex). Panels show the distribution of (a) pulse rate (beats/min), (b) respiratory rate (breaths/min), (c) axillary temperature (°C), (d) systolic blood pressure (mmHg), (e) diastolic blood pressure (mmHg), and (f) iQTcF intervals (ms). The QT intervals were corrected for the effect of heart rate using the Fridericia formula. Boxes show the median and the upper and lower quartiles, and the whiskers define maximum and minimum values excluding outliers

135x190mm (220 x 220 DPI)



. Distributions of laboratory parameters of volunteers participated in EGRESPAR study, stratified by age and sex. Panels a to g are showing the hematological parameters and h to j are showing the biochemistry parameters. Panels show the distribution of (a) white blood cells (103/µl), (b) red blood cells (106/µl), (c) hemoglobin (g/dL), (d) platelets (103/ µl), (e) lymphocytes (103/µl), (f) neutrophils (103/µl), (g) eosinophils (103/µl), (h) alanine aminotransferase (U/L), (i) blood glucose (mg/dL), and (j) creatinine (mg/dL). Boxes show the median and the upper and lower quartiles, and the whiskers define maximum and minimum values excluding outliers.

106x246mm (220 x 220 DPI)

Supplemental Material for:

Assessment of Health Status and Creation of a Registry of Potential Research Participants Age 1.5 to 50 Years on Bioko Island, Equatorial Guinea

López Mikue, MSA et al.

Supplementary Table 1. Inclusion / Exclusion Criteria

Inclusion Criteria: The following are the criteria that allowed the participants to be included in the registry:
1. Age 1.5-50 years at time of anticipated future consent/assent. [Household & Pre-consent]
2. Males and non-pregnant, non-lactating females in general good health. [Household & Pre-consent]
3. Age-appropriate understanding and communication in Spanish for participant or parent/guardian. [Household & Pre-consent]
4. Currently residing in the selected community and willing and able to attend the required appointments at the CRC for the next two years. [Household & Pre-consent]
5. Agree to be contacted by telephone and by home visit. [Household & Pre-consent]
6. Demonstrate understanding of the study by responding correctly to 10 out of 10 true/false statements about the study. (for those who fail to respond correctly to all true/false statements in their first attempt a second and final attempt will be granted). [Town hall - Compliance]
7. Agree (if eligible), to be registered in the database of potential participants for upcoming clinical trial. [Town hall - Compliance]
8. Willing to consider providing consent on pregnancy prevention and breast feeding using highly effective and documented birth control method applicable locally [Town hall - Compliance]
9. Agree to release medical information and inform a study doctor about contraindications for participation in the future studies. [Town hall - Compliance]
10. Agree to be attended to by study doctors and take all medications prescribed during the study period. [Town hall - Compliance]
11. Willing not to take any prescription or herbal medicine obtained outside of the study without first notifying the study team during future studies. [Town hall - Compliance]
12. Agree to provide contact information of a third-party household member and/or close friend to the study team. [Town hall - Compliance]
13. Willing not to participate in another clinical trial during the study period or donate blood during the future study period. [Town hall - Compliance]
14. Agree to undergo HIV, hepatitis B (HBV) and hepatitis C (HCV) testing and all other investigations as stipulated in the protocols or needed for clinical reasons by the investigators. [Town hall - Compliance]
15. Willing to consider not to take drugs with antimalarial activity during the future trials. [Town hall - Compliance]
16. Body Mass Index (BMI) 18-30, growth assessment within +/- 2SD for age. [Town hall - Clinical]
17. Sufficiently healthy as per the clinical judgement based on medical history, examination and investigations done in this study. [Town hall - Clinical] & [CRC]

Exclusion Criteria: The following are the criteria that caused the participants to be excluded from the registry:
1. Previous receipt of an investigational malaria vaccine or drug in the last 5 years. [Household & Pre-consent]
2. Two persons of the same age group are already enrolled from the same household (a maximum of two individuals for each age group may be enrolled in the participants registry from the same household. Participants who are withdrawn or voluntarily withdraw from the registry after enrollment, may also be replaced). [Household & Pre-consent]
3. Regular or planned use of immunoglobulin and/or any blood products in the next 2 years. [Town hall - Clinical]
4. History of drug or alcohol use that meets criteria for “substance abuse” as defined by the WHO (*https://www.who.int/topics/substance_abuse/en/*). [Town hall - Clinical]
5. Known allergic reactions to components of PfSPZ Vaccine (sporozoites, phosphate buffered saline, human serum albumen), normal saline, or artemether/lumefantrine (AL). [Town hall - Clinical]
6. History of anaphylaxis or other life-threatening reaction to a vaccine or drug. [Town hall - Clinical]
7. History of arrhythmias, prolonged QT-interval or other cardiac disease, or clinically significant abnormalities on electrocardiogram (ECG) at screening. [Town hall - Clinical]
8. History of non-febrile seizures or complex febrile seizures. [Town hall - Clinical]
9. History of chronic illness including diabetes mellitus, cancer, HIV/AIDS, tuberculosis, and sickle cell anemia. [Town hall - Clinical]
10. History of clinically significant developmental delay as evaluated by the investigator. [Town hall - Clinical]
11. Any clinically significant deviation from the normal range in biochemistry or hematology tests measured at screening and not resolving.[CRC]
12. HIV, hepatitis B virus or hepatitis C virus tests indicating ongoing infection. [CRC]
13. Positive urine or blood pregnancy test in females of child-bearing potential (9 years old or older at the time of enrollment).[CRC]
14. Signs and symptoms of tuberculosis (e.g., chronic cough, night sweats, chronic fever, enlarged lymph nodes, unintended weight loss).[CRC]
15. Medical history, symptoms, signs and/or laboratory values suggestive of a systemic disorder or chronic illness (including renal, hepatic, blood, cardiovascular, pulmonary, skin, immunodeficiency, psychiatric, and other conditions) which could interfere with the interpretation of the study results or compromise the health of the research participant. [Town hall - Clinical]& [CRC]
16. Any medical, psychiatric, social, behavioral or occupational condition or situation that, in the judgment of the principal investigator, impairs a research subject’s ability to give informed consent, increases the risk to the research subject of participation in the future studies, affects the ability of the research subject to participate fully in the study, or might

negatively impact the quality, consistency, integrity or interpretation of data derived from their participation in the future study. [Town hall - Clinical]& [CRC]

Supplementary Table 2. Criteria associated with failure at household level

		Age groups in years		
	All* (n=4156)	1.5 – 9 (n=1130)	10 – 17 (n=986)	18 – 50 (n=2040)
Did not understand Spanish	139 (3.3)	10 (0.9)	6 (0.6)	123 (6.0)
Plan to stay out of the study area	219 (5.3)	30 (2.7)	39 (4.0)	150 (7.4)
Did not agree to attend screening	156 (3.7)	47 (4.2)	35 (3.5)	74 (3.6)
Received investigational product	4 (0.1)	1 (0.1)	1 (0.1)	2 (0.1)
Totals	518	88	81	349

**A household could have more than one participant providing a reason for failure*

Supplementary Table 3A. Criteria associated with exclusion of screened participants at recruitment venue level (n=418)

		Total (418)	Age groups in years		
			1.5 - 9	10 - 17	18 - 50
Failure to meet both clinical and non-clinical criteria at RV	All	38 of 418	1	3	34
	Male	3	1	0	2
	Female	35	0	3	32
Failure to meet at least 1 non-clinical criterion at RV	All	235	44	38	153
	Male	55	20	9	26
	Female	180	24	29	127
Listing* and occurrence of non-clinical criteria associated with failure at RV					
Not in age 1.5-50 years at time of anticipated future consent/assent.	All	1	0	0	1
	Male	1	0	0	1
	Female	0	0	0	0
Males and non-pregnant, non-lactating females who were not in general good health.	All	5	0	0	5
	Male	0	0	0	0
	Female	5	0	0	5
Currently not residing in the selected community and not willing and not able to attend the required appointments at the CRC for the next two years.	All	55	16	8	31
	Male	29	7	4	18
	Female	26	9	4	13
Did not agree to be contacted by telephone and by home visit	All	61	28	14	19
	Male	25	13	5	7
	Female	36	15	9	12
Did not agree to be in EGRESPAR registry	All	2	0	0	2
	Male	0	0	0	0
	Female	2	0	0	2
Unwilling to provide consent on pregnancy prevention and breast feeding	All	110	NA	16	94
	Male	NA	NA	NA	
	Female	110	NA	16	94
Unwilling not to take any prescription or herbal medicine obtained outside of the study	All	2	0	0	0
	Male	0	0	0	0
	Female	2	0	0	2
Did not agree to undergo HIV, hepatitis B (HBV) and hepatitis C (HCV) tests and all other investigations	All	3	0	0	3
	Male	0	0	0	0
	Female	3	0	0	3
Failure to meet at least 1 clinical criterion at RV	All	202	20	20	162
	Male	66	13	6	47
	Female	136	7	14	115
Listing* and occurrence of clinical criteria associated with failure at RV					
Anthropometric parameters outside reference interval	All	107	4	10	93
	Male	22	3	2	17
	Female	85	1	8	76
Not sufficiently healthy as per the	All	101	10	11	80

clinical judgement based on medical history, examination and investigations done in this study	Male	41	6	5	30
	Female	60	4	6	50
History of drug or alcohol use that meets criteria for “substance abuse”	All	9	0	0	9
	Male	6	0	0	6
	Female	3	0	0	3
History of non-febrile seizures or complex febrile seizures	All	7	0	2	5
	Male	5	0	2	3
	Female	2	0	0	2
History of chronic illness including diabetes mellitus, cancer, HIV/AIDS, tuberculosis, and sickle cell anemia	All	49	8	5	36
	Male	19	5	2	12
	Female	30	3	3	24
History of clinically significant developmental delay as evaluated by the investigator.	All	10	1	5	4
	Male	5	1	2	2
	Female	5	0	3	2
Medical history, symptoms, signs and/or laboratory values suggestive of a systemic disorder or chronic illness	All	104	15	11	78
	Male	48	10	6	32
	Female	56	5	5	46
Any medical, psychiatric, social, behavioral, or occupational condition	All	71	6	9	56
	Male	33	4	5	24
	Female	38	2	4	32

One person can be classified under more than one of the criteria
** see full list at Supplementary Table 1*

Supplementary Table 3B. Criteria associated with failure at CRC level

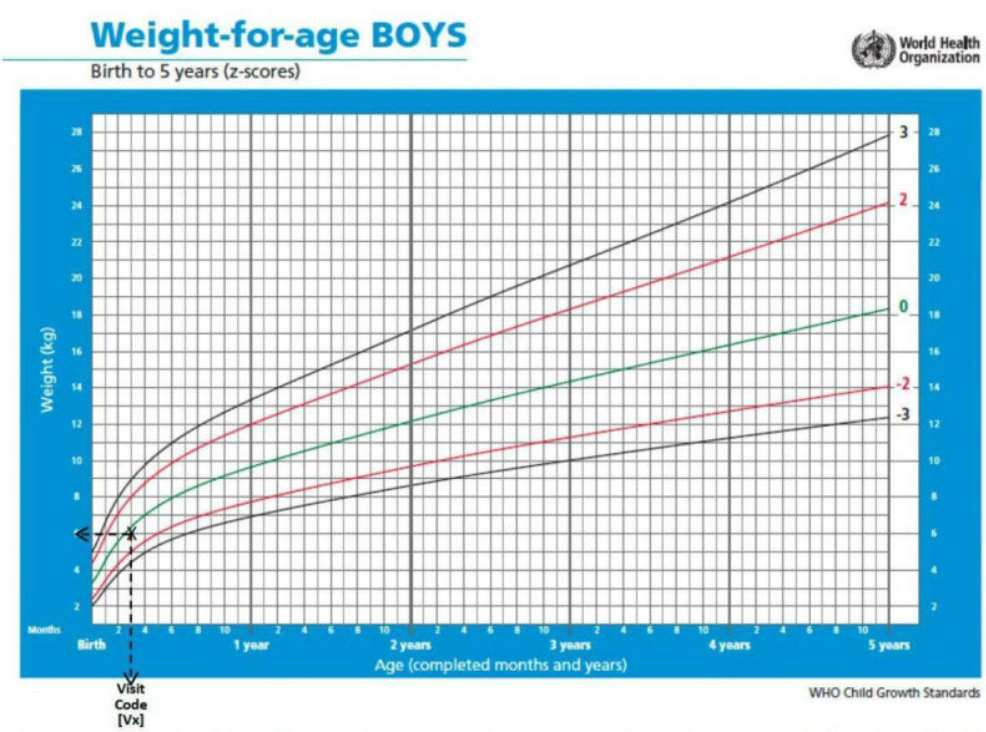
		Total (225)	Age groups in years		
			1.5 - 9	10 - 17	18 - 50
Failure to meet at least 1 non-clinical criterion at CRC	All	8	2	2	4
	Male	3	1	1	1
	Female	5	1	1	3
Currently residing in the selected community and willing and able to attend the required appointments at the CRC for the next two years	All	1	1	0	0
	Male	0	0	0	0
	Female	1	1	0	0
Demonstrate understanding of the study by responding correctly to 10 out of 10 true/false statements about the study. (for those who fail to respond correctly to all true/false statements in their first attempt a second and final attempt will be granted)	All	1	1	0	0
	Male	1	1	0	0
	Female	0	0	0	0
Agree (if eligible), to be registered in the database of potential participants for upcoming clinical trial	All	1	0	1	0
	Male	0	0	0	0
	Female	1	0	1	0
Willing to consider providing consent on	All	1	0	0	1

pregnancy prevention and breast feeding using highly effective and documented birth control method applicable locally	Male	0	0	0	0
	Female	1	0	0	1
History of arrhythmias, prolonged QT-interval or other cardiac disease, or clinically significant abnormalities on electrocardiogram (ECG) at screening	All	3	0	1	2
	Male	2	0	1	1
	Female	1	0	0	1
History of chronic illness including diabetes mellitus, cancer, HIV/AIDS, tuberculosis, and sickle cell anemia	All	1	0	0	1
	Male	0	0	0	0
	Female	1	0	0	1
Failure to meet at least 1 clinical criterion at CRC	All	217	20	34	163
	Male	99	10	16	73
	Female	118	10	18	90
Listing and occurrence of non-clinical criteria associated with failure at					
History of arrhythmias, prolonged QT-interval, or other cardiac disease	All	30	2	2	26
	Male	19	1	2	16
	Female	11	1	0	10
Any clinically significant deviation from the safety data reference interval*	All	29	5	5	19
	Male	11	2	1	8
	Female	18	3	4	11
Positive HIV, hepatitis B virus or hepatitis C virus tests	All	118	5	20	93
	Male	55	2	12	41
	Female	63	3	8	52
Positive urine or blood pregnancy test	All	11	NA	0	11
	Male	NA	NA	NA	NA
	Female	11	0	0	11
Signs and symptoms of tuberculosis	All	1	0	1	0
	Male	0	0	0	0
	Female	1	0	1	0
Medical history, symptoms, signs and/or laboratory values suggestive of a systemic disorder or chronic illness	All	180	13	29	138
	Male	83	5	14	64
	Female	97	8	15	74
Any medical, psychiatric, social, behavioral or occupational condition	All	189	16	29	144
	Male	88	8	13	67
	Female	101	8	16	77

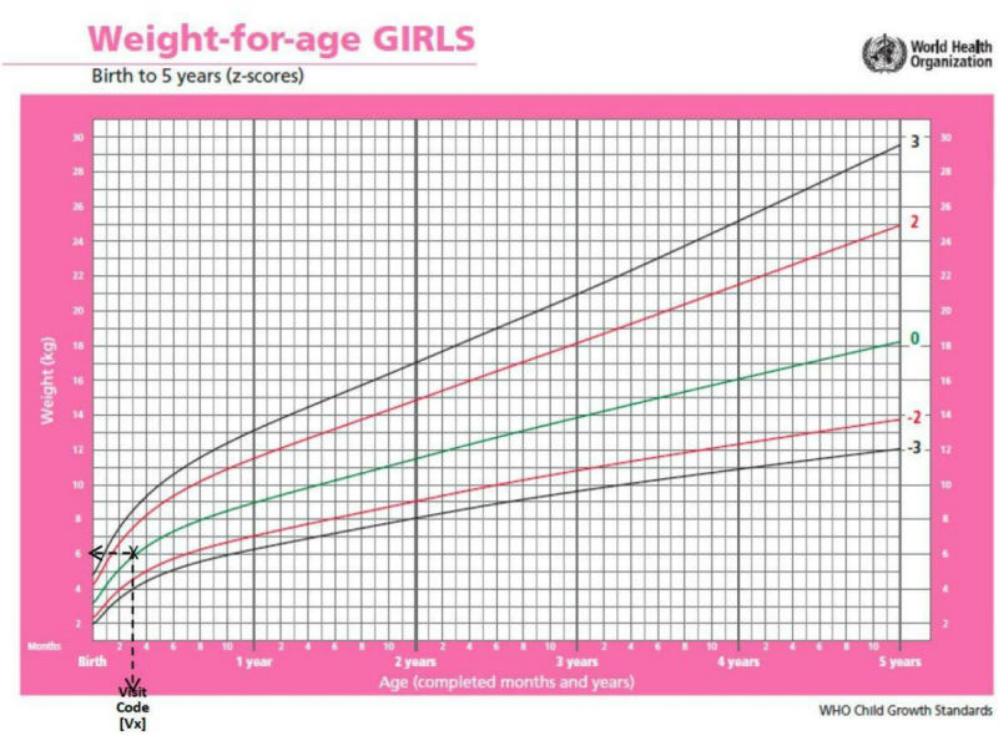
One person can have more than one exclusion criteria
** These 29 clinically significant deviations from reference intervals were for vital signs, ECG screening, infection screening etc., but not from laboratory abnormalities.*

Supplementary Table 4a. Z-score BMI for weight (children <11 years)

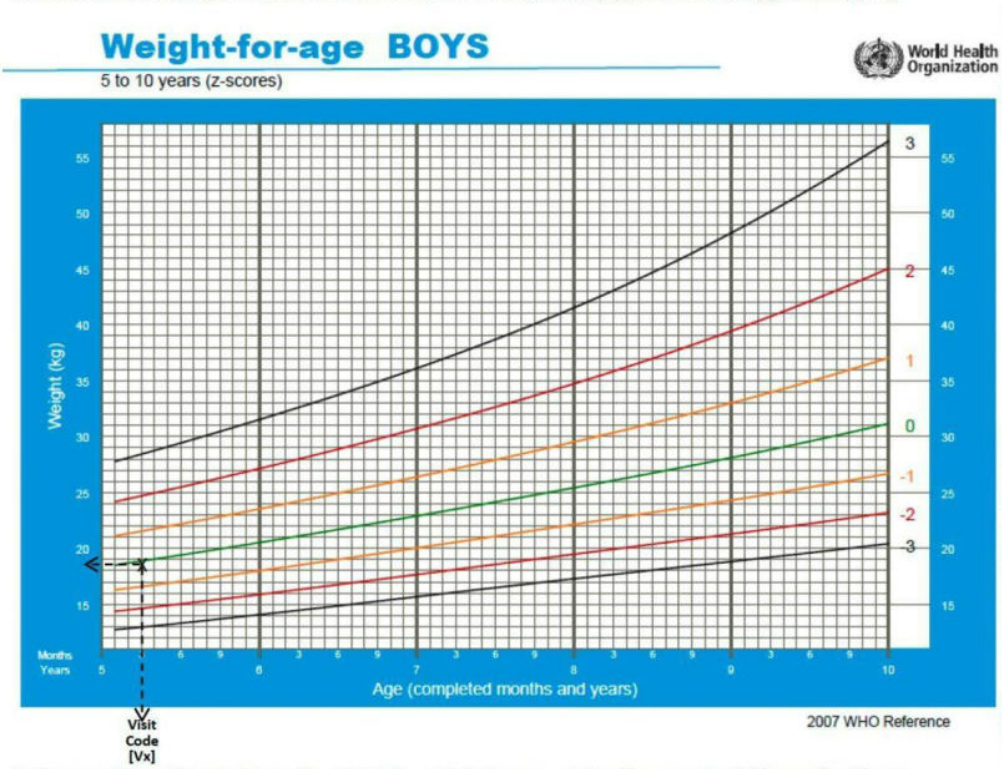
Reference Charts Adopted from the WHO Growth References: http://www.who.int/growthref/en/



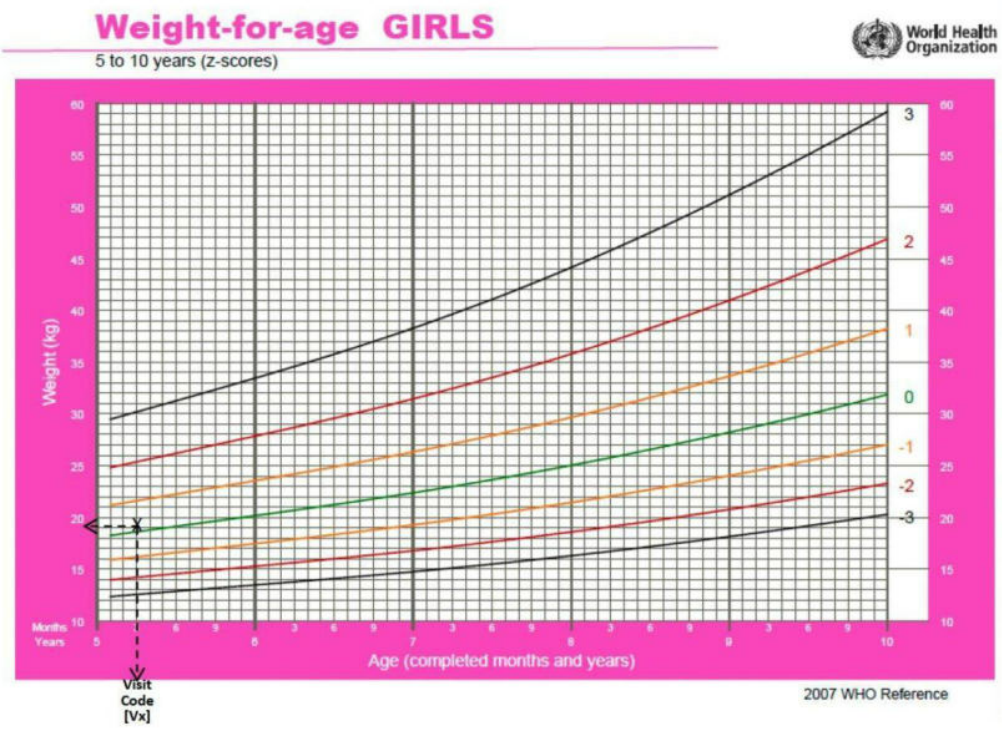
Reference Charts Adopted from the WHO Growth References: http://www.who.int/growthref/en/



Reference Charts Adopted from the WHO Growth References: http://www.who.int/growthref/en/

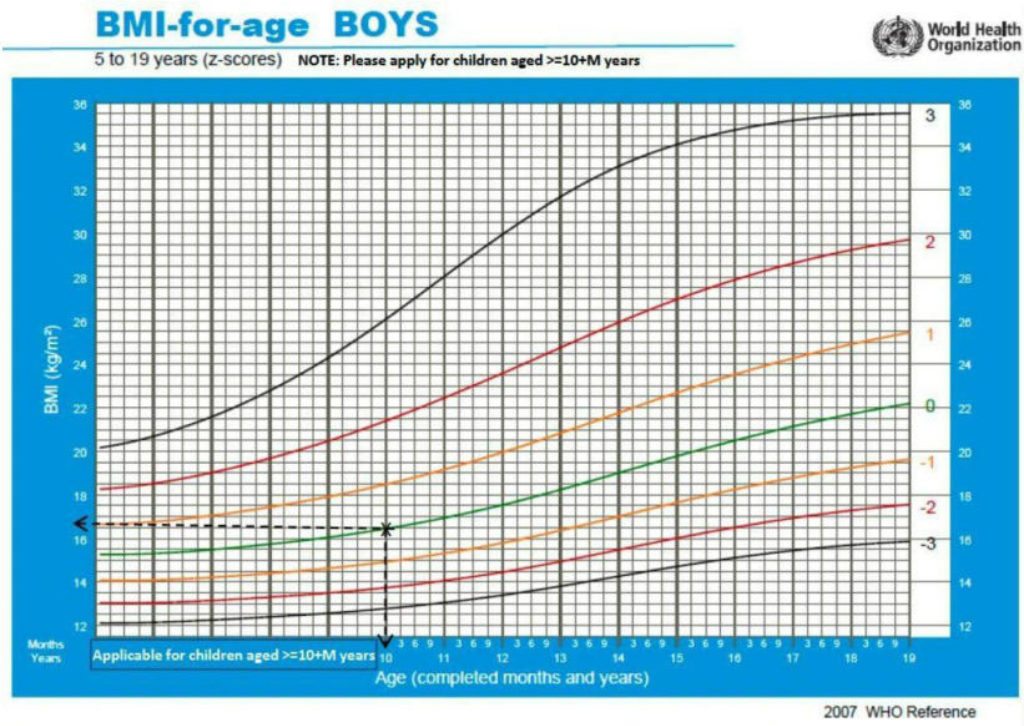


Reference Charts Adopted from the WHO Growth References: http://www.who.int/growthref/en/



Supplementary Table 4b. Z-score BMI for age (children 11-17 years)

Reference Charts Adopted from the WHO Growth References: http://www.who.int/growthref/en/



Reference Charts Adopted from the WHO Growth References: http://www.who.int/growthref/en/

Supplementary Table 5a. Hematology and Chemistry Reference Intervals and Toxicity Grading (Children 6 months to < 11 years)

Reference Intervals: AGE RANGE [Children 6 months to <11 years]		
HEMATOLOGY	**6 Months to <6 Years**	**6 Years to <11 Years**
WBC (Leucocyte count)	[5.1-16.2] 10^3 / µl	[4.5-12.9] 10^3 / µl
LYMPH (Lymphocytes)	[2.1-9.5] 10^3 / µl (30.2-73.2) %	[1.8-6.4] 10^3 / µl (30.8-64.9) %
MONO (Monocytes)	[0.4-1.6] 10^3 / µl (5.1-16.8) %	[0.3-1.1] 10^3 / µl (5.0-14.3) %
NEUT (Neutrophils)	[1.3-6.9] 10^3 / µl (15.8-57.6) %	[1.2-6.2] 10^3 / µl (21.3-59.4) %
EO (Eosinophils)	[0.1-1.6] 10^3 / µl (0.7-17) %	[0.1-2.1] 10^3 / µl (1.0-25.9) %
BASO (Basophils)	[0.01-0.1] 10^3 / µl (0.1-1.2) %	[0.01-0.1] 10^3 / µl (0.1-1.0) %
RBC (Erythrocyte count)	[3.8-5.8] 10^6 / µl	[3.6-5.8] 10^6 / µl
HGB(Hemoglobin)	[8.6-13.2] g/dL	[9.6-14.1] g/dL
HCT (Hematocrit)	[27.5-38.9] %	[28.7-42.3] %
MCV (Mean corpuscular volume)	[56.4-83.1] fL	[61.2-85.1] fL
MCH (Mean corpuscular hemoglobin)	[17.5-28.5] pg/cel l	[20.3-29.2] pg/cell
MCHC (Mean corpuscular hemoglobin concentration)	[30.1-35.6] g/dL	[31.3-36.8] g/dL
RDW_SD (Red-cell distribution width Standard Deviation)	[32.8-48.5] fL	[32.5-44.5] fL
PLT (Platelets)	[91-491] 10^3 / µl	[91-456] 10^3 / µl
MPV (Mean Platelet Volume)	[8.9-13.5] fL	[8.8-14.0] fL
RDW_CV (Red-cell distribution width Coefficient of Variation)	[12.3-22.5] %	[12.0-19.4] %
PDW (Platelet distribution width)	[10.1-21.1] fL	[10.2-23.3] fL
P-LCR (Platelet Large Cell Ratio)	[18.0-51.7] %	[16.8-55.1] %
PCT (Plateletcrit)	[0.15-0.52] %	[0.13-0.45] %

BIOCHEMISTRY	6 Month to <12 Months	1 Year to <6 Years	6 Years to <11 Years
Alanine aminotransferase (ALT/SGPT)	[9-33] U/L	[10-28] U/L	[9-35] U/L
Aspartate aminotransferase (AST/SGOT)	[26-65] U/L	[27-55] U/L	[21-51] U/L
Total Bilirubin (TBIL)	[0.12-1.1] mg/dL	[0.12-0.53] mg/dL	[0.12-0.64] mg/dL
Creatinine	[0.16—0.37] mg/dL	[0.2-0.66] mg/dL	[0.31-0.64] mg/dL
Glucose	[52.3 - 99.1] mg/dL	[54.1-97.3] mg/dL	[48.6-95.5] mg/dL
Albumin	[36-48] g/L	[40-49] g/L	[40-48] g/L
Alkaline Phosphatase	[164-589] U/L	[153-410] U/L	[174-460] U/L
γ- Glutamyltransferase(GGT)	[3.0-34.0] U/L	[3.0-34.0] U/L	[7.0-31.0] U/L
Bilirubin Direct	[0-1.1] mg/dL	[0-0.53] mg/dL	[0.03-0.23] mg/dL
Lactate Dehydrogenase (LDH)	[360-995] U/L	[360-995] U/L	[277-823] U/L
Cholesterol	[2.1-5.7] mmo/L	[2.5-5.3] mmo/L	[2.1-4.9] mmo/L
Creatinine Kinase	[45-227] U/L	[73-248] U/L	[83-294] U/L
Blood Urea Nitrogen (BUN)	[0.7-3.8] mmo/L	[1.3-4.2] mmo/L	[1.4-4.4] mmo/L
Potassium	[4.2-6.5] mmo/L	[3.8-5.9] mmo/L	[3.2-5.2] mmo/L
Sodium	[133-139] mmo/L	[133-141] mmo/L	[134-141] mmo/L
Chloride	[100-107] mmo/L	[100-108] mmo/L	[98-108] mmo/L
Calcium	[2.4-2.8] mmo/L	[2.2-2.7] mmo/L	[2.2-2.6] mmo/L
Magnesium	[0.8-1.1] mmo/L	[0.8-1.0] mmo/L	[0.8-1.0] mmo/L

HEMATOLOGY: Toxicity Grading; (Age Range: 6 months to <11 years)									
Parameters	Age Group	Normal Range			Toxicity Grades				
		Unit	LLN	ULN	Mild (Grade 1)	Moderate (Grade 2)	Severe (Grade 3)	Potentially life Threatening (Grade 4)	Ref

HGB – Hemoglobin	**6mo- <6Y**	g/dL	8.6	13.2	**8- <8.6**	**7-<8**	**<7**	**Cardiac Failure secondary to anemia**	DMID
	6y - <11y	g/dL	9.6	14.1	**8- <9.6**				
PLT – Platelets	**6mo- <6Y**	$10^3/\mu l$	91	491	**75 - <91**	**50 - <75**	**25 - <50**	**<25**	
	6y - <11y	$10^3/\mu l$	91	456					
WBC – White Blood-cell Count Low	**6mo- <6Y**	$10^3/\mu l$	5.1	16.2	**2 - <5.1**	**1.5 - <2**	**1 - <1.5**	**<1**	
	6y - <11y	$10^3/\mu l$	4.5	12.9	**2 - <4.5**				
Neutrophils	**6mo- <6Y**	$10^3/\mu l$	1.3	6.9	**0.8 - <1.3**	**0.6 - <.8**	**0.4 - <.6**	**<0.4**	
	6y - <11y	$10^3/\mu l$	1.2	6.2	**0.8 - <1.2**				
Absolute Neutrophils Count Low	**6mo-12mo**	$10^3/\mu l$	2.1	9.5	**0.6 - <2.1**	**0.5 - <0.6**	**0.35 - <0.5**	**<0.35**	
	6y - <11y	$10^3/\mu l$	1.8	6.4	**0.6 - <1.8**				
Lymphocytes	**6mo-12mo**	$10^3/\mu l$	0.1	1.6	**>1.6 - 2.4**	**>2.4 - 4.8**	**> 4.8**	**Hypereosinophilia**	
	6y - <11y	$10^3/\mu l$	0.1	2.1	**>2.1-3.1**	**>3.2 - 6.3**	**> 6.3**		
BIOCHEMISTRY: Toxicity Grading; (Age Range: 6 months to <11 years)									
Parameters	**Age Group**	**Normal Range**			**Toxicity Grades**				
		Unit	**LLN**	**ULN**	**Mild (Grade 1)**	**Moderate (Grade 2)**	**Severe (Grade 3)**	**Potentially life Threatening (Grade 4)**	Ref
Alanine aminotransferase (ALAT)	**6mo-<12mo**	U/L	9	33	**>33 - 66**	**>66 - 99**	**>99 - 264**	**> 264**	DMID
	1y - <6y	U/L	10	28	**>28 - 56**	**>56 - 84**	**>84 - 224**	**> 224**	
	6y - <11y	U/L	9	35	**>35 - 70**	**>70 - 105**	**>105 - 280**	**> 280**	
Aspartate aminotransferase (ASAT)	**6mo-<12mo**	U/L	26	65	**>65 - 130**	**>130 - 195**	**>195 - 520**	**> 520**	
	1y - <6y	U/L	27	55	**>55 - 110**	**>110 - 165**	**>165 - 440**	**> 440**	
	6y - <11y	U/L	21	51	**>51 - 102**	**>102 - 153**	**>153 - 408**	**> 408**	
Total Bilirubin (TBIL) - when accompanied by any increase in	**6mo - <12mo**	mg/dL	0.12	1.11	**>1.11 - 1.38**	**>1.38-1.65**	**>1.65-1.94**	**> 1.94**	
	1y - <6y	mg/dL	0.12	0.53	**>0.53-0.65**	**>0.65 - 0.78**	**>0.78-0.92**	**> 0.92**	

other Liver function test	**6Y - <11y**	mg/dL	0.12	0.64	**>0.64-0.79**	**>0.79-0.95**	**>0.95-1.13**	**> 1.13**	
Total Bilirubin (TBIL) - when Liver function are normal range	**6mo-12mo**	mg/dL	0	0.11	**>0.11 – 1.66**	**>1.66 – 2.21**	**>2.21-3.33**	**> 3.33**	
	1y - 5y	mg/dL	0	0.52	**>0.52 – 0.78**	**>0.78 - 1.04**	**>1.04 – 1.58**	**> 1.58**	
	6y - <11y	mg/dL	0	0.64	**>0.64-0.95**	**>0.95-1.27**	**>1.27 – 1.93**	**> 1.93**	
CREATININE	**6mo-12mo**	mg/dL	0.16	0.32	**>0.32 - 0.80**	**>0.80 - 1.10**	**>1.10 – 1.50**	**> 1.50**	
	1y - <2y	mg/dL	0.2	0.57	**>0.57 – 0.80**	**>0.80 – 1.10**	**>1.10 – 1.50**	**>1.50**	
	2y - <6y	mg/dL	0.17	0.57	**>0.57-1.0**	**>1.0 – 1.60**	**>1.60 – 2.0**	**> 2.0**	
	6y - <11y	mg/dL	0.27	0.55	**>0.55 – 1.0**	**>1.0 – 1.60**	**>1.60 – 2.0**	**>2.0**	

Supplementary Table 5b. Hematology and Chemistry Reference Intervals and Toxicity Grading (Children 11 to 65 years)

Reference Intervals: AGE RANGE [11 years to 65 years]				
HEMATOLOGY				
WBC (Leucocyte count)	[3.65-9.7] 10^3 / µl			
LYMPH (Lymphocytes)	[1.19 - 3.4] 10^3 / µl			
MONO (Monocytes)	[0.25-1.41] 10^3 / µl (0 – 13.96) %			
NEUT (Neutrophils)	[1.61-5.69] 10^3 / µl (27.9-69.86) %			
EO (Eosinophils)	[0 –0.78] 10^3 / µl (0 – 14.15) %			
BASO (Basophils)	[0 – 0.05] 10^3 / µl (0 – 0.9) %			
RBC (Erythrocyte count)	[3.8-5.67] 10^6 / µl Male		[3.65-5.84] 10^6 / µl Female	
HGB(Hemoglobin)	[12-17.4] g/dL Male		[9.60-14.10] g/dL Female	
HCT (Hematocrit)	[32.8-46.7] % Male		[28.72-42.30] % Female	
MCV (Mean corpuscular volume)	[74.4-92.6] fL			
MCH (Mean corpuscular hemoglobin)	[27.6-34.6] pg/cell			
MCHC (Mean corpuscular hemoglobin concentration)	[35.7-38.8] g/dL			
RDW_SD (Red-cell distribution width Standard Deviation)	[34.97-49.22] fL			

PLT (Platelets)	[124-312] 10^3 / µl
MPV (Mean Platelet Volume)	[8.3 -13.4] fL
RDW_CV (Red-cell distribution width Coefficient of Variation)	[11.7- 16.1] %
PDW (Platelet distribution width)	[9 – 19.14] fL
P-LCR (Platelet Large Cell Ratio)	[11.17 - 51.27] %
PCT (Plateletcrit)	[0 – 0.36] %
BIOCHEMISTRY	
Alanine aminotransferase (ALT/SGPT)	[0 – 45] U/L
Aspartate aminotransferase (AST/SGOT)	[15.2 – 58.7] U/L
Total Bilirubin (TBIL)	[0 – 2.62] mg/dL
Creatinine	[0.54— 1.11] mg/dL
Glucose	[70.3- 109.8] mg/dL
Albumin	[42.7 – 60] g/L
Alkaline Phosphatase	[45 – 186.9] U/L
γ- Glutamyltransferase(GGT)	[8.3 – 108.1] U/L
Bilirubin Direct	[0 – 0.36] mg/dL
Lactate Dehydrogenase (LDH)	[127 – 264] U/L
Cholesterol	[96.7— 200.7] mg/dL
Creatinine Kinase	[77 – 787] U/L
Blood Urea Nitrogen (BUN)	[7—19] mg/dL
Potassium	[3.5 – 5] mmo/L
Sodium	[134 – 142] mmo/L
Chloride	[97 – 107] mmo/L
Calcium²	[1.99 – 2.52] mmo/L
Magnesium²	[0.67 – 0.97] mmo/L

HEMATOLOGY: Toxicity Grading; (Age Range: 11-65 YEARS OLD - Healthy)								
Parameters	**Normal Range**			**Toxicity Grades**				
	Unit	**LLN**	**ULN**	**Mild (Grade 1)**	**Moderate (Grade 2)**	**Severe (Grade 3)**	**Potentially life Threatening (Grade 4)**	Ref

Leukocyte (WBC) Increase	x10^3/µL	3.65	9.7	**>9.7 - 15**	**>15 - 20**	**>20 - 25**	**> 25**	
Leukocyte (WBC) Decrease	x10^3/µL	3.65	9.7	**<3.65 - 2.5**	**<2.5 - 1.5**	**<1.5 - 1**	**< 1**	
Lymphocytes Decrease	x10^3/µL	1.19	3.4	**<1.19 - 0.75**	**<0.75 - 0.5**	**< 0.5 - 0.25**	**< 0.25**	
Neutrophils Decrease	x10^3/µL	1.61	5.69	**<1.61 - 0.9**	**< 0.9 - 0.6**	**< 0.6 - 0.4**	**<0.4**	
Eosinophils - cell/mm3	x10^3/µL	0	0.78	**> 0.78 - 1.5**	**>1.5 - 5**	**> 5**	**Hyper-eosinophilic**	
Hemoglobin (Male)	g/dL	12	17.4	**<12-8.0**	**<8.0 – 7.0**	**<7.0 – 4.0**	**<4.0**	
Hemoglobin (Female)	g/dL	9.6	14.1	**<9.6 – 8.0**	**<8.0 – 7.0**	**<7.0 – 4.0**	**<4.0**	
Platelets Decreased	x10^3/µL	124	312	**75 - <124**	**50 - <75**	**25 – <50**	**< 25**	
BIOCHEMISTRY: Toxicity Grading; (Age Range: 11-65 YEARS OLD - Healthy)								
Parameters	**Normal Range**			**Toxicity Grades**				
	Unit	**LLN**	**ULN**	**Mild (Grade 1)**	**Moderate (Grade 2)**	**Severe (Grade 3)**	**Potentially life Threatening (Grade 4)**	Ref
Alanine aminotransferase (ALAT)	U/L	0	45	**>45 – 106.5**	**>106.5 – 209**	**>209 - 410**	**>410**	
Aspartate aminotransferase (ASAT)	U/L	15.2	58.7	**>58.7-138.8**	**>138.8-272.3**	**>272.3-534**	**>534**	
Total Bilirubin (TBIL) - when accompanied by any increase in other Liver function test	mg/dL	0	2.62	**> 1.82 - 2.27**	**>2.27 – 2.73**	**>2.73 – 3.18**	**> 3.18**	
Total Bilirubin (TBIL) - when Liver function are normal range	mg/dL	0	2.62	**>2.62-3.01**	**>3.01-3.61**	**>3.612-4.19**	**>4.19**	
CREATININE	mg/dL	0.54	1.11	**>1.11-1.70**	**>1.70-2.0**	**>2.0-2.5**	**> 2.5 or require dialysis**	

Supplementary Form 1	**National Malaria Control Program** Bioko Island Malaria Elimination Program	

HOUSEHOLD QUESTIONNAIRE

Instructions: ☑=Tick Appropriate Response **Format**: Time=(*hh:mm*) Hours; Date = (*dd-mmm-yyyy, eg. 11/JAN/2019)*	**Household Number:** ___.___.___.___.___.___.___.___.__ _.___.___.___ **VISIT DATE:** ___.___/___.___.___/20___.__ _

Section A: Down-selection of Potential Participants at Household Level

☐Head of the Household OR ☐Designee	
First name:	
Surname(s):	
Date of birth:	___|___/___|___|___/___|___|___|___|
Actual Age:	Years; |___|___|/ Months; |___|___|
Sex:	☐[1]Male ☐[2]Female
Mobile #:	|___|___|___|___|___|___|___|___|___|___|;☐[1]Own ☐[2]Third Party

Is there any household member meeting any of the following criteria in this household?		**If Yes, please specify number of individuals, else enter N/A**				
Age Group		18mo - 9 Years	10-17 Years		18-50 Years	
Inclusion (1): Aged 18 months to 50 years at "estimated" time of consent/ assent	☐Yes ☐No					
Sex		M&F	M	F	M	F
Inclusion (2): *Applied only for those in* ***(Inclusion 1)*** Male and Non-pregnant, non-Lactating female	☐Yes ☐No					
Inclusion (3): *Applied only for those in* ***(Inclusion 2)*** Age-appropriate understanding and communication in Spanish	☐Yes ☐No					
Inclusion (4): *Applied only for those in* ***(Inclusion 3)*** Plan to stay in Bioko Island and willing to attend required appointments at CRC for at least two years	☐Yes ☐No					

Inclusion (5): *Applied only for those in* ***(Inclusion 4)*** Agree to be contacted by telephone or by home visit	☐Yes ☐No					
Exclusion (1): *Applied only for those in* ***(Inclusion 5)*** Not received investigational malaria vaccine in the last 5 years	☐Yes ☐No					

# of Eligible Potential Household Members for Pilot Study are indicated in Row Exclusion (1)

Please, collect details shown in Section B for each potential participant Identified.

Interviewer Initials: |___|___|___| **Date:** |___|___|-|___|___|___|-|___|___|___|

Reviewer Initials: |___|___|___| **Date:** |___|___|-|___|___|___|-|___|___|___|

Section B: Demographic and Contact Details for individual Potential Participants

PART 1: This section MUST be completed for ALL Potential Participants	
Serial #:	Potential Participant |___|___| of |___|___| In this household
Participant is:	☐¹Adult ☐²Child; ***Part 2 below MUST*** *be completed with Parent/ Guardian details*
First name:	
Surname(s):	
Date of birth:	___|___/___|___|___/___|___|___|___|
Actual Age:	Years; |___|___|/ Months; |___|___|
Sex:	☐¹Male ☐²Female
Mobile #:	|___|___|___|___|___|___|___|___|___|___|;☐¹Own ☐²Same as Parent / Guardian

PART 2: This section must be completed if **PARTICIPANT IS A CHILD** (Else, tick here ☐N/A)		
	Parent / Guardian (1): Same as	**Parent / Guardian (2)**
The adult is:	☐¹Parent ☐²Guardian	☐¹Parent ☐²Guardian
First name:		
Surname(s):		
Date of birth:	___|___/___|___|___/___|___|___|___|	___|___/___|___|___/___|___|___|___|
Actual Age:	Years; |___|___|/ Months; |___|___|	Years; |___|___|/ Months; |___|___|
Sex:	☐¹Male ☐²Female	☐¹Male ☐²Female

Mobile #:	|___|___|___|___|___|___|___|___|___|___| ☐[1]Own; ☐[2]Third party	|___|___|___|___|___|___|___|___|___|___| ☐[1]Own; ☐[2]Third party

Interviewer Initials: |___|___|___| Date of Interview: ___|___/___|___|___/___|___|___|___|

Reviewer Initials: |___|___|___| Date of Review : ___|___/___|___|___/___|___|___|___|

Section C: Disposition of Potential Participant in this household based on Exclusion Criteria (2):

Two persons from the same household are already enrolled, or one person from the same age group	
☐**Yes:** Complete date of termination below	☐**No:** Complete date of townhall visit below
Date of termination from the subsequent procedures **Date** ___|___/___|___|___/___|___|___|___|	Scheduled Date of Potential Participant to Attend to the Townhall meeting **Date** ___|___/___|___|___/___|___|___|___|

Investigator's Initials: |___|___|___| Date of Decision: ___|___/___|___|___/___|___|___|___|

Coordinator's Initials: |___|___|___| Date of Decision: ___|___/___|___|___/___|___|___|___|

References for Supplementary material

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2. Bagamoyo Research and Training Centre, Ifakara Health Institute, Tanzania
3. TzSTG = Tanzania Standard Treatment Guidelines
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5. Saathoff E, Schneider P, Kleinfeldt V, GeisS, Haule D, et al. (2008) Laboratory reference values for healthy adults from southern Tanzania. Trop Med Int Health 13: 612-625.
6. PfSPZ Vaccine IV trial in Mali 15 May 2014 Version 5.0

CHAPTER 6: Optimization of tools for safety assessment through application of local population parameters

CHAPTER 6: Optimization of tools for safety assessment through application of local population parameters

6.1 General overview

This section provides details substantiating the description provided for thesis aim 3 in section 2.32.2. The clear ICH-GCP statement on the protection of the rights, safety and well-being of participants being the most important considerations during clinical trials, indicates the need to ensure that, sites are well capable to comply with such requirement. This role is primarily for clinical investigators who should perform the assessments, interpret the results and provide relevant care or further reporting to sponsor or regulatory authorities if needed (these will potentially cover the rights, safety and well-being). It is critical not to undermine the important role of decisions made through investigator's clinical judgement since there is no known potential decision matrix that can fully take this role in the clinical trials. It is, however, important that the sponsor and the team of clinical investigators involved in the same clinical trial ensure the reliability of clinical decisions that are made by different investigators through clinical judgement. Ruling out whether or not the abnormal results are clinically significant is where potential differences in opinion may occur, not only between investigators themselves, but also between investigators and sponsor's medical monitors. In most cases, it should be possible to reach consensus if the event is captured and reported in time and therefore, the likelihood for such uncertainties to occur, must be considered during the risk assessment depending on the local capabilities. As described in section 2.3, the notion that, standardized toxicity assessment scales have been widely used may not be entirely correct when it comes to clinical trials conducted in sub-Saharan Africa where less than 10% of clinical trials are conducted (Edem et al., 2021). The key rationale for uniformity in categorizing toxicities is, the improvement on the issues associated with subjective clinical judgement. While the rationale is strong and acceptable, the process of adopting the standardized toxicity grading systems to local African population may be challenging. The description on the process and related challenges in developing the population-based toxicity grading manual is provided in section 6.1.1.

6.1.1 Overview on the development of population-based toxicity grading manual and associated challenges

Within the scope of this thesis the population-based toxicity grading system refers to the listing of parameter limit values for severity grades defining results of abnormal clinical and laboratory safety parameters beyond the reference intervals that are relevant for local population. Such a list is constructed in reference to the known standardized toxicity grading systems (FDA 2005D-0155, 2007; NCI, 2017; NIH-DAIDS, 2017; NIH, 2005). The standardized toxicity grading systems provide guidance in two main forms, presented as, pre-defined parameter limit values or multiples of lower / upper limits of the reference intervals. In essence, both forms are linked to the assessment of change from baseline following IP administration estimated from data collected from multiple sources.

The process to adopt the standard toxicity grading system has three key steps:

1. Selection of relevant standard toxicity grading for healthy subjects (FDA 2005D-0155, 2007) or cancer or AIDS (NCI, 2017; NIH-DAIDS, 2017; NIH, 2005) patients
2. Identification of relevant reference interval for respective population to be assessed
3. Integration of relevant reference interval to the selected standard toxicity grading system for generation of population-based severity grades

The selection of relevant standardized toxicity grading systems, was based on the target population of participants in terms of study population (healthy or those with cancer or HIV). To generate population-based reference intervals, we used the guidance from the Clinical and Laboratory Standards Institute's (CLSI) document; "Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Guideline-(C28-A3)". Following that guidance, we used clinical and laboratory data collected from screening of volunteers in previous studies including the pilot studies we conducted as described in section 5.1.1.2. Based on the CLSI guidance we selected the reference population adequate to meet the sample size needed for individual parameters, and followed the

process for identification of outliers, and perform the calculations necessary to generate valid reference intervals presented in section 6.2. Developing the reference intervals based on data from the local population would ensure a more efficient clinical care of participants. Following the availability of population-based reference interval and the identification of relevant standard toxicity grading systems, our data and statistical unit applied the relevant calculations to generate the toxicity grades. Through the process of validation by the QA unit and investigators, it became apparent that, additional steps were needed to generate a usable listing. Fundamental to the challenges in adopting standardized systems to local African population are; the misalignment of parameter limit values defining local reference interval versus those defining severity grades; and discontinuity between severity grades resulting from the application multiplication factors defined in the standard toxicity grading systems respectively. Potential reason for this challenge is the way the parameter limit values were chosen, indicated to be, either based on available published information or on clinical experience (FDA 2005D-0155, 2007), raising the question on the degree to which data from African population was included in choosing the parameter limit values. The procedures applied in addressing these challenges are outlined in the site manual developed for population-based reference intervals and toxicity grading in section 6.2.

6.2 Population-based manual of reference interval and toxicity grading system

This section presents the QA-controlled, manual optimized for assessing the severity of clinical and laboratory abnormalities in volunteers enrolled in clinical trials conducted within the scope of this thesis. The need for uniform criteria for categorizing toxicities, application in defining a particular study's stopping rules and potential to improve comparisons of safety data among groups within the same study and also between different studies have been mentioned as potential rationales for the recommended use of standardized toxicity grading systems (FDA 2005D-0155, 2007; NCI, 2017; NIH-DAIDS, 2017; NIH, 2005). Site level application of such manual for the trials conducted in Tanzania

and Equatorial Guinea, proved to beneficial considering that, the ultimate goal of safety assessment in clinical trials is to ensure that, safety assessment procedures are adequate for screening, safe administration of IP, safety follow-up and clinical care and safety reporting. Thus similar process may be applicable to other sites in Africa.

TITLE: Manual of Reference Intervals and Grading of Selected Abnormal Values	Code: BRTC_RITox_001_V3.1	Page 1 of 19

WRITTEN BY:			REVISED BY:		
Name	**Date**	**Signature**	**Name**	**Date**	**Signature**
Said Jongo	01-Feb-2022 | 17:58:48 CET	DocuSigned by: 7B4CEB6C4618459...	Florence Milando	02-Feb-2022 | 04:26:08 PST	DocuSigned by: ADA1323B6C0948F...
Kamaka Kassim	02-Feb-2022 | 00:41:33 PST	DocuSigned by: 464A3BA53C99430...	Hussein Mbarak	02-Feb-2022 | 04:31:43 PST	DocuSigned by: 687A7BF6DBBC48E...
Ummi Abdul	02-Feb-2022 | 11:15:07 CET	DocuSigned by: 980049C0BC784CF...	Mohammed Rashid	02-Feb-2022 | 13:36:37 CET	DocuSigned by: 6A7B89B3BE7C401...

APPROVED BY			AUTHORISED BY:		
Name	**Date**	**Signature**	**Name**	**EFFECTIVE DATE**	**Signature**
Alwisa Urassa	02-Feb-2022 | 19:06:20 CET	DocuSigned by: 490CCAC6783346F...	Sarah Mswata	03-Feb-2022 | 01:53:17 PST	DocuSigned by: 3272191976C64B...

ORIGINAL LANGUAGE:	

TRANSLATED BY:			TRANSLATION VERIFIED BY		
Name	**Date**	**Signature**	**Name**	**Date**	**Signature**
Not applicable			Not applicable		

CHANGES

DATE	CHANGE	REASON FOR CHANGE
01JAN2018	Creation	
19AUG2021	Update	**Additional parameters added as required**
06OCT2021	Update	**Additional Parameters added and graded**
01FEB2022	Update	**Additional Parameters added and graded with minor correction of typing errors for clarity**

Contents

Abbreviations

aPTT	Activated partial thromboplastin time
BASO	Basophils
D	Days
DMID	Division of Microbiology and Infectious Diseases
EO	Eosinophils
HCT	Haematocrit
HGB	Haemoglobin
INR	International Normalized ratio
LLG	Lower Limit of Grade
LLN	Lower Limit of Normal
LYMPH	Lymphocytes
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
Mo	Months
MONO	Monocytes
MPV	Mean Platelet Volume
NA	Not applicable
NEUT	Neutrophils
PCT	Plateletcrit
PDW	Platelet distribution width
P-LCR	Platelet Large Cell Ratio
PLT	Platelets
PT	Prothrombin Time
RBC	Erythrocyte count
RDW_CV	Red-cell distribution width Coefficient of Variation
RDW_SD	Red-cell distribution width Standard Deviation
TT	Thrombin Time
ULG	Upper Limit of Grade
ULN	Upper Limit of Normal
WBC	Leucocyte count
Y	Years

Clinical and Laboratory Reference Intervals

Determination of Reference Values

These reference intervals were obtained based on guidance from the Clinical and Laboratory Standards Institute (CLSI) guidelines [1]. The team including clinicians, data unit, laboratory and statisticians either used available local data or reviewed available published literature from the populations most likely to have similar biological characteristics. Geographical vicinity is therefore primarily considered for the reference to be selected. First to be reviewed for selection were those derived from Bagamoyo population [2, 3] in which case data was obtained from the IDEA project (a cross sectional study conducted at Bagamoyo Research and Training Centre, Ifakara Health Institute, Bagamoyo District from 2010-2013) and from pilot study conducted between 2013 and 2014 at BRTC. Reference intervals by age groups were estimated based on the likelihood for individuals within the same age groups to have similar biological characteristics. From the IDEA study, a total of 1030 children were screened in which 231 were excluded from the analysis of reference range due to malaria, 68 the hematological test was not done, 25 has temperature of more than 37.5°C,49 had poor nutritional status based on ±2SD score and 6 had age of less than 6 months. Data from a total of 751 subjects was used in the analysis of reference interval. Sample size may be less than 751 for some parameters due to final number obtained after detection of outliers [1]. For the pilot study data from approximately 450 individuals was used.

For parameters lacking data from Bagamoyo, the next priority were reference intervals derived from Tanzanian population or treatment guidelines [4, 5, 6]; then from other African countries [7-11]; and if no eligible publication then from internationally recognized institutions [12-14]. For each specific parameter on the reference interval tables, relevant reference is provided.

Statistical Method

Calculation of reference intervals was performed using the 2.5^{th} and 97.5^{th} centile of the distribution with 90% CI based on Clinical and Laboratory Standards Institute (CLSI) guidelines.

Parameters with sample size ≥120; reference intervals were estimated using STATA version 11.0 software (Stata Corp LP; College Station, Texas, USA). Only one Parameter had a sample size less than 120; in which reference interval was estimated by Robust method with Medical calculator software as recommended [3]. The possibility of calculating separate intervals for different groups (Partition test) between age group and gender as suggested by CLSI was performed by Quantile regression (reference). Parameters which shows statistical and clinical significant association with age group or/and gender their range calculated separately.

Detection of outlier was performed by using D test (one third rule), where D is the difference the absolute difference between an extreme observation and the next largest (or smallest) observation and R is the range difference between the largest and the smallest observation. The Cut off was done if D is equal or greater than 1/3, thus the extreme observation excluded [1]

LISTING 1 of 6: Clinical Reference Intervals by Age Categories

PARAMETERS	Units	Age Categories					Ref.
		(0 – 12) months	(1 – 3) Years	(3 – 6) Years	(6 – 12) Years	> 12 years	
Heart Rate	Beats/ Minute	80 - 120	70 - 110	65 - 110	60 - 95	56 - 96	2,3,14
Blood Pressure-Systolic	mmHg	80 - 100	90 - 105	95 - 110	100 - 120	105 - 136	2,3,14
Blood Pressure-Diastolic	mmHg	55 - 65	55 - 70	60 - 75	60 - 75	58 - 84	2,3,14
Respiratory Rate	Breaths/ Minute	25 - 40	20 - 30	20 - 25	14 - 22	12 - 20	2,3,14
Temperature Axillary	°C	36.0 – 37.5					4

LISTING 2 of 6: Haematology Reference Intervals, by Age Groups and Sex

PARAMETERS	Units	Age Categories											
		(3 to 5) Months		Ref.	6 Months to 5 Years		Ref.	(6 to 10) Years		Ref.	>10 Years		Ref.
WBC	10^3 / µl	4.74-14.77		7	5.1-16.2		3	4.5-12.9		3	3.48 – 9.11		2
RBC-ALL SEX	10^6 / µl	3.63-5.15		10	3.8-5.8		3	3.6-5.8		3			2
RBC-Male	10^6 / µl										4.39 – 6.5		2
RBC-Female	10^6 / µl										3.65-5.84		2
HGB-ALL SEX	g/dL	8.1-13.8		7	8.6-13.2		3	9.6-14.1		3			2
HGB-Male	g/dL										12.6 – 17.3		2
HGB-Female	g/dL										9.60-14.10		2
HCT-ALL SEX	(%)		(24.8-41.9)	7		(27.5-38.9)	3		(28.7-42.3)	3			2
HCT-Male	(%)											(38.94 – 49.5)	2
HCT-Female	(%)											(28.72-42.30)	2
MCV	fL	57-100		7	56.4-83.1		3	61.2-85.1		3	70.09 – 95.34		2
MCH	pg/cell	21.5–28.9		10	17.5-28.5		3	20.3-29.2		3	22.85 – 32.78		2
MCHC	g/dL	31.2-34.7		7	30.1-35.6		3	31.3-36.8		3	30.67 – 36.33		2
PLT	10^3 / µl	74-765		7	91-491		3	91-456		3	107 – 396.2		2
RDW_SD	fL			7	32.8-48.5		3	32.5-44.5		3	34.97 – 49.22		2
RDW_CV	(%)			7		(12.3-22.5)	3		(12.0-19.4)	3		(11.7 16.1)	2
PDW	fL			7	10.1-21.1		3	10.2-23.3		3	9 – 19.14		2
MPV	fL			7	8.9-13.5		3	8.8-14.0		3	8.3 13.4		2
P-LCR	(%)			7		(18.0-51.7)	3		(16.8-55.1)	3		(11.17 – 51.27)	2
PCT	(%)			7		(0.15-0.52)	3		(0.13-0.45)	3		(0 – 0.36)	2
NEUT	10^3 / µl (%)	0.57-3.53	(10.9–61.0)	7,11	1.3-6.9	(15.8-57.6)	3	1.2-6.2	(21.3-59.4)	3	1.18 – 5.46	(27.9 – 69.86)	2
LYMPH	10^3 / µl (%)	3.06-9.04	(33.6–82.5)	7,11	2.1-9.5	(30.2-73.2)	3	1.8-6.4	(30.8-64.9)	3	1.19 – 3.4	(21.4 – 55.1)	2
MONO	10^3 / µl (%)	0.38-1.89	(1.7–11.9)	7,11	0.4-1.6	(5.1-16.8)	3	0.3-1.1	(5.0-14.3)	3	0 – 0.85	(0 – 13.96)	2
EO	10^3 / µl (%)	0.07-0.70		7	0.1-1.6	(0.7-17)	3	0.1-2.1	(1.0-25.9)	3	0 – 0.78	(0 – 14.15)	2
BASO	10^3 / µl (%)	0.01-0.06		7	0.01-0.1	(0.1-1.2)	3	0.01-0.1	(0.1-1.0)	3	0 – 0.05	(0 – 0.9)	2
PT	Seconds										9.9 – 11.8		13
aPTT	Seconds										21 – 30		13
TT	Seconds										15.4 – 19.4		13
INR	Seconds										0.9 – 1.1		12

LISTING 3 of 6: Biochemistry Reference Intervals by Age Groups

PARAMETERS	Units	Age Categories					
		1 to 11 Months	1 to 5 Years	6 to 10 Years	Ref	> 10 Years	Ref
Albumin	g/L	36-48	40-49	40-48	5	42.7 – 60	2
Alkaline Phosphatase	U/L	164-589	153-410	174-460	5	45.4 – 153.1	2
γ- Glutamyltransferase (GGT)	U/L	3.0-34.0	3.0-34.0	7.0-31.0	8	8.3 – 108.1	2
Alanine aminotransferase (ALT/SGPT)	U/L	9-33	10-28	9-35	5	3.5 – 46.8	2
Aspartate aminotransferase (AST/SGOT)	U/L	26-65	27-55	21-51	5	12.3 – 74.7	2
Bilirubin Direct	µmol/L	0-19	0-9	0.6-3.9	5,9	0 – 6.2	2
Total Bilirubin (TBIL)	µmol/L	2-19	2-9	2-11	5	3.1 – 31.1	2
Glucose	mmol/L	2.9-5.5	3.0-5.4	2.7-5.3	5	3.9 – 6.0	2
Lactate Dehydrogenase (LDH)	U/L	360-995	360-995	277-823	8	127 – 264	6
Cholesterol	mmol/L	2.1-5.7	2.5-5.3	2.1-4.9	5	2.5 – 5.6	2
Creatinine Kinase	U/L	45-227	73-248	83-294	5	77 – 787	6
Creatinine	µmol/L	12-28	15-50	24-49	5	49 – 95.3	2
Blood Urea Nitrogen (BUN)	mmol/L	0.7-3.8	1.3-4.2	1.4-4.4	5	2.5 – 6.7	2
Potassium	mmol/L	4.2-6.5	3.8-5.9	3.2-5.2	5	3.5 – 5	2
Sodium	mmol/L	133-139	133-141	134-141	5	136 – 146	2
Chloride	mmol/L	100-107	100-108	98-108	5	97 – 107	2
Calcium	mmol/L	2.4-2.8	2.2-2.7	2.2-2.6	5	1.99 – 2.52	6
Magnesium	mmol/L	0.8-1.1	0.8-1.0	0.8-1.0	5	0.67 – 0.97	6
Bicarbonate - Male	mmol/L					22 - 32	16
Bicarbonate - Female	mmol/L					14 - 29	16

References for Reference Intervals

1 CLSI (2008) Defining, establishing, and verifying reference intervals in the clinical laboratory: approved guideline - third edition. CLSI Document C28-A3. Wayne, PA: Clinical and Laboratory Standards Institute.

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16 Miri-Dashe T, Osawe S, Tokdung M, Daniel N, Choji RP, et al. (2014) Comprehensive Reference Ranges for Hematology and Clinical Chemistry Laboratory Parameters Derived from Normal Nigerian Adults. PLOS ONE 9(5): e93919. https://doi.org/10.1371/journal.pone.009391

Grading of Laboratory Parameters:

- These grading tables for infants, young and older children consist of parameters, with severity grading to be used in clinical trials for safety data reporting to maintain accuracy and consistency in the evaluation of AEs
- When a single factor, guide or normal range is not appropriate for grading an AE across age groups, separate entries with specified age ranges are provided. If no distinction between age categories has been made, the listed entry is used in grading across all groups
- Applicable local normal range is prioritized, and therefore, any laboratory value that is between the LLN (for low values) or ULN (for high values) and grade 1 at the time of adopting grading guides is graded as grade 1
- Whenever the severity of an AE found to be in either one of two AE grades (i.e., the severity of an AE could be either grade 2 or grade 3), the value is allocated to the higher of the two grades
- Estimating Severity Grade for Parameters Not Identified in any of the guides for Toxicity Grading will be based on clinical or specific protocol guidance

LISTING 4 of 6: Grades for Selected Abnormal HEMATOLOGY PARAMETERS

PARAMETERS	Units	Age Range	NR / Source	Grade 1 Mild	Grade 2 Moderate	Grade 3 Severe	Grade 4 Life Threatening
HGB Decrease (ALL SEX)	gm/dL	3Mo to 5Y	TzSTG	9 to 11	7 to < 9	< 7	Na
	gm/dL	3Mo to 5Mo	8.1 to 13.8	7.8 to < 8.1	7 to < 7.8	< 7	< 4 or < 7 with signs of cardiac failure
	gm/dL	6Mo to 5Y	8.6 to 13.2	8 to < 8.6	7 to < 8		
	gm/dL	6Y to 10Y	9.6 to 14.1	9 to < 9.6	7 to < 9		
HGB Decrease (Female)	gm/dL	> 10Y	9.6 to 14.1				
HGB Decrease (Male)	gm/dL	> 10Y	12.6 to 17.3	9 to < 12.6			
WBC Decrease	cell/mm3	> 7D to 10Y	1000¦DMID	2,000 to 2,499	1,500 to 1,999	1,000 to 1,499	< 1,000
	10^3 / µl	3Mo to 5Mo	4.74 to 14.77	2 to < 4.74	1.5 to 1.999	1 to 1.499	< 1
	10^3 / µl	6Mo to 5Y	5.1 to 16.2	2 to < 5.1			
	10^3 / µl	6Y to 10Y	4.5 to 12.9	2 to < 4.5			
	cell/mm3	> 10Y	1000¦FDA	2,500 to 3,500	1,500 to 2,499	1,000 to 1,499	< 1,000
	10^3 / µl	> 10Y	3.48 to 9.11	2.5 to < 3.48	1.5 to 2.499	1 to 1.499	< 1
WBC Increase	cell/mm3		Site & 1000¦FDA	Values between ULN and ULG2(FDA)		20,001 to 25,000	> 25,000
	10^3 / µl	3Mo to 5Mo	4.74 to 14.77	> 14.77 to 17	> 17 to 20	> 20 to 25	> 25
	10^3 / µl	6Mo to 5Y	5.1 to 16.2	> 16.2 to 18	> 18 to 20		
	10^3 / µl	6Y to 10Y	4.5 to 12.9	> 12.9 to 16	> 16 to 20		
	cell/mm3	> 10Y	1000¦FDA	10,800 to 15,000	15,001 to 20,000	20,001 to 25,000	> 25,000
	10^3 / µl	> 10Y	3.48 to 9.11	> 9.11 to 15	> 15 to 20	> 20 to 25	> 25
PLT Decreased (Platelets)	10^9/L		CTCAEv5	< LLN to 75	< 75 to 50	< 50 to 25	< 25
	10^3 / µl	3Mo to 5Mo	74 to 765	62 to < 74	50 to < 62	25 to < 50	< 25
	10^3 / µl	6Mo to 5Y	91 to 491	75 to < 91	50 to < 75		
	10^3 / µl	6Y to 10Y	91 to 456				
	10^3 / µl	> 10Y	107 to 396.2	75 to < 107			
NEUT (Neutrophils) Decrease		Decrease	Site & DMID	Values between LLN and LLG3			
	10^3 / µl	< 6Mo	0.57 to 3.53	0.51 to < 0.57	0.45 to < 0.51	0.4 to < 0.45	< 0.4
	cells/mm3	>= 6Mo	1000¦DMID	800 to 1,000	600 to 799	400 to 599	< 400
	10^3 / µl	6Mo to 5Y	1.3 to 6.9	0.8 to < 1.3	0.6 to 0.799	0.4 to 0.599	< 0.4
	10^3 / µl	6Y to 10Y	1.2 to 6.2	0.8 to < 1.2			
	10^3 / µl	> 10Y	1.18 to 5.46	0.8 to < 1.18			
LYMPH (Lymphocytes) Decrease	10^9/L		CTCAEv5	< LLN to 0.8	< 0.8 to 0.5	< 0.5 to 0.2	< 0.2
	10^3 / µl	3Mo to 5Mo	3.06 to 9.04	0.8 to < 3.06	0.5 to < 0.8	0.2 to < 0.5	< 0.2
	10^3 / µl	6Mo to 5Y	2.1 to 9.5	0.8 to < 2.1			
	10^3 / µl	6Y to 10Y	1.8 to 6.4	0.8 to < 1.8			
	10^3 / µl	> 10Y	1.19 to 3.4	0.8 to < 1.19			
EO (Eosinophils)	factor		CPI(*ULN)	0.5x10^9 to 1.5	1.5 to 3	> 3	
	10^3 / µl	3Mo to 5Mo	0.07 to 0.70	> 0.7 to 1.1	> 1.1 to 2.1	> 2.1	Hypereosinophilic
	10^3 / µl	6Mo to 5Y	0.1 to 1.6	> 1.6 to 2.4	> 2.4 to 4.8	> 4.8	
	10^3 / µl	6Y to 10Y	0.1 to 2.1	> 2.1 to 3.2	> 3.2 to 6.3	> 6.3	
	cell/mm3	> 10Y	1000¦FDA	650 to 1500	1501 to 5000	> 5000	Hypereosinophilic
	10^3 / µl		0 to 0.78	> 0.78 to 1.5	> 1.5 to 5	> 5	Hypereosinophilic
Activated Partial Thromboplastin Time (aPTT)	factor		CPI(*ULN)	1.1 to 1.3	1.3 to 1.5	> 1.5 or minor bleeding	
	Seconds	> 10Y	21 to 30	> 30 to 39	> 39 to 45	> 45 or minor bleeding	
Prothrombin Time (PT) Increase	Factor	> 10Y	FDA (*ULN)	1.0 to 1.1	1.11 to 1.2	1.21 to 1.25	> 1.25
	Seconds	> 10Y	9.9 to 11.8	> 11.8 to 13	> 13 to 14.2	> 14.2 to 14.8	> 14.8
International Normalized Ratio (INR) Increase (not on anticoagulation therapy)	Factor	No age	DMID(*ULN)	1.1 to < 1.5	1.5 to < 2.0	2.0 to < 3.0	≥ 3.0
	Seconds	> 10Y	0.9 to 1.1	> 1.1 to 1.7	> 1.7 to 2.2	> 2.2 to 3.3	≥ 3.3

LISTING 5 of 6: Grades for Selected Abnormal BIOCHEMISTRY PARAMETERS

PARAMETERS	Units	Age Group	NR/ Source	Grade 1	Grade 2	Grade 3	Grade 4
Alanine aminotransferase (ALT/SGPT)	Factor	1Mo to 10Y	DMID(*ULN)	1.25 to < 2.5	2.5 to < 5.0	5.0 to < 10.0	≥ 10.0
	U/L	1Mo to 11Mo	9 to 33	> 33 to < 82.5	82.5 to < 165	165 to < 330	≥ 330
		1Y to 5Y	10 to 28	> 28 to < 70	70 to < 140	140 to < 280	≥ 280
		6Y to 10Y	9 to 35	> 35 to < 87.5	87.5 to < 175	175 to < 350	≥ 350
	Factor	> 10Y	FDA(*ULN)	1.1 to 2.5	2.6 to 5.0	5.1 to 10	≥ 10.0
	U/L	> 10Y	3.5 to 46.8	> 46.8 to 117	> 117 to 234	> 234 to 468	≥ 468
Aspartate aminotransferase (AST/SGOT)	Factor	No Age	DMID(*ULN)	1.25 to < 2.5	2.5 to < 5.0	5.0 to < 10.0	≥ 10.0
	U/L	1Mo to 11Mo	26 to 65	> 65 to < 162.5	162.5 to < 325	325 to < 650	≥ 650
		1Y to 5Y	27 to 55	> 55 to < 137.5	137.5 to < 275	275 to < 550	≥ 550
		6Y to 10Y	21 to 51	> 51 to < 127.5	127.5 to < 255	255 to < 510	≥ 510
	Factor	> 10Y	FDA(*ULN)	1.1 to 2.5	2.6 to 5.0	5.1 to 10	≥ 10.0
	U/L	> 10Y	12.3 to 74.7	> 74.7 to 186.75	> 186.75 to 373.5	> 373.5 to 747	≥ 747
Total Bilirubin	Factor	> 28D	DMID(*ULN)	1.1 to < 1.6	1.6 to < 2.6	2.6 to < 5.0	≥ 5.0
	µmol/L	1Mo to 11Mo	2 to 19	> 19 to < 30.4	30.4 to < 49.4	49.4 to < 95	≥ 95
		1Y to 5Y	2 to 9	> 9 to < 14.4	14.4 to < 23.4	23.4 to < 45	≥ 45
		6Y to 10Y	2 to11	> 11 to < 17.6	17.6 to < 28.6	28.6 to < 55	≥ 55
	Factor	> 10Y	FDA(*ULN)	1.1 to 1.5	1.6 to 2.0	2.0 to 3.0	> 3.0
	µmol/L	> 10Y	3.1 to 31.1	> 31.1 to 46.65	> 46.65 to 62.2	> 62.2 to 93.3	> 93.3
Total Bilirubin – *when accompanied by any increase in Liver Function Test*	Factor	> 10Y	FDA(*ULN)	1.1 to 1.25	1.26 to 1.5	1.51 to 1.75	> 1.75
	µmol/L	> 10Y	3.1 to 31.1	> 31.1 to 38.875	> 38.875 to 46.65	> 46.65 to 54.425	> 54.425
Creatinine	factor	1Mo to 10Y	DMID(*ULN)	1.1 to 1.3	> 1.3 to 1.8	> 1.8 to < 3.5	≥ 3.5
	µmol/L	1Mo to 11Mo	12 to 28	> 28 to 36.4	> 36.4 to 50.4	> 50.4 to < 98	≥ 98
		1Y to 5Y	15 to 50	> 50 to 65	> 65 to 90	> 90 to < 175	≥ 175
		6Y to 10Y	24 to 49	> 49 to 63.7	> 63.7 to 88.2	> 88.2 to < 171.5	≥ 171.5
	mg/dL	> 10Y	88.4¦FDA	1.5 to 1.7	1.8 to 2.0	2.1 to 2.5	> 2.5 or requires dialysis
	µmol/L	> 10Y	49 to 95.3	> 95.3 to 150.28	> 150.28 to 176.8	> 176.8 to 221	> 221 or requires dialysis
Blood Urea Nitrogen (BUN)	mg/dL	> 10Y	0.3571¦FDA	23 to 26	27 to 31	> 31	Requires dialysis
	mmol/L	> 10Y	2.5 to 6.7	> 6.7 to 9.3	> 9.3 to 11.1	> 11.1	Requires dialysis
Potassium – Hypokalemia	mmol/L		TzSTG	2.5 – 3		< 2.5	NA
			DMID	3.0 to < 3.4	2.5 to < 3.0	2.0 to < 2.5	< 2.0
		> 10Y	3.5 to 5.0	3.0 to < 3.5	2.5 to < 3.0	2.0 to < 2.5	< 2.0
Potassium – Hyperkalemia	mmol/L	> 10Y	FDA	5.1 to 5.2	5.3 to 5.4	5.5 to 5.6	> 5.6
		> 10Y	3.5 to 5	> 5 to 5.2	> 5.2 to 5.4	> 5.4 to 5.6	> 5.6
Glucose -Hypoglycemia	mg/dL	> 10Y	FDA	65 to 69	55 to 64	45 to 54	< 45
	mmol/L	> 10Y	3.9 to 6.0	3.6 to 3.8	3.1 to 3.5	2.5 to 3.0	< 2.5
Random Glucose -Hyperglycemia	mg/dL	> 10Y	FDA	110 to 125	126 to 200	> 200	Insulin requirements or hyperosmolar coma
	mmol/L	> 10Y	3.9 to 6.0	6.1 to 6.9	7.0 to 11.1	> 11.1	
Sodium – Hyponatremia	mmol/L	1Mo to 10Y	DMID	130 to < 135	125 to < 130	121 to < 125	≤ 120
		1Mo to 11Mo	133 to 139	130 to < 133	125 to < 130	121 to < 125	≤ 120
		1Y to 10Y	133 to 141				
		> 10Y	FDA	132 to 134	130 to 131	125 to 129	< 125
		> 10Y	136 to 146	132 to < 136	130 to < 132	125 to < 130	< 125
Sodium – Hypernatremia	mmol/L	No age	DMID	146 to < 150	150 to < 154	154 to < 160	≥ 160
		1Mo to 11Mo	133 to 139	> 139 to < 150	150 to < 154	154 to < 160	≥ 160
		1Y to 10Y	133 to 141	> 141 to < 150			
		> 10Y	FDA	144 to 145	146 to 147	148 to 150	> 150
		> 10Y	136 to 146	> 146 to 145	> 145 to 147	> 147 to 150	> 150
Calcium – hypocalcemia	mg/dL	≥ 7D	0.25¦DMID	7.8 to < 8.4	7.0 to < 7.8	6.1 to < 7.0	< 6.1
	mmol/L	1Mo to 11Mo	2.4 to 2.8	1.95 to < 2.4	1.75 to < 1.95	1.525 to < 1.75	< 1.525
		1Y to 5Y	2.2 to 2.7	1.95 to < 2.2			
		6Y to 10Y	2.2 to 2.6				
		> 10Y	1.99 to 2.52	1.95 to < 1.99			
Calcium – hypercalcemia	mg/dL	≥ 7 days of age	0.25¦DMID	10.6 to < 11.5	11.5 to < 12.5	12.5 to < 13.5	≥ 13.5
	mmol/L	1Mo to 11Mo	2.4 to 2.8	> 2.8 to < 2.9	2.9 to < 3.1	3.1 to < 3.4	≥ 3.4
		1Y to 5Y	2.2 to 2.7	> 2.7 to < 2.9			
		6Y to 10Y	2.2 to 2.6	> 2.6 to < 2.9			
		> 10Y	1.99 to 2.52	> 2.52 to < 2.9			
Bicarbonate	mmol/L		CTCAEv5	< LLN and no intervention initiated	-	-	-
		Male	22 to 32				
		Female	14 to 29				
Chloride	mmol/L	Increase in serum chloride	Suetrong,2016	Δ[Cl-] = [Cl-]max – initial chloride concentration			
				< 2.1	2.1 to < 5	5 to 5.1	> 5.1

LISTING 6 of 6: Reference and Grades for Selected Abnormal Urinalysis PARAMETERS

PARAMETERS	Units	Age Group	Normal/ Source	Grade 1 Mild	Grade 2 Moderate	Grade 3 Severe	Grade 4 Life Threatening
Protein	NA	> 10Y	Site & FDA	Values beyond Negative			
		> 10Y	Negative	Trace	1+	2+	> 3+ or require dialysis
Glucose	NA	> 10Y	Site & FDA	Values beyond Negative			
		> 10Y	Negative	Trace	1+	2+	> 3+ or requires hospitalization for hyperglycemia
Blood (microscopic)	rbc/hpf	> 10Y	Site & FDA	Values beyond Negative			
		> 10Y	Negative	1-10	11-50	> 50 and/or gross blood	Hospitalization or requires packed red blood cells transfusion

Reference for Laboratory Parameters Grading:

- **TzSTG** = Standard Treatment Guidelines and Essential Medicines List
- **Sibile et al =** Sibille M et al. A safety grading scale to support dose escalation and define stopping rules for healthy subject first-entry into man. Be J Clin Pharmacol 2010; 70: 736-48.
- **FDA** = US Food and Drug Administration. Guidance for industry: Toxicity grading scale for healthy adult and adolescent volunteers enrolled in preventive vaccine clinical trials. 2007. Available at https://www.fda.gov/regulatory-information/search-fda-guidance-documents/toxicity-grading-scale-healthy-adult-and-adolescent-volunteers-enrolled-preventive-vaccine-clinical (last accessed 03 OCTOBER 2021).
- **DAIDS =** Division of AIDS (DAIDS) Table for Grading the Severity of Adult and Paediatric Adverse Events Corrected Version 2.1 July 2017
- **CTCAEv5 =** Common Terminology Criteria for Adverse Events (CTCAE), Version 5.0. Published: November 27, 2017, U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES, National Institutes of Health, National Cancer Institute
- **CPI = Club Phase I.** a French non-profitable association of clinical pharmacologists. https://afpt-clubphase1.com/

Growth Reference Charts

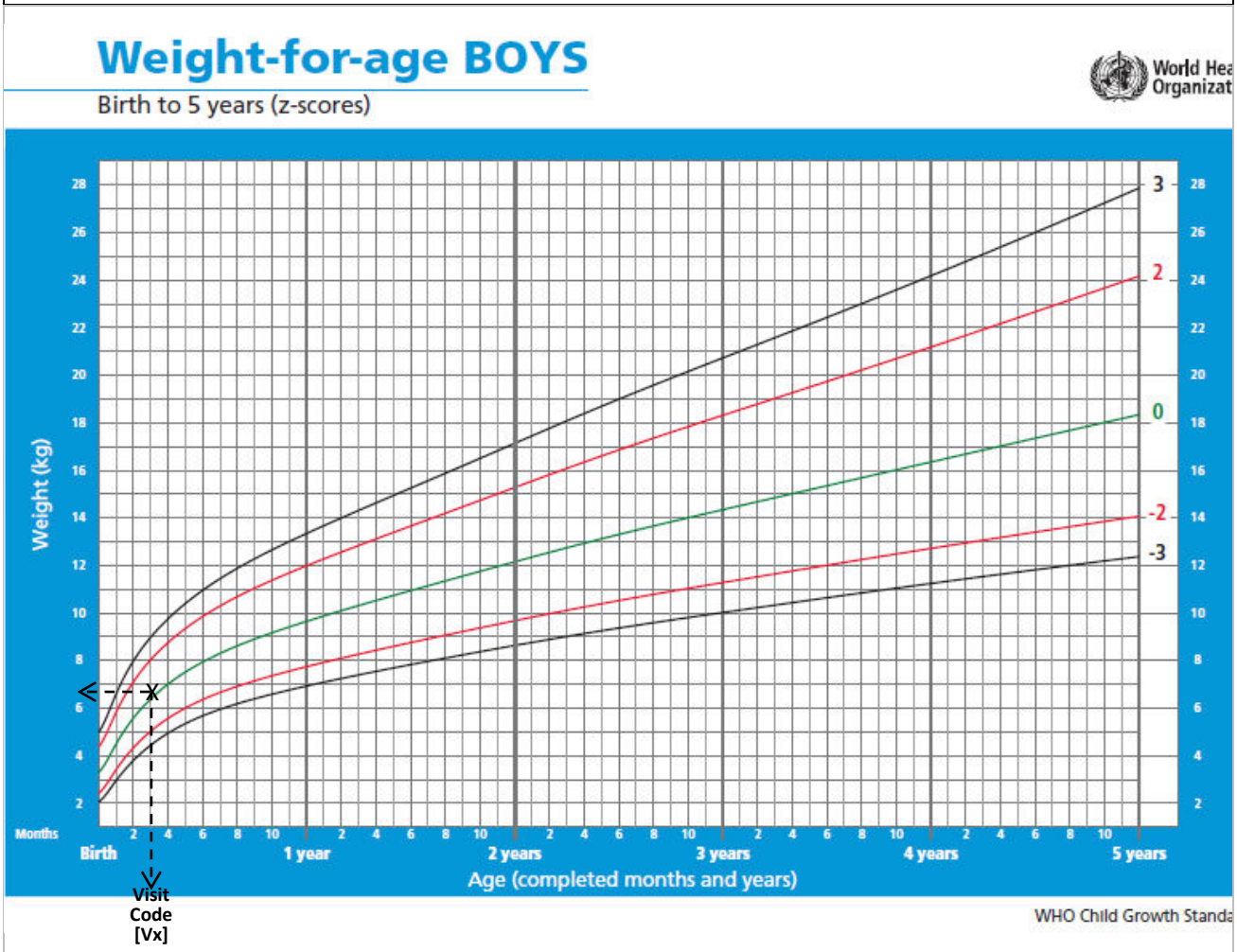
Instructions on using growth reference charts

In order to plot points, one needs to understand the following:

- x-axis – the horizontal reference line at the bottom of the graph. In the Growth Record
    - In the graphs below, the x-axis shows age (completed months and years). Plot points on vertical lines corresponding to completed age (in months, or years and months).
- y-axis – the vertical reference line at the far left of the graph. In the Growth Record
    - In the graph, the y-axis shows weight. Plot points on or between horizontal lines corresponding to weight as precisely as possible.
- Plotted point – the point on a graph where a line extended from a measurement on the x axis
    - Intersect by dote the value of age on the x-axis with a line extended from a measurement of weight on the y-axis.
    - When points are plotted for two or more visits, connect adjacent points with a straight line to better observe the trend.
- Judge whether a plotted point seems sensible, and if necessary, re-measure the child.

CHART 1 of 6: Weight-for-age Z-scores: Birth to 5 years: BOYS

Study Code ¦_____¦: Subject Initials ¦___|___|___¦: Subject ID ¦_____¦



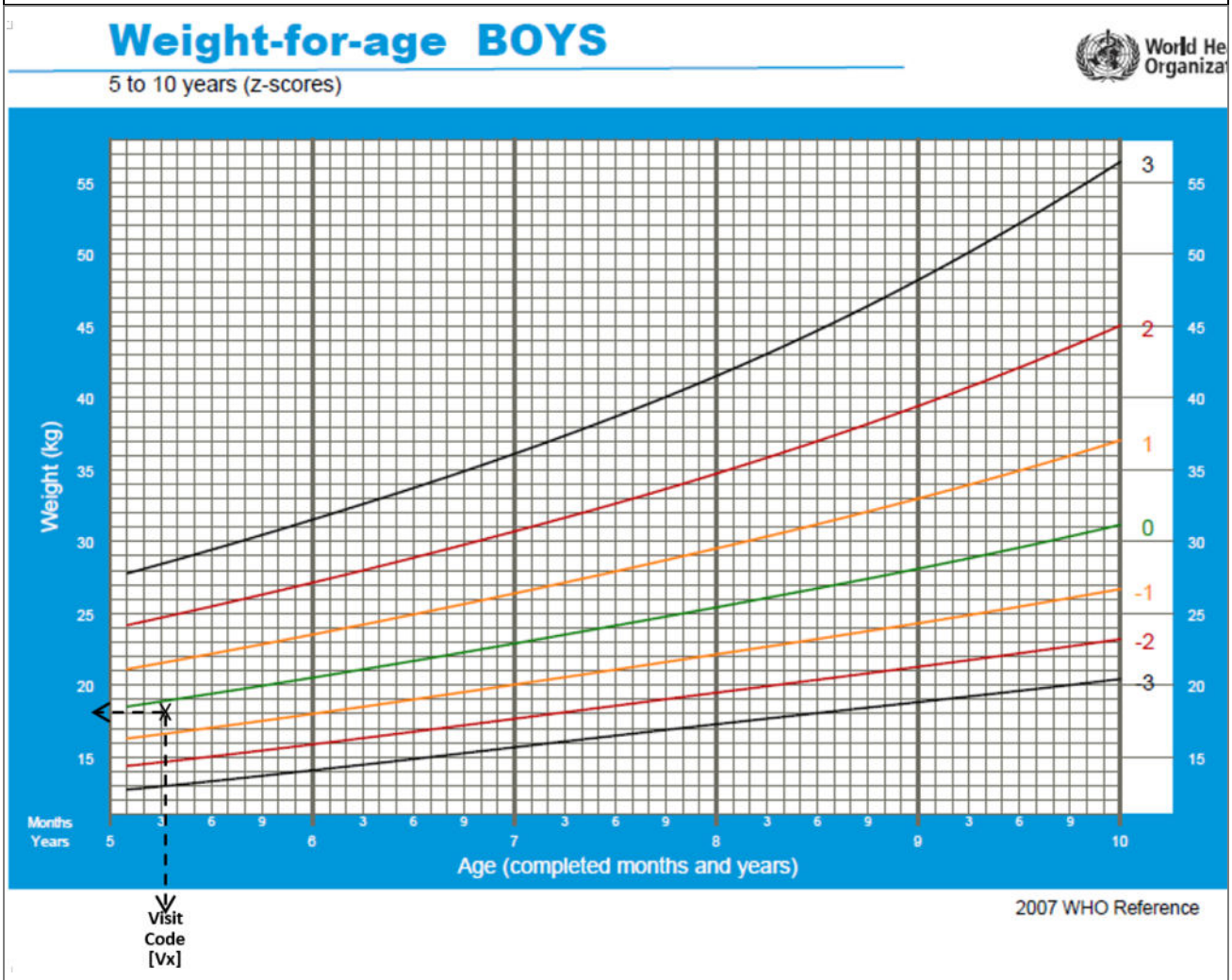
[See EXAMPLE above: For a Child aged 3 months with weight of 6kg]

Visit Code	DATE	COMMENT	INITIALS
Vx	dd/MMM/yyyy	Normal Growth	XYZ

Comments for Unscheduled Nutritional Assessments:

CHART 2 of 6: Weight-for-age Z-scores: 5 to 10 years BOYS

Study Code ¦_____¦: Subject Initials ¦___|___|___¦: Subject ID ¦_____¦



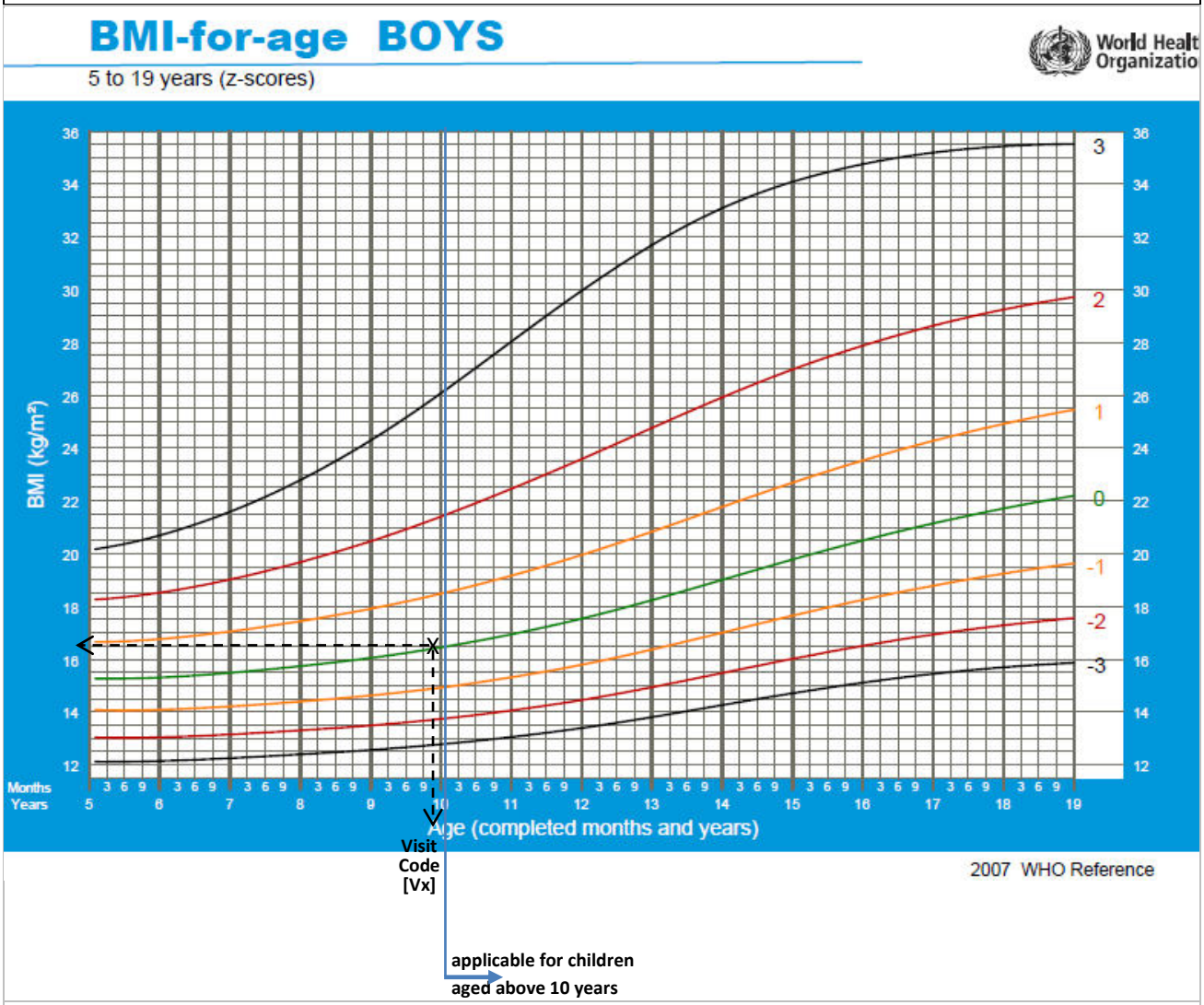
[*See EXAMPLE above: For a Child aged 5 years and 3 months with weight of 19kg*]

Visit Code	DATE	COMMENT	INITIALS
Vx	dd/MMM/yyyy	Normal Growth	XYZ

Comments for Unscheduled Nutritional Assessments:

CHART 3 of 6: BMI-for-age Z-scores: 5 to 19 years BOYS

Study Code ¦_____¦: Subject Initials ¦___|___|___¦: Subject ID ¦_____¦

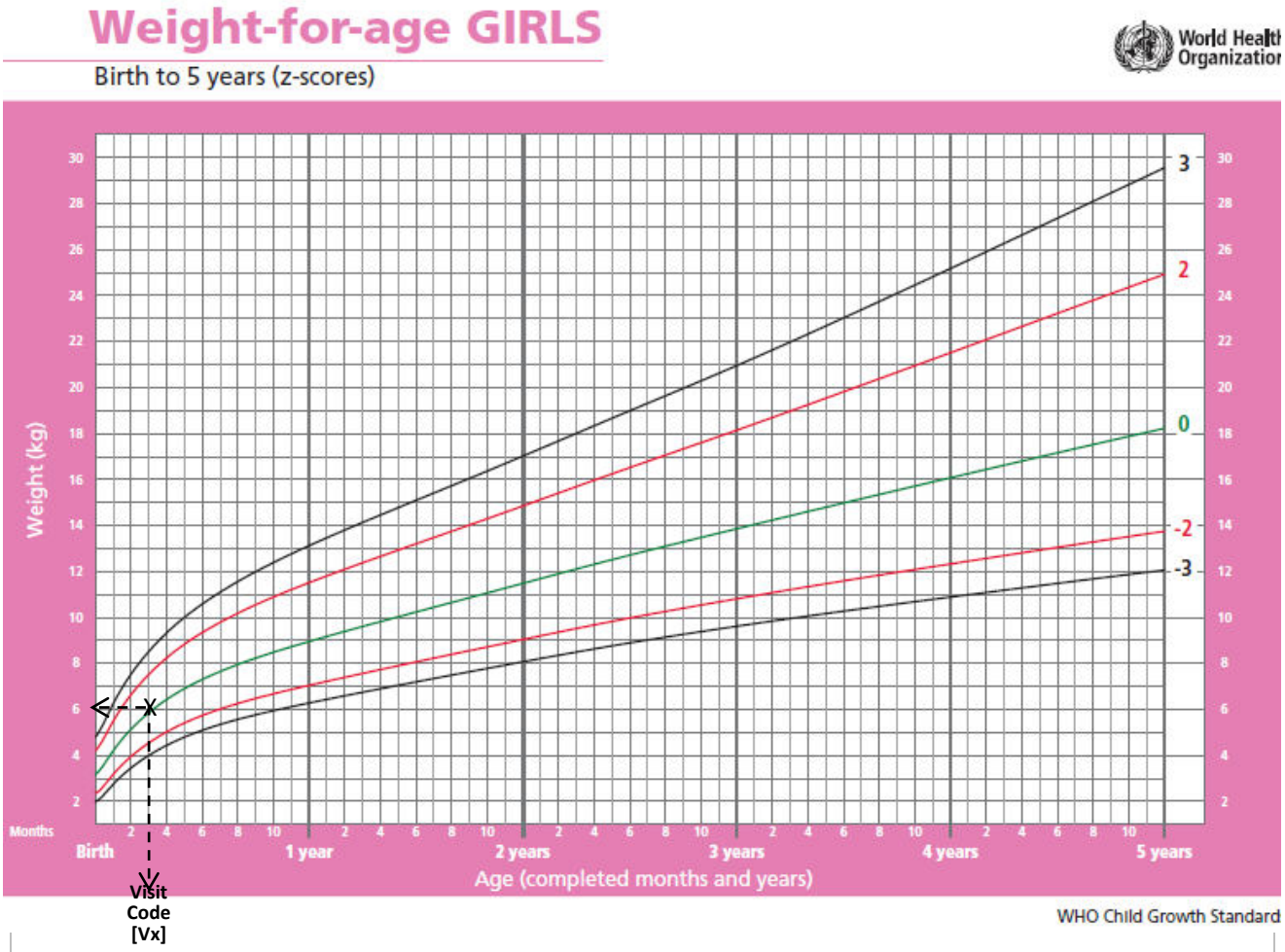


INSTRUCTIONS [*See EXAMPLE above: For a Child aged 9 years and 9 months with BMI of 16.5*]

Visit Code	DATE	COMMENT	INITIALS
Vx	dd/MMM/yyyy	Normal Growth	XYZ

CHART 4 of 6: Weight-for-age Z-scores: Birth to 5 years GIRLS

Study Code ¦_____¦: Subject Initials ¦___|___|___¦: Subject ID ¦_____¦



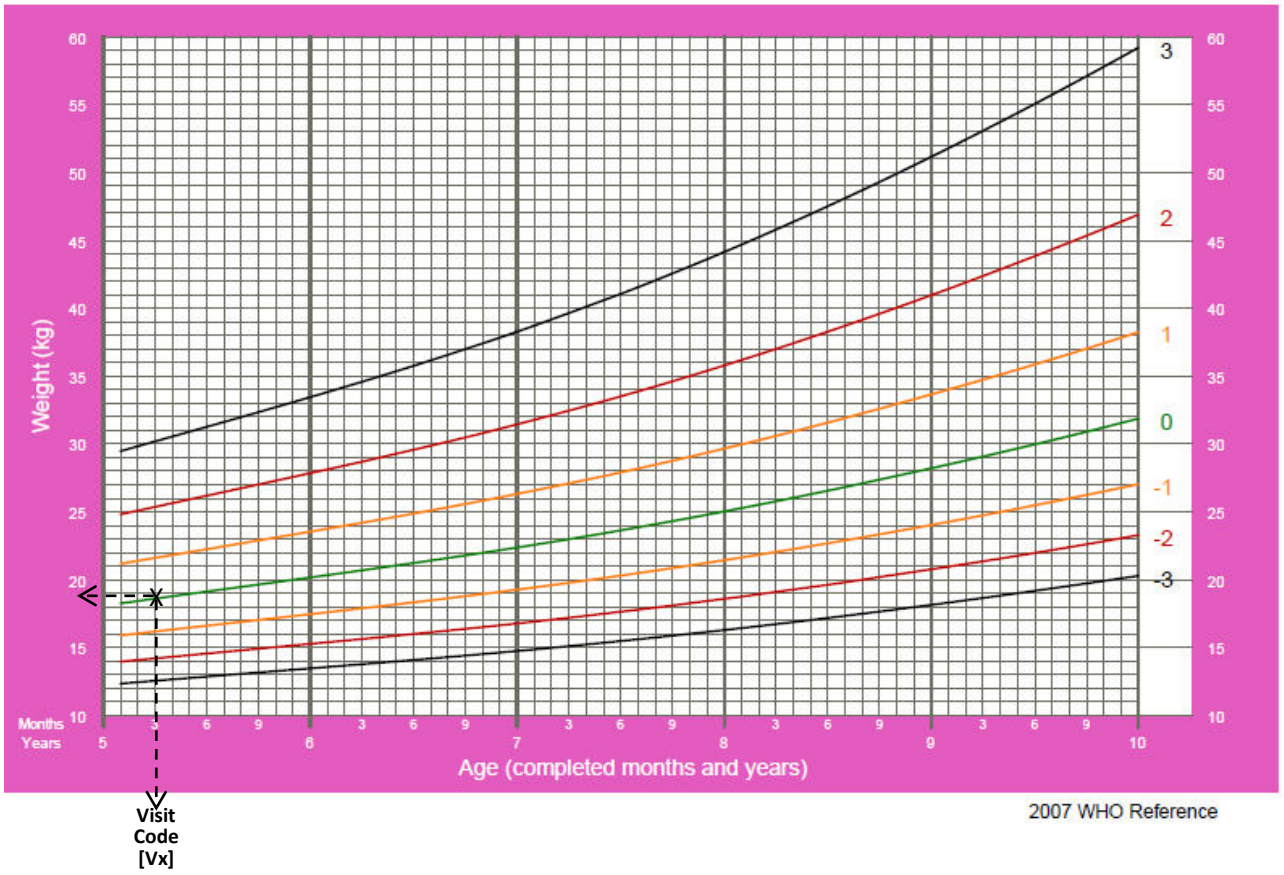
INSTRUCTIONS [*See EXAMPLE above: For a Child aged 3 months with weight of 6kg*]

Visit Code	DATE	COMMENT	INITIALS
Vx	dd/MMM/yyyy	Normal Growth	XYZ

Comments for Unscheduled Nutritional Assessments:

CHART 5 of 6: Weight-for-age Z-scores: 5 to 10 years GIRLS

Study Code ¦_____¦: Subject Initials ¦___|___|___¦: Subject ID ¦_____¦



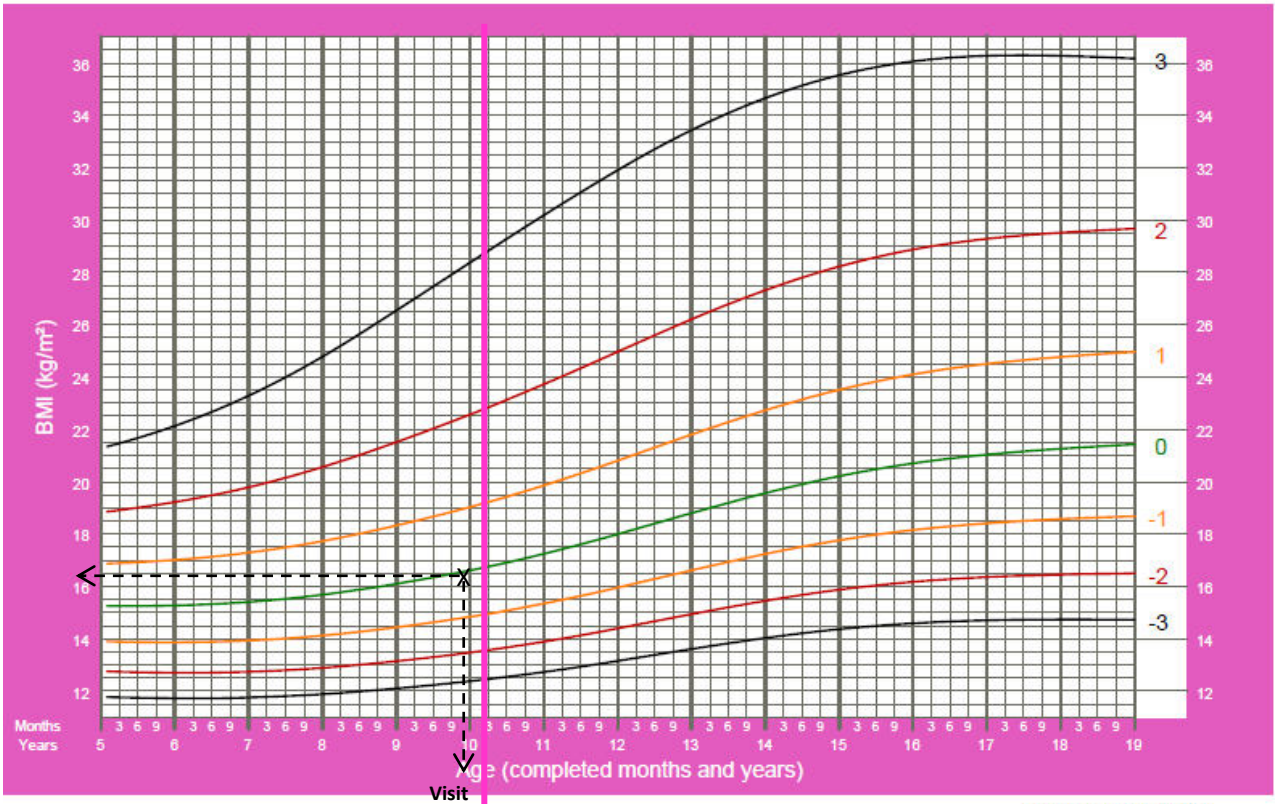
[See EXAMPLE above: For a Child aged 5 years and 3 months with weight of 19kg]

Visit Code	DATE	COMMENT	INITIALS
Vx	dd/MMM/yyyy	Normal Growth	XYZ

Comments for Unscheduled Nutritional Assessments:

CHART 6 of 6: BMI-for-age Z-scores: 5 to 19 years GIRLS

Study Code ¦_____¦: Subject Initials ¦___|___|___¦: Subject ID ¦_____¦



INSTRUCTIONS [*See EXAMPLE above: For a Child aged 9 years and 9 months with BMI of 16.5*]

Visit Code	DATE	COMMENT	INITIALS
Vx	dd/MMM/yyyy	Normal Growth	XYZ

Comments for Unscheduled Nutritional Assessments:

CHAPTER 7: Discussion, conclusion and recommendations

7.1 General discussion

This chapter discusses the key safety components, outcomes and the related key optimizations undertaken to support implementation of GCP compliant malaria vaccine studies performed in the context of my PhD thesis. Fundamental to this works is our hypothesis that, the safety profile of malaria vaccine candidates is determined by the product itself rather than the type of African population in which it is tested. We aligned our three aims to with the goal to demonstrate the potential reproducibility and reasonable justification for application, of processes and results across sites; (i) formation of South-South/ North-South collaborations implementing a series of clinical trials among African clinical research sites; (ii) standardization of optimization-approach for enrolment practices that are tailored to local context while maintaining the key components of product specific eligibility criteria; (iii) standardization of optimization-approach for interpretation of results and assessment of abnormal clinical and laboratory parameters in reference to the clinical characteristics of the local population. Strategic partnerships created an enabling platform for interactions with multiple regulatory authorities, ethical committees and IRBs, a critical component reflecting independent reviews for the protocols and reporting of results. Ultimately, this approach leads to the generation of clinical trial safety data that is adequate to enable proper selection of relevant study participants, justify safe administration of interventions, support clinical care of participants and meet reporting requirements to regulatory authorities among trials conducted in different African communities. Through this implementation, we identified key challenges and solutions related to safety evaluation, enrolment process, eligibility criteria and standardization of safety assessment among trials conducted in different African communities.

We evaluated the PfSPZ based candidates in clinical trial centres located in malaria endemic regions on the eastern and western coasts of Africa namely Bagamoyo, Tanzania and Bioko Island, Equatorial Guinea, among the target communities for the PfSPZ-based malaria vaccine candidates. We bring together, the process of safety assessment tailored to the protocol components in the context of the local African population and healthcare systems, to answer questions on the key early

safety concerns about the PfSPZ-based malaria vaccine candidates across different age categories. We generated pivotal safety data for subsequent safety evaluation and dose optimization among African communities including; adults living with or without HIV, adolescents, children and infants (Chapter 4). In addition, we discuss the fundamental elements for optimizations to the recruitment and enrolment processes targeting to not only achieve recruitment targets but also to build and strengthen the knowledge base and ensure skill transfer among the African sites and investigators. (Chapter 5). Furthermore, we discuss the need and the potential challenges, of adopting standard toxicity grading systems as well as the process we have adopted and optimizations needed to develop population-based toxicity grading limit values as a tool for standardized reporting of safety results to ensure proper clinical care of participants and safety reporting to the respective local clinical trials regulatory agencies (Chapter 6). We also give recommendations for further research and practical application for optimizations achieved as the contribution of the current PhD work in the field of malaria vaccine development.

7.1.1 Overall development strategy and the safety profile of PfSPZ Vaccine candidates

The clinical development plan of PfSPZ vaccine candidate products in Africa is implemented through South-South, North-South collaboration in partnership through a platform of i-PfSPZ consortium. With the understanding that, reporting, analysis and interpretation of safety data in clinical trials require careful examination of data, this platform enabled a program of work that facilitated sharing of results of safety evaluations among clinical trials in several clinical research sites in Africa. This setup has enabled detailed evaluation of our hypothesis that, the safety profile of malaria vaccine candidates is determined by the product itself rather than the type of African population in which it is tested. Sanaria® PfSPZ Vaccine is comprised of aseptic, purified, cryopreserved, Pf sporozoites (PfSPZ) that are manufactured according to cGMPs for parenteral injection. PfSPZ are diluted in phosphate buffered saline (PBS) and human serum albumen (HSA) to obtain the desired dose and

concentration, and are administered by DVI without an adjuvant. The PfSPZ of PfSPZ Vaccine are attenuated by exposure to radiation during manufacture and are unable to cause parasitemia in humans or undergo transmission to mosquitoes that might bite an individual following immunization. The long-term objective for PfSPZ Vaccine is deployment for mass vaccination programs (MVPs), in which entire populations in defined geographic areas will be immunized (excluding infants less than 5 months of age). The strategy to combine mass drug administration (MDA) with MVPs to prevent reinfection has the potential to halt malaria transmission and eliminate malaria from the target population.

The first-in-humans Phase 1 clinical trial of PfSPZ Vaccine was conducted in malaria-naïve adults at the Naval Medical Research Center and the University of Maryland USA, via ID or SC routes (Epstein et al., 2011b). Administration via such routes in human subjects was well tolerated and indicated no breakthrough blood stage infections, with immune responses that protected several volunteers against controlled human malaria infection (CHMI). In non-human primates, administration via IV route greatly increased PfSPZ-specific immune responses. Clinical testing of PfSPZ Vaccine using the IV route was similarly safe and well tolerated following dose escalated in VRC 312 trial conducted in the US and indicated a vaccine dose response with regard to antibody and T cell following CHMI VE 100% (6/6) in subjects who received 5 doses (Ishizuka et al., 2016c). These initial studies conducted in non-immune populations in the U.S established tolerability, safety, immunogenicity and efficacy data were followed by the intention to demonstrate early on if there are differences in safety, immunogenicity and efficacy of this whole parasite vaccine between naïve and endemic populations. Multiple research groups joined the International PfSPZ Consortium (I-PfSPZ-C), subsequently used DVI as standard for administration, where the vein is punctured directly with a 25-gauge needle and the vaccine in 0.5 mL of diluent is injected over a few seconds. The results that we reported in Paper I, have shown that, PfSPZ Vaccine has been safe and well tolerated in African adults: the rates of AEs (including laboratory abnormalities) have been similar in study subjects receiving PfSPZ Vaccine and NS placebo, indicating that PfSPZ Vaccine has not caused any side effects at the doses studied

among the trials we reported in this thesis with similar observations made in studies conducted among adults elsewhere in Africa (Oneko et al., 2021; Sissoko et al., 2017b). The first pediatric trial was conducted in Tanzania (Paper II), and assessed the safety of PfSPZ vaccine in adolescents, children and infants, through age de-escalation / dose-escalation for safety, indicated that, the vaccine was safe and well tolerated in all age groups. Safety data generated allowed the initiation of second pediatric trial in western Kenya (Oneko et al., 2021) and a third pediatric study in Equatorial Guinea addressing safety and immunogenicity, showed PfSPZ Vaccine to be safe and well tolerated in infants and children. Results indicating important key milestones are presented in Paper III, that, increasing the dose of PfSPZ in PfSPZ Vaccine from 9.0x10^5 to 1.8x10^6 (3 doses at 8-week intervals) in Tanzanian adults significantly reduced vaccine efficacy (VE) against controlled human malaria infection (CHMI) from 100% to 33%. There were no safety concerns related to higher doses. Prior to these findings, we have pursued an empiric development process in which we have altered PfSPZ/dose, and dose numbers and intervals to identify an optimal immunization regimen. Interestingly, in both USA/EU and some African studies, 9.0x10^5 PfSPZ appeared to be the optimal dose, and higher doses were less protective. In the second Equatorial Guinea trial, less than 50% of adults were protected after receiving three injections at 8-week intervals with a still higher dose, 2.7x10^6 PfSPZ of PfSPZ Vaccine (Paper IV). On the other hand, the 1.8x10^6 PfSPZ dose achieve 100% protection in Mali; in the Mali trial, performed in a location where naturally acquired immunity (NAI) is strongly developed, NAI may have reduced the effective dose by neutralizing a proportion of the injected PfSPZ. Thus it may turn out that higher doses are needed in those with heavy prior malaria exposure than are needed for malaria-naïve individuals. The results that we reported in paper V on a study of PfSPZ Vaccine we conducted in HIV- and HIV+ adults in Tanzania, showed that, the vaccine was safe and well tolerated indicating that screening for HIV may not be necessary when the vaccine is deployed in MVPs. Further assessment is ongoing among immunocompromised individuals to understand the immune responses that may improve efficacy among immunocompromised individuals while maintaining the good safety profile. PfSPZ Vaccine was similarly tolerable and safe

when given in condensed multi-dose prime regimens ranging from 3-dose regimen (days 1, 8 and 29) to 5-dose regimen (whether boosts at 29 days or 113 days) and a boost apparently improve vaccine efficacy (Paper VI). These observations, indicated that the most optimal multi-prime regimen is 9x10^5 PfSPZ given on days 1, 8 and 29 and has been adopted for use in subsequent trials testing PfSPZ Vaccine. Overall there have been no observed breakthroughs following injection with over 5,000 doses of PfSPZ administered to date. Fever has only rarely been reported as a solicited AE and the few reports to date were generally attributed to other causes. Randomized, double-blind, placebo-controlled trials in African adults, children and infants, have also found no differences in the rates of local or systemic reactogenicity between vaccine recipients and controls after unblinding (Jongo et al., 2019; Oneko et al., 2021). Most importantly, there were no age or dose effects on the rates of AEs with doses as high as 1.8x10^6 PfSPZ in infants and young children and there also has not been an increase in AEs with repeated dosing (i.e., the rates of AEs after second or third doses have been similar to the rates after first dose). (Jongo et al., 2022a; Jongo et al., 2018b; Oneko et al., 2020; Oneko et al., 2021; Sissoko et al., 2017b). In addition, there have been no allergic reactions linked to the vaccine. A few volunteers have developed urticarial rashes in the days following immunization, but in no cases have these rashes been clearly linked to the vaccine. It is thus not clear that PfSPZ Vaccine has caused any adverse reactions at the doses tested to date. The work that we conducted in two clinical research centres in Africa (Bagamoyo, Tanzania and Bioko island, Equatorial Guinea) formed the basis of internal validation, that was used as a reference to application of procedures and safety results in trials conducted in other African sites as external validation, strongly agrees to our hypothesis that, the safety profile of malaria vaccine candidates is determined by the product itself rather than the type of African population in which it is tested. This breakdown of internal-externa validation is demonstrated by the forest plots showing the side effect profile of PfSPZ Vaccine recorded in 18 Phase 1 and 2 clinical trials in Africa. However, the side effect profile could evolve with the performance of the phase 3 trials, which will increase the power to detect small differences in the rates of AEs between vaccinees and placebo recipients, and which has a higher likelihood of

revealing uncommon adverse effects due to the larger sample size. Until such studies can definitively rule out side effects, the study investigators will need to continue to describe the signs and symptoms that are typically caused by less well tolerated vaccines or by malaria, as theoretical concerns.

7.1.2 Optimizations to the recruitment and enrolment processes through understanding of the local African population

Extrapolations of recruitment criteria from clinical trial protocols previously implemented outside Africa, may be part of the efforts to standardize procedures between trials that are linked by design or IP being investigated. However, the GCP guidance that stipulates human subject protection in clinical research and those promoting enrollment practices reflect the population may potentially be compromised during such extrapolations (FDA-2019-D-1264, 2020). The dominance of subjective judgement or lack of proper skills in determining how and when the criteria optimizations are needed across clinical trials sites among African communities may further lessen the value of recruitment criteria extrapolation. With the goal to demonstrate the potential reproducibility and reasonable justification for application, of processes and results across sites, we targeted to establish, the standardization of optimization-approach for enrolment practices that are tailored to local context while maintaining the key components of product specific eligibility criteria. The measure of successful recruitment, is beyond achieving the required sample size, but rather, should focus on how well the recruitment criteria will perform in reference to GCP compliance (including local regulation), protocol compliance and relevance of clinical and laboratory criteria defining participant health status. Better performance of recruitment criteria is key to the participant retention and quality data collection. The quest for the more objective approach in constructing and applying relevant recruitment criteria only begins with understanding the constellation of challenges facing clinical research sites and investigators among African communities. These challenges range from the competency level of the local regulatory and ethical infrastructure to handling of guidance stipulated in the GCP and clinical trials regulation. Handling of such challenges must be tailored to the local context that will provide

potential options to support successful execution of each recruitment criteria. While clinical trials volunteers in some countries may be invited to attend clinical trials via internet, the local infrastructure may not allow majority to be reached via internet, and therefore, locally applicable methods of advertisements must be adopted and receive approval prior to their use. Both, study advertisement and ICF must use local language. Additional optimizations may be needed in; volunteer identification, mitigation for over recruitment, pregnancy prevention approach linked with the IP risk assessment including the indications for using highly effective methods of birth control, re-embursement for time and transport, participants' retention and creation of volunteer database. Criteria for the assessment of compliance to GCP must be reflected in the local regulatory requirements and must not be diluted simply because the trial is conducted in Africa, as they focus on key issues related to the responsibilities of the sponsor, the investigator and the rights of individual trial participants. Criteria optimizations are fundamentally linked with equally relevant optimizations targeting the sites and the teams of local investigators. Efforts must be made by the sponsor and investigators to ensure that those involved in conducting a trial are qualified by education, training and experience to perform their respective tasks (ICH-GCP). Subsequent to this setup, are the community engagement activities. Optimizations are required to ensure proper engagement with the local community, involving local community leaders on every aspect. The work in this thesis, included the capacity strengthening in Bagamoyo and capacity building in Bioko Island to ensure that resources are made available for local investigators to implement GCP compliant procedures. In some cases, especially for the sites, the capacity building may be required at regulatory level. The concept of optimization that we described in this thesis, is based on the practical experience and is a means for skill transfer on the decision matrix that may potentially support investigators and sponsors to make informed decisions, not only on the recruitment criteria that is extrapolated for implementation of similar trials in Africa but also for the development of a new set of criteria for new protocols that are implemented for the first time in Africa. As more and more African sites are lined to participate in conducting first in man trials, this is a

significant step towards achieving synchronization of processes and will allow timely execution of program portfolios and introduction of new interventions to policy.

7.1.3 Optimization of Tools for safety assessment based on data generated from the local population

The ultimate goal of safety assessment in clinical trials is to ensure that, safety assessment procedures are adequate for screening, safe administration of IP, safety follow-up or care and safety reporting. Regulatory guidance is available on the safety reporting requirements related to certain types of adverse events, reporting for which need to be expedited (TMDA, 2017). Furthermore, stopping rules are used to define the threshold of events for investigator to report to sponsor and safety oversite committees. These and other related scenarios require that, the protocol reporting instructions are primarily referenced to the applicable regulatory requirements and the delegated investigators are trained to properly evaluate the reporting thresholds. With the goal to demonstrate the potential reproducibility and reasonable justification for application, of processes and results across sites, we targeted to establish standardization of optimization-approach, for interpretation of results and assessment of abnormal clinical and laboratory parameters in reference to the clinical characteristics of the local population.

Severity grading systems that are based on the investigator's clinical judgement, are subjective and may cause significant delays in recognition of an event that has actually met the reporting requirements. In addition to minimization of investigator's clinical judgement this optimization is necessary to support our hypothesis through standardization in the severity assessment of results for abnormal clinical and laboratory parameters, among investigators from different sites. One way to address this, is to use recommended standard toxicity grading systems which provide recommendations on assessing the severity of clinical and laboratory abnormalities in volunteers enrolled in clinical trials (FDA 2005D-0155, 2007; NCI, 2017; NIH-DAIDS, 2017; NIH, 2005). Such uniformity in categorizing toxicities can improve the issues associated with subjective clinical

judgement. It is important to also recognize that, determination of parameter limit values and multiplication factors for severity grades were based on published information or clinical experience (FDA 2005D-0155, 2007), raising a question of how well, the standardized toxicity grading systems align with local reference intervals. For this reason, this optimization, is perhaps the most complicated single optimization at site level especially when the parameter limit values are defined and may require the exercise of clinical judgment. The first step in adopting this system was to select the appropriate standard toxicity grading of reference based on the study population. The second step was to generate reference intervals based on local clinical and laboratory data and the third step was to integrate the local reference intervals to the standard toxicity severity grades. This approach can be employed in many settings in Africa where known conditions associated with samples or clinical data collected may be retrieved as required by the CLSI. Additional optimization measures not mentioned in the recommendations and may require clinical judgement include; the presence of overlapping values between grades and the presence of values that cannot be located to any severity grade. What we achieved in this work, is a description and application of the process we have adopted. The challenges that we experienced and described with regards to the application of standard toxicity grading systems in African population, are primarily due to the fact that, the available standard toxicity grading systems have been generated from the different populations. The next steps for African clinical research sites / centers should focus on further optimization, through generation of standard toxicity grading systems using data collected from African population.

7.2 Contribution of the current thesis

This thesis has systematically addressed matters beyond the individual study benefits through activities that led to transformation in research and development agenda for the local clinical research scientific communities through the knowledge base in terms of utilizing data generated locally for optimizing subsequent stages of clinical development plan. Through this work, we performed; (1) The first demonstration of PfSPZ Vaccine efficacy against PfSPZ Challenge in healthy adults population

(PAPER I), the first demonstration of safety and tolerability of PfSPZ Vaccine in infants and children along with demonstration of the safety and exploration of efficacy of Sanaria's PfSPZ vaccine using PfSPZ-CHMI in African adults (Paper II and Paper III); (2) the demonstration of safety and tolerability of Sanaria's chemo-attenuated vaccine (PfSPZ-CVac) in Equatorial Guinea (Paper IV); (3) the first demonstration of safety and tolerability of PfSPZ Vaccine and exploration of efficacy of Sanaria's PfSPZ vaccine using PfSPZ-CHMI in individuals living with HIV (Paper V); (4) the demonstration of safety and tolerability of accelerated vaccination regimens of PfSPZ Vaccine and down-selection of optimal regimen for pivotal trials in Africa (Paper VI).

Through this work, we demonstrated the broader concept of safety profiling, as the process to achieve safety assessment beyond mere findings from the results of individual parameters following exposure to IMP, but rather, to generate safety data adequate to justify safe administration of interventions, support clinical care of participants and meet reporting requirements to regulatory authorities in reference to the baseline epidemiolocal and clinical characteristics of the local population being assessed. The process we have demonstrated, provides fundamental bridge between the intervention being assessed and the local clinical trial regulation system, by enabling proper use of local resources as well as the timely and relevant policy decisions to be made. We generated early safety and tolerability data related to the administration of PfSPZ based malaria vaccine candidates in endemic populations residing in different geographical locations of Africa, including early assessment in healthy adults with or without HIV and in target pediatric population of healthy adolescents, children and infants. These data, not only contributed to address early safety concerns related to the PfSPZ vaccine products, but also played critical role in supporting further exploration of immunization regimens during clinical development of PfSPZ vaccine. Overall, PfSPZ Vaccine and normal saline show equivalent adverse event profile across these populations, regardless of the site from which they reside in Africa. We were able to achieve down-selection of the vaccine regimen for PfSPZ Vaccine for pivotal clinical trials. Thus far, findings from this work have significantly contributed to the wide testing of PfSPZ based vaccine candidates in 7 African countries. The pilot epidemiological

studies conducted in parallel, were specifically designed to characterize baseline epidemiological and clinical parameters of the malaria endemic populations. Such studies enabled piloting and subsequent optimization of recruitment strategies and tools for safety assessment by accommodating relevant elements for local population and healthcare systems. Through such optimizations we were able to screen over 6000 individuals and created site registry of potential research participants with additional development and improvement of site-based community engagement and recruitment manuals / SOPs. Furthermore, for standardized interpretation and reporting of results of safety assessment among local population, we developed quality-controlled site-specific manuals of reference intervals and adopted toxicity grading systems for sites located in Bagamoyo and Bioko Island. Such optimizations have enabled safety assessment processes that, adequately address matters related to safe administration of study interventions, proper care to trial participants and meet the reporting requirements to regulatory authorities. In addition, this work has spearheaded the establishment of the clinical research capacity for conduct of regulatory trials in Equatorial Guinea, further strengthened clinical research capacity for conduct of regulatory trials in Tanzania, and used the clinical trials platform to support several masters and PhD training of colleagues in both, Tanzania and Equatorial Guinea.

7.3 Conclusion

Evaluation of potential relevant health interventions tailored to collaborations that involve South-South / North-South partnerships linking multiple sites in Africa, can be the peculiar platform to demonstrate and promote the potential reproducibility of processes and results, with reasonable justification for their application across sites. Through such a platform, standardizations that we made on the optimization-approaches for enrolment practices and for interpretation of results and assessment of abnormal clinical and laboratory parameters, played essential role enabling reproducibility of procedures across sites. Fundamental to the benefit of such standardizations, is the potentially reasonable justification for application of results from evaluations across sites. In the scope of this

thesis, the assessments that we performed, provided results indicating that, the safety profile of malaria vaccine candidates is determined by the product itself rather than the type of African population in which it is tested. Strategic partnerships created an enabling platform for interactions with multiple regulatory authorities, ethical committees and IRBs, a critical component reflecting independent reviews for the protocols and reporting of results. With the spectrum of available malaria vaccine candidates including the PfSPZ based, these findings will enable accelerations of clinical development plans not only for clinical trials accessing malaria vaccines but all other related interventions. Our perspective is to maintain the portfolio of activities and skill base that will streamlined implementation for subsequent programs of work.

7.4 Recommendations

Several recommendations could be derived on further research and practical applications for concepts delineated from our work as outlined below.

7.4.1 Further research

- The role of partially preventive vaccines to be further explored through field testing and clinical trials iterating with modeling
- Proof of concept demonstrating the benefits of concomitant use of antimalarials prior to the administration of PfSPZ Vaccine
- Improvement on Sanaria's first generation, radiation attenuated PfSPZ Vaccine by developing a genetically attenuated, late liver stage-arresting replication competent (LARC). As compared to PfSPZ Vaccine, the PfSPZ-LARC vaccines are expected to provide better protection against malaria. The novel platform trial comparing different PfSPZ candidate products or any combination of interventions could be useful.

7.4.2 Applications

- Development of standard toxicity grading systems based on data originating from the African population
- Expand the use of toxicity grading systems among trials conducted in Africa. This needs participation of local regulatory authorities or even the WHO Africa region
- Safety process we have described is potentially applicable in clinical trials assessing newer interventions such as genetically modified PfSPZ Vaccine and monoclonal antibodies
- Maintaining the South-South, North-South and North-North collaborations that worked together to accomplish this task
- With the projected increase in cost on malaria control, it is important to ensure that local involvement and strengthening of the local healthcare systems is encouraged in clinical development plans for better priority setting and resource allocation. Such decentralization approach will strengthen the capacity of African sites to take a driving seat on the agenda portfolio.
- Sustainability in maintaining research teams with individuals who have gained substantial experience in clinical trials, this must be led by the local senior investigators in collaboration with local and international partner institutions. It is important that, African clinical research centres operate through the portfolio of clinical research focusing on interventional products for disease of public health importance for different trial phases and age groups representing internal (population) and external (sponsor) value

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CHAPTER 8: PhD supplementary work and Curriculum Vitae

8.1 Appendices

During the course of this PhD work, several contributions were made to the research work published. Two articles on malaria diagnostics studies were published, in Malaria Journal (comparison of ultrasensitive and conventional rapid diagnostic test) and Nature-Scientific Reports (multiplex qPCR approach for detection of pfhrp2 and pfhrp3 gene deletions in multiple strain infections of Plasmodium falciparum). Five articles were published on characterization of immune responses among African participants immunized with the PfSPZ Vaccine (Malaria Journal (1), Nature (2), Elife (1), Journal of Immunology (1)). One article was published in Virology Journal aiming to evaluate the role of viral Co-infection among immunized participants and one article was published in Malaria Journal on epidemiological characterization of malaria. These articles were linked to the primary work in my thesis, my contribution included data sharing and samples sharing as Principal Investigator and reviewing of manuscripts.

Malaria Journal

RESEARCH Open Access

Diagnostic performance and comparison of ultrasensitive and conventional rapid diagnostic test, thick blood smear and quantitative PCR for detection of low-density *Plasmodium falciparum* infections during a controlled human malaria infection study in Equatorial Guinea

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Abstract

Background: Progress towards malaria elimination has stagnated, partly because infections persisting at low parasite densities comprise a large reservoir contributing to ongoing malaria transmission and are difficult to detect. This study compared the performance of an ultrasensitive rapid diagnostic test (uRDT) designed to detect low density infections to a conventional RDT (cRDT), expert microscopy using Giemsa-stained thick blood smears (TBS), and quantitative polymerase chain reaction (qPCR) during a controlled human malaria infection (CHMI) study conducted in malaria exposed adults (NCT03590340).

Methods: Blood samples were collected from healthy Equatoguineans aged 18–35 years beginning on day 8 after CHMI with 3.2×10^3 cryopreserved, infectious *Plasmodium falciparum* sporozoites (PfSPZ Challenge, strain NF54) administered by direct venous inoculation. qPCR (18s ribosomal DNA), uRDT (Alere™ Malaria Ag P.f.), cRDT [Carestart

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Malaria Pf/PAN (PfHRP2/pLDH)], and TBS were performed daily until the volunteer became TBS positive and treatment was administered. qPCR was the reference for the presence of *Plasmodium falciparum* parasites.

Results: 279 samples were collected from 24 participants; 123 were positive by qPCR. TBS detected 24/123 (19.5% sensitivity [95% CI 13.1–27.8%]), uRDT 21/123 (17.1% sensitivity [95% CI 11.1–25.1%]), cRDT 10/123 (8.1% sensitivity [95% CI 4.2–14.8%]); all were 100% specific and did not detect any positive samples not detected by qPCR. TBS and uRDT were more sensitive than cRDT (TBS vs. cRDT p = 0.015; uRDT vs. cRDT p = 0.053), detecting parasitaemias as low as 3.7 parasites/μL (p/μL) (TBS and uRDT) compared to 5.6 p/μL (cRDT) based on TBS density measurements. TBS, uRDT and cRDT did not detect any of the 70/123 samples positive by qPCR below 5.86 p/μL, the qPCR density corresponding to 3.7 p/μL by TBS. The median prepatent periods in days (ranges) were 14.5 (10–20), 18.0 (15–28), 18.0 (15–20) and 18.0 (16–24) for qPCR, TBS, uRDT and cRDT, respectively; qPCR detected parasitaemia significantly earlier (3.5 days) than the other tests.

Conclusions: TBS and uRDT had similar sensitivities, both were more sensitive than cRDT, and neither matched qPCR for detecting low density parasitaemia. uRDT could be considered an alternative to TBS in selected applications, such as CHMI or field diagnosis, where qualitative, dichotomous results for malaria infection might be sufficient.

Keywords: Malaria, Rapid diagnostic test, Controlled human malaria infection, Thick blood smear, Low parasite density infections, Malaria pre-exposure

Background

Over the past decade, malaria treatment and vector control interventions have significantly decreased malaria burden worldwide. The global incidence rate of malaria decreased between 2010 and 2018 from 71 to 57 cases per 1000 people at risk [1]. However, during 2020, the first full year of the COVID-19 pandemic, the World Health Organization (WHO) World Malaria Report documented an increment of 14 million annual malaria cases and 69,000 additional deaths compared to 2019, much of this attributable to COVID-19-related interruption of malaria control and medical services [2]. A significant challenge faced by malaria control and elimination projects is addressing transmission potential from low parasite density carriers with mild or no symptoms. Low parasite density carriers are estimated to account for 20–50% of human-to-mosquito transmission [3]. It would be beneficial to have a rapid test able to identify these infections.

Current widely deployed diagnostic tools such as conventional rapid diagnostic tests (cRDTs) are affordable and have user-friendly formats and function. In 2018, 259 million cRDTs were distributed mainly in sub-Saharan Africa and utilized to examine suspected malaria cases [1]. cRDTs test for the presence of histidine-rich protein 2 (PfHRP2), an antigen specific to *Plasmodium falciparum*, and many iterations of the test also include a pan-malaria antigen (PAN) common to all 4 major malaria species such as lactate dehydrogenase (LDH). While cRDTs are affordable, provide quick and readable results, and require little training to operate, they cannot detect low-density infections, which can occur frequently in low transmission areas [3–5]. As a result, efforts are underway to develop advanced malaria rapid diagnostic tests that are more sensitive and effective at identifying low-density *P. falciparum* infections [6, 7].

Laboratory-based techniques such as thick blood smear (TBS) and polymerase chain reaction (PCR) are considered to have greater sensitivity than cRDTs [8]. Historically, TBS has been the gold standard for malaria diagnosis. When performed by expert microscopists reading 0.5 μL of blood, detection may range typically between 10 and 50 parasites per microlitre (p/μL), and under carefully controlled CHMI and TBS preparation conditions, expert microscopists can quantify parasite densities at the theoretical lower limit of detection for this blood volume, 2 p/μL. In contrast, cRDTs have reported detection limits of 100–200 p/μL of blood in field studies [9–11]. TBS has the additional advantages of diagnosing infections even in the presence of parasites carrying PfHRP2 deletions, a challenge that RDT manufacturers are currently facing [12, 13], and allowing detection of all species of malaria parasite. In recent years, the further development of the quantitative polymerase chain reaction (qPCR) method has enabled the detection of low parasite densities that frequently go undetected by cRDTs and TBS [14, 15]. Utilizing qPCR, reservoirs of low parasite density cases can be identified and treated, which is an essential component for elimination in low transmission areas [8, 16–18]. The drawback of both TBS and qPCR is the need for specialized laboratory equipment, materials and well-trained staff, which are often in short supply in low income countries, making them unfeasible for use under field conditions on a large scale [19, 20]. TBS can also lead to false negative results when performed by a non-competent microscopist, the parasitaemia is low or there are multiple-species co-infections [21, 22]. An ideal diagnostic tool would

combine the sensitivity of qPCR with the affordability and simplicity of the cRDT.

An ultrasensitive RDT (uRDT; Alere™ Malaria Ag P.f.) has recently been developed and offered as a reliable diagnostic tool that can be used in clinical studies and in field operations [23–25]. Similar to conventional RTDs [cRDTs, such as Carestart Malaria Pf/PAN (PfHRP2/pLDH)] in form and function, the uRDT detects PfHRP2 in *P. falciparum*, but at greater sensitivity, identifying densities as low as 0.1–1.0 p/μL in culture-derived samples [7, 23]. This more sensitive RDT, which has been described as being capable of detecting low parasite density infections, could target these low-density infections for treatment [26]. If the sensitivity reported is confirmed, it could also be used in clinical trials of antimalarial drugs or vaccines to document protection following CHMI or natural *P. falciparum* exposure [27–29]. To date, limited literature exists systematically examining the performance of the uRDT in direct comparison to cRDTs, TBS and qPCR in samples collected from individuals with low parasite density [30, 31]. Existing investigations have been conducted predominantly in the field where it is difficult to monitor factors such as timings of infectious mosquito bites and the waxing and waning of parasite densities [24, 31–35].

A new approach to conducting controlled human malaria infection (CHMI) using *P. falciparum* sporozoites (PfSPZ) has become available over the past five years, based on administering aseptic, purified, cryopreserved, infectious PfSPZ (Sanaria® PfSPZ Challenge) [36]. In this model, PfSPZ are administered by syringe, replacing mosquito bite administration, and the study subjects are then monitored in the standard way [37]. The advantages are that CHMI can be performed by institutions without an insectary or without the need to import infected mosquitoes, the dose of PfSPZ can be standardized, and CHMI can be administered at any time without coordinated mosquito infections. The use of cryopreserved infectious PfSPZ is thus similar to the use of cryostabilates for induced blood stage malaria (IBSM), a controlled human infection model that bypasses the SPZ and liver stages and is similarly free of constraints [38]. CHMI using PfSPZ Challenge is now being utilized extensively to evaluate efficacy of anti-malarial drugs and vaccines in malaria-naive and malaria pre-exposed populations [39–45].

CHMI using injectable PfSPZ provides an opportunity to assess malaria diagnostics under carefully controlled conditions in malaria exposed populations. The exact exposure time is known, the induced infections gradually increase in density, and the time of first detection and associated prepatent period can be precisely determined. The aim of this study was to systematically evaluate and compare uRDT (Alere™ Malaria Ag P.f.) performance against three other commonly used malaria diagnostic tools using whole blood samples collected daily from malaria pre-exposed individuals undergoing CHMI.

Methods

Study site

The Bioko Island Malaria Elimination Program (BIMEP) focuses on developing malaria vaccines and other interventions to decrease malaria-attributable morbidity and mortality on Bioko Island, Equatorial Guinea [46]. BIMEP performs various activities on Bioko such as in-depth epidemiological studies and clinical trials of the malaria vaccine candidates Sanaria® PfSPZ Vaccine and PfSPZ-CVac [44, 47, 48] to collect data on safety and efficacy to support vaccine licensure. In 2018, the BIMEP conducted a regimen optimization trial of PfSPZ Vaccine (ClinicalTrials.gov ID: NCT03590340) at the Baney Research Facility that involved 104 healthy Equatoguinean adults male and female, aged 18–35 years [49]. Study volunteers were recruited from Baney district and the city of Malabo, and were enrolled after providing informed consent. Homologous (the strain of *P. falciparum* in PfSPZ Challenge, PfNF54, was the same as in the vaccine) CHMI with 3.2×10^3 PfSPZ was administered to 95 eligible individuals, 6 to 7 weeks after last vaccination and the observation period was conducted in a hotel at the La Paz Hospital beginning eight days after PfSPZ Challenge injection.

Malaria rapid diagnostic tests

Malaria rapid diagnostic tests were performed with frozen venous whole blood samples anticoagulated with EDTA and stored at − 80 °C. Blood samples were allowed to thaw slowly and equilibrate to ambient temperature for at least 30 min before performing the RDT. The commercially available cRDT [Carestart malaria Pf/PAN (PfHRP2/pLDH) Ag Combo, ACCESSBIO, USA (Lot# MR18F63, expiration: 30th Nov, 2020)] and uRDT [the Alere™ Malaria Ag Pf, Standard Diagnostic Inc., Republic of Korea (Lot# 05LDE001A, expiration: 20th Feb, 2020)] were used throughout the study. Both tests have similar manufacturer instruction workflows, requiring 5 μL blood to be applied to the RDT, followed by addition of the assay buffer and incubation for 20 min. After incubation, diagnosis was determined by the appearance of lines in the test result window. A single control line was considered malaria negative, a line for both control and test were considered malaria positive, and no lines, neither control nor test, was considered an invalid test. Two to three readers were involved in determining each RDT result, the third added when there were discrepancies between the first two readers. The samples were analysed

in batches of 10 by a first reader and the developed RDTs were then given to a second reader to confirm the reading of the first reader. Any discrepancy between first and second readers was resolved using a third reader within a period of 20 ± 1 min. Results were recorded in the database as binary figures with zero representing negatives and one representing positives. Five fresh known malaria positive and five fresh known negative samples were used for quality control for the performance of the assay and for training the technicians prior to conducting the study.

Thick blood smear for quantification of *Plasmodium falciparum*

Two millilitres of fresh whole venous blood from study participants were used for the preparation of TBS. The TBS was prepared by evenly spreading 10 µL of fresh whole venous blood into a 1 cm × 2 cm rectangle. The smears were air dried, stained for 45 min using 4% Giemsa stain and rinsed with buffered water, pH 7.2. The slides were dried and read using a light microscope with a high-power field (immersion oil, 100× objective) of 0.18 mm diameter. 6 × 1 cm passes equivalent to 0.54 µL of blood or 24 × 1 cm passes equivalent to 2.14 µL of blood for symptomatic volunteers were read before a TBS was declared positive or negative. The slides were read by two independent expert microscopists and any discrepancies were resolved by a third microscopist. For Giemsa staining quality control, known positive and negative thin blood smears were included at the beginning of the day and analysed for both parasites and cell staining colour and quality according to a standard operating procedure. Only Giemsa stain that passed the quality control procedures was allowed to be used for the slide staining on that day. Microscopes were maintained on a daily basis.

Quantification of *P. falciparum* parasite density by polymerase chain reaction (qPCR)

DNA was extracted directly from 180 µL of freshly collected venous whole blood using Quick-DNA Miniprep kits (Zymo Research, Irvine, USA) and eluted with 50 µL of elution buffer as recommended by manufacturer. DNA samples were kept at − 20 °C until analysis using the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA). The PlasQ assay previously described by Schindler et al. [50] was used for quantification of *Plasmodium spp.* and *P. falciparum* parasites in the venous blood sample. This multiplex assay targets two independent *Plasmodium* genes namely the Pan-*Plasmodium* 18 S rDNA sequence (Pspp18S) and the *P. falciparum*-specific acidic terminal sequence of the *var* genes (PfvarATS). The human Ribonuclease P gene (HsRNaseP) was used as a DNA extraction and qPCR amplification control. All qPCR assays were run in duplicate and both non-template control (molecular grade nuclease-free water) and *P. falciparum* 3D7 DNA were included in each PCR run as negative and positive controls, respectively. For the parasite density estimation, a serial dilution was made according to the 1st WHO International Standard for *P. falciparum* DNA Amplification Technique (NIBSC code: 04/176) to establish a calibration curve with the parasite densities ranging between 0.01 and 10,000 p/µL. The actual parasite density of the tested sample by qPCR was then estimated from the calibration curve's y-intercept and slope. The lower limit of detection for this qPCR assay was 50 copies/mL. The sample was considered *P. falciparum* positive if each of the two replicates for both PfvarATS and 18 S RNA gene targets had quantitation cycles (Cq) < 40 and Cq < 28 for qPCR amplification control (HsRNaseP). In case of a discrepancy between duplicates, the assay was repeated, with at least two positive replicates out of four considered a positive result. The final results were used for the qPCR-based estimate of parasite density.

Controlled human malaria infection (CHMI)

From October 2018 to March 2019, 95 healthy Equatoguinean adults underwent CHMI [49]. Prior to CHMI, a full 3-day course of artemether/lumefantrine treatment was given to all volunteers. Eligibility criteria for CHMI were met if volunteers had received a complete regimen of PfSPZ Vaccine and were negative for malaria infection at the time of CHMI. During the ward observation period, volunteers were monitored daily for *P. falciparum* parasitaemia starting on day 8 to detect the parasite early and prevent the development of symptoms. Two millilitres of venous whole blood were collected in EDTA tubes daily on days 8–20 post-infection and transported to the laboratory in cooling boxes (4–8 °C) within 30 min of collection. One mL blood was used for examining malaria parasites positivity and density by TBS and qPCR within 4 h of collection and 1 mL was stored at − 80 °C for 8 months before retrospectively analysing samples using uRDT and cRDT. The standard artemether/lumefantrine treatment was given to subjects once malaria parasites were detected by TBS or on day 28 post CHMI for volunteers who remained negative throughout the post-CHMI follow-up period. Positive TBS results confirmed by qPCR were used as the end-point for initiating participant malaria treatment and termination of ward visits and further diagnostic sample collection. Volunteers diagnosed as malaria positive by TBS during 28 days of CHMI follow-up were considered eligible for participation in this malaria diagnostics study.

Sampling and statistical analyses

The aim of this study was to demonstrate differences in performance sensitivity between TBS, cRDT, and uRDT methods in detecting low density malaria infection, using qPCR as the gold standard for sensitivity. To obtain sufficient samples, Epi Info 7 software was used to calculate the sample size assuming the following parameters: minimum sensitivity of 90% for a reference method (qPCR) and 80% for cRDT, a 95% confidence and 90% power to detect a maximum sensitivity difference of 10%. cRDT was used to determine sample size due to its wider application in the field [51]. This gave a minimum required sample size of 267 samples. Sensitivity of the various tests was compared using Fisher's exact test.

Participating individuals were to be observed from day 8 after challenge until the day of first positivity by TBS. Tests were not performed on samples collected after TBS diagnosis since volunteers were treated and positive results could still occur for RDTs and qPCR due to residual parasite material, which would confound results. Out of the eligible volunteers, a subset of 24 individuals were to be randomly selected to meet sample size requirements while maintaining the distribution of parasite densities observed using qPCR. Considering the low sensitivity of most cRDTs at 100 p/μL, a stratified random sampling method was selected, whereby samples with parasite density > 100 p/μL and < 100 p/μL as detected by qPCR were put into two different strata. Using Microsoft Excel (2016), simple random sampling was performed within each stratum to obtain a total of 24 individuals.

To assess the distributions of the complete set, sampled and unsampled subsets were examined to ensure that they presented similar structure. Sampled individuals provided a total of 279 observed individual sample time points.

For this analysis, a multiplex qPCR targeting Pspp18S and PfvarATS was designated as the reference for detection of infection against which TBS, uRDT and cRDT positivity could be compared. A two-tailed Fishers exact test was used to determine the significant differences between the sensitivities of the various diagnostic tests. In this study, all samples were included in the sensitivity analysis of diagnostics. Only positive samples by either TBS, uRDT and cRDT were included in the analyses of the overall geometric mean (geomean) of parasite density of positive results and the geomean of parasite density at time of first detection (prepatent period). If a TBS, uRDT or cRDT test was negative, the respective sample point was deemed not applicable for the geomean parasite density and time to first detection analysis. TBS and qPCR both provided density measurements but TBS was considered to be more reliable as it did not involve conversion from gene copy number using a reference standard.

Results were recorded by trained and qualified laboratory staff on case report forms (CRFs) during the CHMI ward observation period and later entered onto an Excel spreadsheet (Microsoft, Office 2019 Ver 16). All samples were assigned a sample specific number that was linked to each volunteer ID. No personal information was recorded on laboratory CRFs and for the laboratory staff the connection of each sample with the corresponding donor volunteer was not possible. Retrospective RDT results were recorded on the same Excel spreadsheet. The geomean and geomean confidence intervals of parasite densities were calculated using R 4.0.1. Sensitivity and 95% confidence intervals for all diagnostic methods were calculated in R 4.0.1 using the epiR package [52].

Results

Overview

A total of 48 volunteers were diagnosed positive for malaria by reference method (qPCR), qualifying them for inclusion in the malaria diagnostic comparison. Individuals had an average of 12 time points of observation-days (range 8 to 17), with each day-test-record representing an independent observation since each was obtained from a newly collected whole blood sample. Out of the eligible volunteers, 24 (50%) individuals were randomly selected while maintaining the distribution of parasite densities observed using qPCR. The subset of selected samples was evaluated against unselected samples by parasite density distribution and variance and the selected subset was determined to be an appropriate representation (Additional file 1: Figs. S1–S3).

A total of 279 samples were collected from the 24 selected study participants; 123 and 156 samples were positive and negative for *P. falciparum* infection by qPCR, respectively. All 156 samples negative for *P. falciparum* by qPCR were also negative by TBS, uRDT and cRDT demonstrating 100% specificity for these tests. In total, 24 of 123 positive samples were detected by TBS, 21 by uRDT and 10 by cRDT, providing sensitivities of 19.5% (95% CI 13.1–27.8%), 17.1% (95% CI 11.1–25.1%), and 8.1% (95% CI 4.2–14.8%), respectively. qPCR detected more positives than any of the other tests ($p < 0.001$) and TBS and uRDT were both more sensitive than cRDT (TBS vs. cRDT, $p = 0.015$ by Fishers Exact two-tailed; uRDT vs. cRDT, $p = 0.053$). TBS detected 61.9% (13/21) of uRDT positive infections, while uRDT detected 54.2% (13/24) of TBS positive infections. The uRDT detected 100% (10/10) of cRDT positive infections while TBS detected 90% (9/10). The cRDT detected 47.6% (10/21) of uRDT positive infections and 37.5% (9/24) of TBS positive infections. The summary of the findings are depicted in a Venn-diagram (Fig. 1).

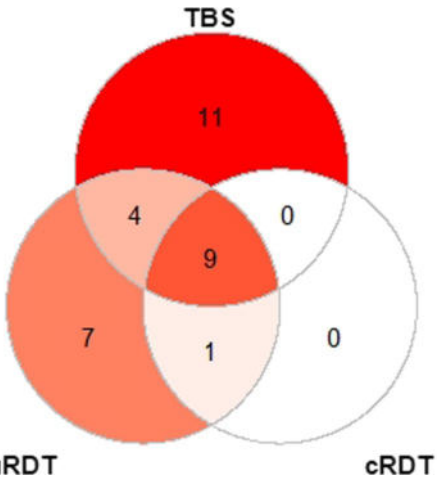

Fig. 1 Similarities and discrepancies in detection of *P. falciparum* cases by different diagnostic methods. Venn-diagram showing distribution of positive results according to the diagnostic test used with qPCR as the reference method. All samples were sorted by thick blood smear (TBS), ultrasensitive rapid diagnostic test (uRDT), and conventional rapid diagnostic test (cRDT). All cases were low parasite density *P. falciparum* infections that occurred during CHMI

Parasite density by qPCR was calculated using a standard curve generated using a WHO reference sample that related copy number to density, and ranged from 0.14 to 603.8 p/μL, with a geomean of 2.57 p/μL. Using this scale TBS detected parasites in the range of 5.9–603.8 p/μL (geomean 97.6 p/μL) and uRDT detected parasites in the range of 0.8–603.8 p/μL (geomean 103.4 p/μL), compared to cRDT, which detected parasites in the range of 2.6–603.8 p/μL (geomean = 149.4 p/μL) (Table 1).

Parasite density by TBS ranged from 3.7 to 201.8 p/μL (geomean 12.81 p/μL). Using this scale, uRDT detected parasites in the range of 3.7–201.8 p/μL (geomean 20.6 p/μL), compared to cRDT, which detected parasites in the range of 5.6–201.8 p/μL (geomean = 30.2 p/μL) (Table 2).

Examining just infections positive by both qPCR and TBS, the geomean ratio established from parasite density of qPCR and TBS (qPCR/TBS) was 7.62 p/μL (Table 3). PCR detects gene copy number in a specimen and each *P. falciparum* genome has at least 5–8 copies [53], and in addition qPCR can detect free DNA in a specimens. These variables make it difficult to calculate parasite density accurately using qPCR, even when using a standard curve to convert copy numbers to density based on a WHO reference sample. TBS detects parasites, regardless of how many genes and nuclei are present, but is hindered by the possible loss of significant numbers of parasites during processing [54]. All these factors were suspected to have contributed to the higher densities found using qPCR compared to TBS.

Table 1 Overall geomean and sensitivity of TBS, uRDT, and cRDT compared to the qPCR method

Diagnostic test	TBS (+)	TBS (−)	uRDT (+)	uRDT (−)	cRDT (+)	cRDT (−)	Total
PCR Pf (+)	24	99	21	102	10	113	123
PCR Pf (−)	0	156	0	156	0	156	156
Total	24	255	21	258	10	269	279
Range of positive samples (p/μL by qPCR)	5.9–603.8		0.8–603.8		2.6–603.8		
Geomean of positive samples (p/μL by qPCR)	97.6		103.4		149.4		
Sensitivity (%)	19.5% (13.1–27.8)		17.1% (11.1–25.1)		8.1% (4.2–14.8)		

Number of positive and negative samples, overall geomean and sensitivity of TBS, uRDT, and cRDT compared to the reference qPCR method. All cases were low parasite density infections that occurred during CHMI. Parasite densities of positive samples by qPCR ranged from 0.14–603.84 p/μL

Table 2 Overall geomean and sensitivity of uRDT and cRDT compared to the TBS method

Diagnostic test	uRDT (+)	uRDT (−)	cRDT (+)	cRDT (−)	Total
TBS (+)	13	11	9	15	24
TBS (−)	8	247	1	254	255
Total	21	258	10	269	279
Geomean of positive samples (p/μL by TBS)	20.6		30.2		
Sensitivity (%)	54.2% (33.2–73.8)		37.5% (19.6–59.2)		

Overall geomean, sensitivity and number of positive and negative samples of uRDT, and cRDT compared to TBS. All cases occurred during CHMI. Parasite densities of positive samples by TBS ranged from 3.7 to 201.8 p/μL

Table 3 Overall geomean, ranges and the ratio of parasite density established from qPCR and TBS

	qPCR (Pf/μL)	TBS (Pf/μL)	Ratio qPCR/TBS
GeoMean	97.57	12.81	7.62
Range	[5.86–603.84]	[3.70–201.80]	[0.07–53.84]

Comparison of geomean, ranges and the ratio of parasite density established from qPCR and TBS using paired samples in which both tests were positive. All cases were low parasite density infections that occurred during CHMI. The Ratio was determined by dividing the geomean of qPCR by geomean of TBS (qPCR/TBS). N = 24

Next, the diagnostic test sensitivities of TBS, uRDT and cRDT were stratified by ranges of parasite density of qPCR (Table 4). None of the three diagnostic tests detected *P. falciparum* infections below 1 p/μL as determined by qPCR. For TBS, the lowest parasite density detected as quantified by qPCR was 5.86 p/μL and as quantified by TBS was 3.7 p/μL. At parasite densities of 1–50 p/μL, uRDT and TBS appeared roughly equally sensitive and both appeared more sensitive than cRDT. At parasite densities of 51–100 p/μL by qPCR, uRDT appeared more sensitive (71% [95% CI 29–96%]) compared to TBS and cRDT with, 29% (4–71%) and 29% (4–71%) sensitivity, respectively. However, numbers were small and differences in sensitivity amongst the tests across the density categories were not statistically significant.

Finally, the uRDT and cRDT diagnostic test sensitivities were stratified by ranges of parasite density measured by TBS (Table 5). At parasite densities range between 1 and 50 p/μL, uRDT had higher sensitivity compared to cRDT; 33% (95% CI 12–62) for 1–10 p/μL and 100% (95% CI 48–100) at 11–50 p/μL compared to 13% (95% CI 2–40) for 1–10 p/μL and 80% (95% CI 28–99) at 11–50 p/μL respectively. Above 50 p/μL, both uRDT and cRDT had the same sensitivity but again numbers were too small to allow a meaningful comparison.

Finally, the range and distribution of parasite densities of samples determined to be positive by qPCR (n = 123), by TBS (n = 24), by uRDT (n = 21) and by cRDT (n = 10) were examined over the follow up period for the 24 volunteers who were TBS positive. TBS and uRDT recorded a trend for lower geomean parasite densities detected compared to cRDT, which did not reach statistical significance [p = 0.19 and p = 0.26, respectively] (Fig. 2).

Time to first detection

To investigate the efficiency of cRDT and uRDT to detect asexual blood stage parasites during CHMI follow-up, the median times to first detection of parasites (in days) by qPCR, TBS, uRDT and cRDT were compared. There

Table 4 Comparison of TBS, uRDT and cRDT sensitivity stratified by parasite density (p/μL) as determined by qPCR

Group density (p/μL)	# samples qPCR (+) (reference)	TBS (+)	TBS sensitivity (95% CI)	uRDT	uRDT sensitivity (95% CI)	cRDT	cRDT sensitivity (95% CI)
< 1	37	0	–	0	–	0	–
1–10	38	1	3% (0–14)	1	3% (0–14)	1	3% (0–14)
11–50	25	7	28% (12–49)	4	16% (5–36)	0	–
51–100	7	2	29% (4–71)	5	71% (29–96)	2	29% (4–71)
> 100	16	14	88% (62–98)	11	69% (41–89)	7	44% (20–70)

Number of samples and sensitivity of TBS, uRDT and cRDT stratified by parasite density (p/μL). All cases were low parasite density samples that occurred during CHMI follow-up and were 100% specific compared to qPCR

Table 5 Comparison of uRDT and cRDT sensitivity stratified by parasite density (p/μL) as determined by TBS

Group density (p/μL)	# samples TBS (+) (reference)	uRDT (+)	uRDT sensitivity (95% CI)	cRDT (+)	cRDT sensitivity (95% CI)
< 1	0	0	–	0	–
1–10	15	5	33% (12–62)	2	13% (2–40)
11–50	5	5	100% (48–100)	4	80% (28–99)
51–100	2	2	100% (16–100)	2	100% (16–100)
> 100	2	1	50% (1–99)	1	50% (1–99)

Number of samples and sensitivity of uRDT and cRDT stratified by parasite density (p/μL). Diagnostic methods are compared to TBS as reference and all cases were low parasite density samples that occurred during CHMI follow-up

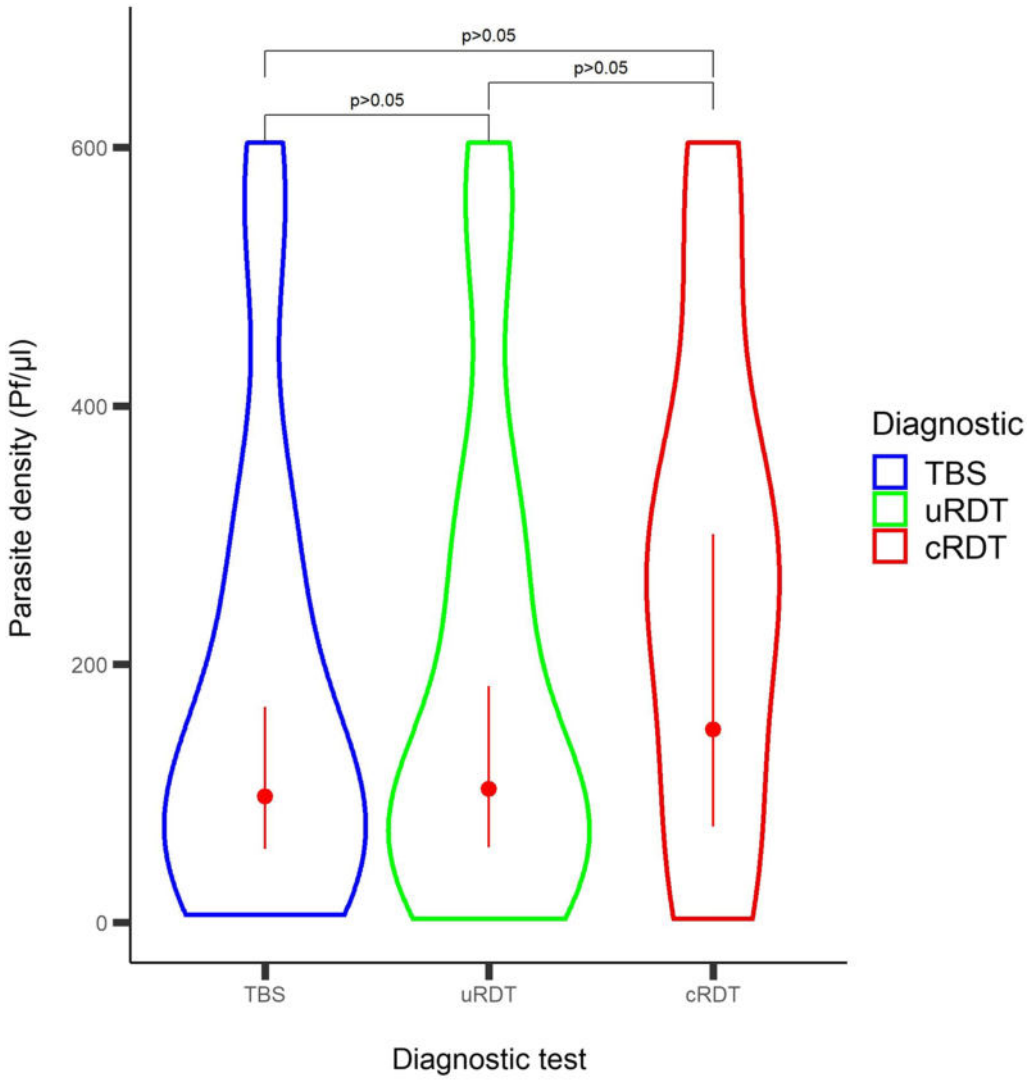

Fig. 2 Distribution of parasite density by qPCR of all malaria positive samples by TBS, uRDT, and cRDT. Red dots represent the geomean and error bars represent the 95% confidence intervals of each respective diagnostic test. Significance values were calculated using two-tailed Wilcoxon-test

was no evidence to support differences in prepatent period when using TBS, uRDT and cRDT since these methods all reported a median of 18.0 days to first parasite detection. The median days to detection of asexual blood stage parasitaemia by qPCR was 14.5, 3.5 days earlier than TBS, uRDT and cRDT ($p < 0.001$ log-rank test) (Fig. 3).

Discussion

As progress against malaria is made, asymptomatic infections at lower parasite densities become a significant challenge for malaria control and elimination efforts due to their contribution to ongoing transmission [3, 4, 55, 56]. Mass testing of a population with treatment of those found positive is one approach to address this problem [57]. However, it is difficult to diagnose low density parasitaemias and the most sensitive and, therefore, the best method, qPCR, is expensive, requires special laboratories and skilled personnel. Thus, the development of an inexpensive rapid test with equivalent sensitivity would be of great benefit, especially as conventional rapid diagnostic tests (cRDTs) are significantly less sensitive. Other applications could also benefit from a simple, rapid test that is more sensitive than cRDTs, such as detection of parasitaemia following sporozoite or blood stage CHMI, both important procedures for evaluating vaccine and drug efficacy [44, 45, 58–61] or for exploring innate and acquired immunity [62–66]. PfSPZ CHMI in particular is

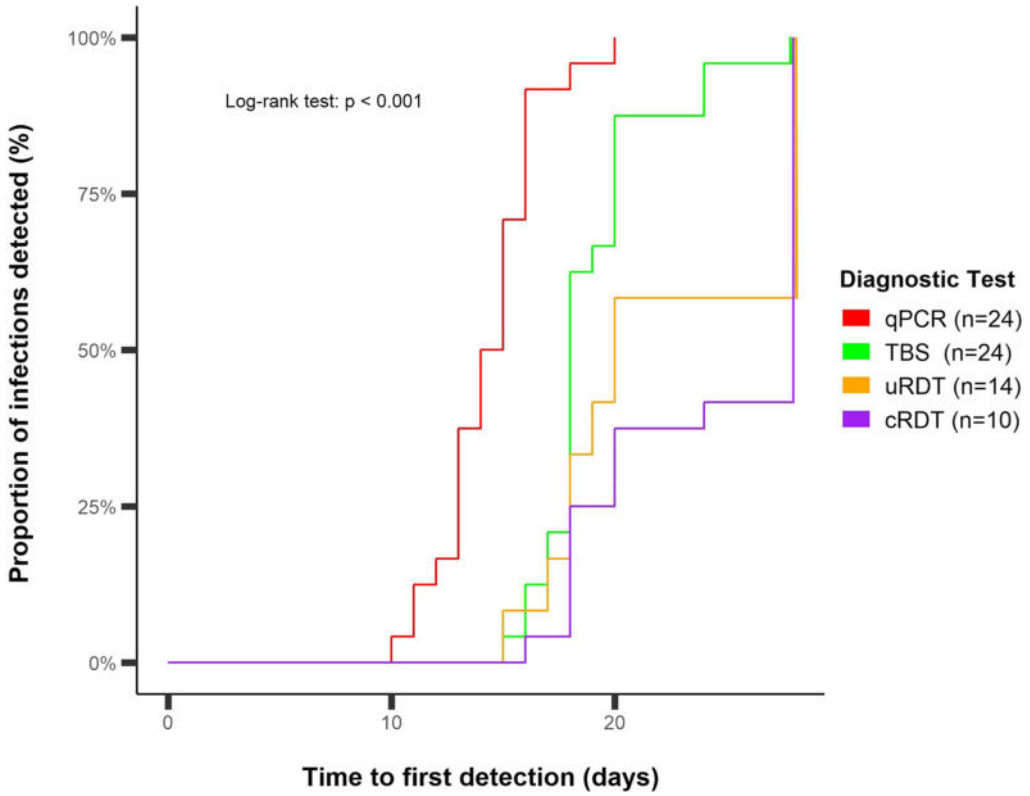

Fig. 3 Comparison of time to detection of parasites. Kaplan–Meier plot of the number of infections detected by time since CHMI qPCR (N = 24), TBS (N = 24), uRDT (N = 14) and cRDT (N = 10). All cases were low parasite density Pf infections that occurred during CHMI. P-value < 0.001 calculated using the log-rank test

now gaining attention by investigators, since it can now be used by any clinical centre without the need for infectious mosquitoes [67–69]. In CHMI, detection of low parasite densities is useful because it allows the identification and treatment of positive study subjects earlier in the course of their parasitaemia thereby preventing or ameliorating clinical manifestations. Therefore, on many fronts, there is a need to develop simpler, highly sensitive methods to diagnose low parasite densities that could augment the success of mass testing and treatment, promote epidemiological studies and simplify and lessen the costs associated with CHMI.

cRDTs have been a tremendous boon to diagnosing clinical malaria, where parasite densities are relatively high and the tests adequately sensitive. An uRDT has recently been developed, and might extend the usefulness of RDTs particularly in low to moderate transmission areas, in pre-elimination settings, and in experimental uses such as CHMI follow-up. For example, it has been reported that the uRDT is significantly more sensitive than cRDTs and TBS, detecting PfHRP2 at parasite densities as low as 0.1–1.0 p/μL in culture-derived samples [7, 23]. For this reason, the current study tested the uRDT and a cRDT during follow-up in a CHMI trial, and compared their sensitivities to those of TBS, using qPCR as the reference standard.

This study indeed found that both TBS and the uRDT were more sensitive than the cRDT. However, the data demonstrated that in samples from malaria-experienced subjects undergoing CHMI with parasites that contained PfHRP2, the sensitivity of uRDT (17.1%) was about threefold lower than that reported for pretreatment specimens from an IBSM study (47%) and in samples from a low transmission setting (44%), and fivefold lower than that reported in samples from a high transmission setting (84%) [23]. None of 37 specimens less than 1.0 p/μL by qPCR were identified. In 15 specimens that had 1–10 p/μL by TBS, uRDT identified 5 (33%), and cRDT 2 (13%). In 5 specimens with 11–50 parasites/μL by TBS, uRDT identified all 5 as positive (100%) and cRDT identified 4 (80%). Overall, uRDT and TBS gave similar results, and both tests were more sensitive than cRDTs in a setting of CHMI with PfHRP2-containing parasites.

Currently, CHMI requires highly trained clinical and laboratory staff including expert microscopists. Considering the comparable outcomes of TBS and uRDT

in this study, uRDT could be considered to replace TBS microscopy, especially in settings with inexperienced microscopists. However, parasite density estimation using qPCR has become a standard method utilized in many malaria studies [70, 71] and has been particularly useful in CHMI follow-up where it can detect parasitaemia earlier than TBS and allow treatment before signs and symptoms of clinical malaria develop [72–74]. Thus, uRDT would need to show advantages over TBS in the early identification of positive study subjects, as TBS itself is now being supplanted by qPCR. As expected, the study showed a significant difference in prepatent period amongst qPCR on the one hand and TBS, uRDT and cRDT on the other, confirming that qPCR is the most sensitive diagnostic method. The study further demonstrated that the median time to first malaria parasite detection by qPCR was 3.5 days earlier (14.5 days) compared to the other tests, and also that TBS, uRDT and cRDT were substantially equivalent to each other, each providing a prepatent period of 18 days. Similar to these findings, the CHMI studies conducted in semi-immune participants [44, 58, 73, 75] and in malaria naïve participants [76] have reported comparable prepatent periods using qPCR as the reference method. The fact that in this setting of progressively rising parasitaemias, the prepatent periods calculated by TBS, uRDT and cRDT were similar even though positive samples diagnosed by uRDT had a lower overall geomean of parasite density by qPCR than did TBS, suggests that uRDT may not have any particular advantage over TBS other than reduced costs and easier performance, or even over cRDT, as in this study the day of treatment would not have been affected had it been cRDT- rather than TBS-based. It would be expected that the same relative detection abilities would hold for blood stage CHMI, although this was not evaluated in this study, and Das et al. reported that uRDT detected parasitaemia 1.5 days earlier than cRDT in this setting [23].

In a field setting, the greater sensitivity of the uRDT over cRDTs could allow the detection of more asymptomatic carriers. This question was not directly examined in this CHMI-based study. However, the results showed that despite the uRDT being hailed as a significant improvement in malaria diagnostics, leading to increased sensitivity and specificity, satisfactory RDT performance for parasite density infections < 10 p/μL remains elusive. Field studies of mass testing and treatment are needed to further explore the potential contribution of the uRDT in identifying and treating asymptomatic carriers with low parasite densities contributing to ongoing transmission.

One important consideration for evaluating RDTs based on the detection of PfHRP2 is the increase in prevalence of parasites carrying PfHRP2 deletions, not only in Southeast Asia [77], but within the study area as well [12]. In 2018, approximately 65% of all suspected malaria cases in public health facilities in sub-Saharan African were tested with RDTs (~ 150 million cases) [1]. During CHMI, a standardized infectious PfSPZ dose of PfSPZ Challenge (NF54) was used, a parasite that expresses PfHRP2 to initiate the infection. Conducting a similar study in hospitals and field environment with important confounders, such as a deleted *Pfhrp2* gene, would likely have had different results.

Limitations

Anticoagulated (EDTA) fresh whole blood was used for to prepare samples for qPCR and TBS assessments. Anticoagulated (EDTA) cryopreserved (temperature of − 80 °C) whole blood held for 8 months and thawed was used to prepare samples for uRDT and cRDT. It is possible, but unlikely that HRP2 degraded during storage. Whole blood samples were temperature monitored during storage. When proper procedures are followed for long-term storage of whole blood, the quality of DNA, RNA or HRP2 is not compromised [7, 78]. Considering the strict temperature monitoring in this study and the fact that samples were only thawed once for processing, the difference in quality of samples over time is unlikely to have been different.

Another limitation is the discrepancy in parasite densities estimated by qPCR and TBS. qPCR may have overestimated parasite density due to variable numbers of copies of the amplification target and the persistence of nucleic acid from non-viable parasites [79], and TBS may have underestimated parasite density due to the loss of parasites during processing.

Because the research subjects were semi-immune, they may have had variable levels of anti-HRP2 or anti-LDH antibodies, which could have affected results [80, 81]. Some may also have had ongoing infections at the time of clearance with artemether/lumefantrine prior to CHMI. Although the current study did not measure the level of HRP2 in the participants before and after CHMI, none of the individuals were RDT positive between days 8 and 14 after CHMI. Therefore, it is likely that this factor did not affect the current performance comparison.

This study was designed to evaluate the performance of malaria diagnostic tests in independent samples and was analysed accordingly, even though several samples were collected from each individual post CHMI. This was based on the reasoning that, since each test was measuring a different parameter (DNA for qPCR, whole parasites for TBS, HRP2 for uRDT, HRP2/LDH for cRDT) and these parameters would vary independently from

day to day due to the presence of multiple clones of NF54 parasites released from individual hepatocytes over several days each with its own asynchronous 48 h reproductive/sequestration cycle, it would be difficult to propose a biological metric characterizing an individual that could introduce bias or similarities in observations. Nevertheless, such a bias or similarities could exist and might have affected the data.

Conclusions

TBS has been the classical approach to malaria diagnosis for clinical use, malaria control programs, research studies such as CHMI and field epidemiology. TBS can distinguish the five malaria species that infect humans, which cannot yet be achieved by using RDTs or a single reaction qPCR, and provides a reasonable estimate of parasite density. TBS, however, requires laboratories that support and maintain microscopes, staining solutions and human resources with the requisite microscopy skills. qPCR, with much greater sensitivity, is now supplanting TBS for many applications such as detection of parasitaemia following CHMI, but also requires a high level of laboratory capability and involves higher costs than TBS. cRDTs have, therefore, been a welcome addition to malaria diagnostics and in many places have supplanted TBS for the clinical diagnosis of malaria, where parasite densities are high, but have not been useful for applications requiring greater sensitivity. This study compared qPCR, TBS, a cRDT to a new uRDT advertised as rivaling qPCR in sensitivity, to assess its value for detection of parasitaemia following PfSPZ CHMI, an application where early diagnosis and treatment is important to reduce the severity of adverse events. The major conclusions were that for this specific application, while the uRDT was better than the cRDT, and approached TBS in sensitivity, it did not close the gap with respect to qPCR, and thus could be considered for replacing TBS only in studies unable to use qPCR or TBS due to resource limitations. The added value of the uRDT in field studies, particularly in mass testing and treatment, requires further study.

Abbreviations

cRDT: Conventional rapid diagnostic test; PfHRP2: Histidine-rich protein 2; PAN: Pan-malaria antigen; TBS: Thick blood smear; p/µL: Parasites per microlitre; qPCR: Quantitative polymerase chain reaction; uRDT: Ultra-sensitive rapid diagnostic test; CHMI: Controlled human malaria infection; DVI: Direct venous inoculation; BIMEP: Bioko Island malaria Elimination Project; CRFs: Clinical report forms.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-022-04103-y.

Additional file 1: Figure S1. Trend ofparasite density over time between individuals in selected and unselected groups.Trend of parasite density over time between selected and unselectedparticipants. Parasite density was determined by quantitative polymerase chainreaction assays (qPCR). **Figure S2.** Overall distribution of parasite densityamong groups of selected and unselected participants. Scatter plots of parasitedensity measured by quantitative polymerase reaction assays (qPCR) betweenselected and unselected data points. **Figure S3.** Distribution of parasitedensity by individuals participants in selected and unselected groups. Barplotsof parasite density as measured by quantitative polymerase reaction assays(qPCR) between selected vs. unselected individual volunteers.

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Authors' contributions

SLH posed the research question and the applicability of the CHMI follow-up as a means to evaluate a potential uRDT application. MM and CAD conceived and designed the evaluation plan for this study. MM, JR, AD, LA, EN, MdCOD, TS, VU, AM, AH, MSAL, BP, and MAOE were responsible for collecting and processing volunteer samples. MRR, CCF, GAG, RC, ES, PFB, LWPC, BKLS, TLR, BM, MT, SA, CM, SLH, SJ sponsored and oversaw the management of the PfSPZ Vaccine trial and CHMI. SJ was the principal investigator. CAD managed all laboratory activities and supervised this study. JCM managed quality control and quality assurance of all documentation. TCS programmed and conducted the statistical analysis of the data and generated all tables and figures. MM and TCS wrote the manuscript in consultation with PFB, SLH and TLR. TLR and CAD contributed equally on this work. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysis during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Ethics approval was obtained to conduct the malaria vaccine trial from the Comité Ética Nacional de Guinea Ecuatorial (CENGE) in Equatorial Guinea, Ifakara Health Institute (IHI) in Tanzania, Ethikkommission Nordwest- und Zentralschweiz (EKNZ) in Switzerland, and Prime IRB in the USA. Included in this approval was the use of participant biological samples for future use if informed consent was provided. No identifying information was accessible to laboratory staff and was not included in any database or analysis for this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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A multiplex qPCR approach for detection of *pfhrp2* and *pfhrp3* gene deletions in multiple strain infections of *Plasmodium falciparum*

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The rapid and accurate diagnosis of *Plasmodium falciparum* malaria infection is an essential factor in malaria control. Currently, malaria diagnosis in the field depends heavily on using rapid diagnostic tests (RDTs) many of which detect circulating parasite-derived histidine-rich protein 2 antigen (PfHRP2) in capillary blood. *P. falciparum* strains lacking PfHRP2, due to *pfhrp2* gene deletions, are an emerging threat to malaria control programs. The novel assay described here, named qHRP2/3-del, is well suited for high-throughput screening of *P. falciparum* isolates to identify these gene deletions. The qHRP2/3-del assay identified *pfhrp2* and *pfhrp3* deletion status correctly in 93.4% of samples with parasitemia levels higher than 5 parasites/µL when compared to nested PCR. The qHRP2/3-del assay can correctly identify *pfhrp2* and *pfhrp3* gene deletions in multiple strain co-infections, particularly prevalent in Sub-Saharan countries. Deployment of this qHRP2/3-del assay will provide rapid insight into the prevalence and potential spread of *P. falciparum* isolates that escape surveillance by RDTs.

Malaria is an infectious disease with an estimated 219 million cases globally and was responsible for 435'000 deaths in 2017. More than 90% of these malaria cases and deaths occurred in sub-Saharan Africa with *Plasmodium falciparum* as the most pathogenic malaria parasite species, accounting for the vast majority of clinical malaria cases[1].

Advances have been made in malaria control which have contributed to the decline in malaria prevalence observed worldwide with improved diagnostic tests and better access to malaria treatment contributing significantly to this development[1]. The rapid and accurate diagnosis and treatment of malaria cases is an essential factor in the control of malaria. Rapid diagnostic tests (RDTs) are becoming the most widely used method to diagnose malaria infections in the field with 245 million RDTs distributed worldwide in 2017[1]. In sub-Saharan Africa an estimated 75% of malaria tests conducted in 2017 were based on RDTs[1]. Malaria RDTs are based on an immuno-chromatographic assay using a lateral-flow device which allows the detection of malaria antigens in usually 5 to 15 µL of capillary blood[2]. RDTs provide results within 20 minutes and can be employed by inexperienced health workers operating in resource-limited settings[3]. RDTs recognizing circulating histidine-rich protein 2 (PfHRP2) for sensitive and specific detection of *P. falciparum* make up more than 90% of RDTs currently in

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Target gene	Size	Oligo name	Oligo sequence [5′ to 3′]	Fluorophores	Conc. in 5 × PrimerMix[a]
pfrnr2e2 (PF3D7_1015800)	107 bp	IC-PfRNR2E2 fwd	AGTATCCAAAACACTATAATTCCAAGTAC	—	1.5 µM
		IC- PfRNR2E2 rev	ATTTTCTCCTTTCTTAACAGTTTCTTCC	—	1.5 µM
		IC-PfRNR2E2 Cy5	CCTTTTAGTTGGCCCGAATTTACAA	Cy5-BHQ2	1.125 µM
pfhrp2 (PF3D7_0831800)	286 bp	PfHRP2 fwd[b]	GTATTATCCGCTGCCGTTTTTGCC	—	1.5 µM
		PfHRP2 rev[b]	TCTACATGTGCTTGAGTTTCG	—	1.5 µM
		PfHRP2 TxRd	TTCCGCATTTAATAATAACTTGTGTAGC	TexasRed-BHQ2	0.375 µM
pfhrp3 (PF3D7_1372200)	289 bp	PfHRP3 fwd	ATATTATCCGCTGCCGTTTTTGCT	—	1.5 µM
		PfHRP3 rev	CCTGCATGTGCTTGACTTTCGT	—	1.5 µM
		PfHRP3 YY	CTCCGAATTTAACAATAACTTGTTTAGC	YakimaYellow-BHQ2	0.75 µM

Table 1. Oligonucleotide sequences used for qHRP2/3-del assay. [a]All oligonucleotides are premixed as a 5× primer mix. [b]Oligonucleotide sequences obtained from Abdallah *et al.*[32].

use[4]. The relatively high abundance and stability of PfHRP2 in the blood of infected patients and expression by *P. falciparum* during the erythrocytic stage makes this antigen a valuable biomarker for malaria infection[5]. PfHRP3, a protein also expressed by *P. falciparum* with high level of structural similarity to PfHRP2, might be also recognized by some of the monoclonal antibodies used in the RDTs[6]. RDTs are critical diagnostic tools for identifying symptomatic malaria infections; however, due to the reduced performance in infections with low parasite density, its use for the diagnosis of malaria infection in asymptomatic individuals is rather limited[7].

Recent studies report on reduced diagnostic performance of PfHRP2-based RDTs which were attributed to genetic diversity of the *pfhrp2/3* genes[6], differences in expression level of PfHRP2/3 antigen in parasite field strains[8] or isolates lacking *pfhrp2* and/or *pfhrp3* genes[9]. *P. falciparum* isolates lacking *pfhrp2* and/or *pfhrp3* genes are found around the world, with different proportions of the circulating *P. falciparum* population affected. The regions with the highest proportions of *P. falciparum* strains carrying *pfhrp2* deletions are South America and sub-Saharan Africa[10]. Since malaria control programmes depend on reliable diagnosis of malaria cases using RDTs, parasites lacking *pfhrp2/3* genes pose a threat to malaria control and local elimination efforts[11].

The presence or absence of *pfhrp2/3* genes is usually determined by amplifying these genes by polymerase chain reaction (PCR). Several different (nested) PCR protocols have been published and a deletion is reported if there is no amplification of the *pfhrp2/3* genes in the presence of an amplification in at least two *P. falciparum* single copy genes[12]. The conventional nested PCR methods are time consuming, requiring separate reactions for each target gene amplification as well as gel electrophoresis for visualization of the PCR products. Additionally, there are methodological issues related to this approach which assumes identical PCR performance of the *pfhrp2/3* and the reference genes. Particularly at lower parasitemia levels with a small number of DNA target molecules present, unavoidable stochastic effects can play a major role and might lead to false reporting of *pfhrp2/3* deletions. Furthermore, none of the published methods detecting *pfhrp2/3* deletions can identify "masked" deletions in multiple strain infections with only one out of several *P. falciparum* strains carrying a *pfhrp2* and/or *pfhrp3* gene deletion[13]. These limitations of recommended molecular monitoring methods could result in an underestimation of the prevalence of *P. falciparum* strains with *pfhrp2/3* deletions, especially in regions with high proportions of multiple strain co-infections.

This paper presents a novel, quantitative PCR-based method for detecting *pfhrp2* and *pfhrp3* gene deletions suitable for high throughput screening of *P. falciparum* isolates. The qHRP2/3-del (quantitative detection of *pfhrp2* and *pfhrp3* deletion) assay was developed as a multiplex assay, with the ability to amplify individually and specifically the *pfhrp2* and *pfhrp3* genes together with a single copy gene, the *P. falciparum* ribonucleotide reductase R2_e2 (*pfrnr2e2*)[14], as an internal reference. The quantitative nature of the qHRP2/3-del assay provides the basis for estimating the proportions of *P. falciparum* strains carrying *pfhrp2* and *pfhrp3* deletions in regions with multi-clonal malaria infections.

Results

Design and evaluation of the novel qHRP2/3-del assay. We aimed at improving the detection of *pfhrp2* and *pfhrp3* gene deletions by developing a quantitative PCR-based assay able to detect and quantify *pfhrp2* and *pfhrp3* genes in a single reaction. Given the high nucleotide sequence similarity and the repetitive structure of the *pfhrp2* and *pfhrp3* genes, nucleotide regions serving as targets for primers and probes were limited (Supplementary File 1). The primer and probe combinations selected for our assay (Table 1) bind to a region spanning exon 1 and exon 2 of both genes. Absence of amplification will therefore indicate a deletion of the entire genes or partial gene deletions including exon 1, the intron and first 96 base pairs of exon 2. Although there are chromosome breaking points outside the amplified regions, in particular the section that contains the repeats and epitopes detected by RDTs, analysis of field isolates suggest that the selected regions are highly predictive for *pfhrp2/3* deletions in field strains[9,12,15,16].

We designed a multiplex qPCR assay using three differently labelled TaqMan assays detecting the *pfhrp2* (PF3D7_0831800) and *pfhrp3* (PF3D7_1372200) genes with the single copy gene *pfrnr2e2* (PF3D7_1015800) as the internal control. The sequence alignment of the *pfhrp2* and *pfhrp3* genes highlighting the oligo binding regions is shown in Supplementary File 1.

The multiplexed assays correctly identify *P. falciparum* strains carrying known deletions of *pfhrp2* (PfDD2 strain) and *pfhrp3* (PfHB3 strain) as well as a strain without deletion (PfNF54 strain) (Fig. 1A). The multiplexed assays show comparable characteristics in terms of sensitivity and qPCR performance. Using DNA extracted from

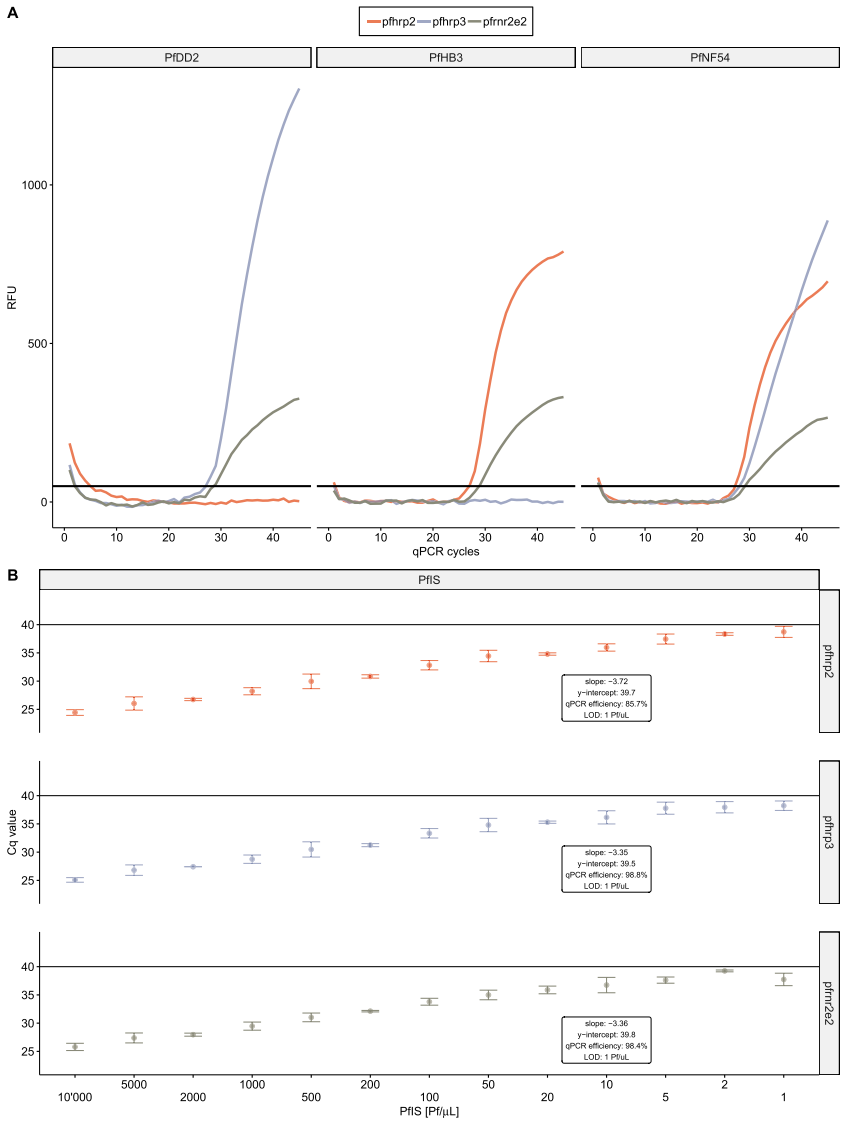

Figure 1. Multiplex detection of *pfhrp2* and *pfhrp3* genes using the qHRP2/3-del assay. (**A**) The qHRP2/3-del assay amplifies *pfhrp2*, *pfhrp3* and *pfrnr2e2* target sequences in a multiplex qPCR reaction and correctly identifies strains carrying either a *pfhrp2* deletion (PfDD2), a *pfhrp3* deletion (PfHB3) or no deletion (PfNF54). (**B**) Performance characteristic of each individual amplification assay, run within the multiplex qHRP2/3-del assay, is shown. Correlation with high linearity between serially diluted WHO international standard for *P. falciparum* NATs (PfIS) and Cq values was obtained and used to calculate the qPCR efficiency. Cq values above 40 (black line) are considered negative.

cultured parasites, all three targets are detected in samples with parasitemia as low as 1 parasite/µL and an inverse linear correlation between Cq values and parasite densities ranging from 1 to 10'000 parasites/µL was observed. The qPCR efficiencies were calculated as 85.7%, 98.8% and 98.4% for the amplification of *pfhrp2*, *pfhrp3* and *pfrnr2e2*, respectively (Fig. 1B). The qHRP2/3-del assay was next tested using purified DNA from eight culture adapted *P. falciparum* strains from Africa (Pf3D7, PfNF54, PfNF166.C8), South and Central America (Pf7G8, PfHB3), South East Asia (PfNF135.C10, PfDD2) and Papua New Guinea (PfFC27) with known deletion status of the *pfhrp2* and *pfhrp3* genes. The Cq values for amplification of *pfrnr2e2* were comparable between the eight strains amplified and no significant differences of Cq values for the *pfhrp2* gene and *pfhrp3* gene across the strains carrying the genes was observed. Sequence alignments of PfNF135.C10, Pf3D7, Pf7G8, PfNF54 and PfNF166.C8 did not reveal sequence variation in the oligo binding regions of *pfhrp2* (Supplementary File 2) or *pfhrp3* (Supplementary File 3) supporting these findings. DNA derived from five non-*falciparum Plasmodium* species (*P. ovale curtisi*, *P. ovale wallikeri*, *P. malariae*, *P. knowlesi*, *P. vivax*) was tested with the qHRP2/3-del assay and did not result in amplification of any target demonstrating the specificity for *P. falciparum*. In summary, we developed a *P. falciparum*-specific multiplex qPCR assay that allowed the simultaneous amplification of the *pfhrp2*, *pfhrp3* and *pfrnr2e2* genes in a single reaction with high efficiency and ability to correctly identify *pfhrp2* and *pfhrp3* gene deletions.

Sample set	Description of sample set	Number of *P. falciparum* positive samples[b]	Parasitemia in parasites/µL (Median/IQR)	Amplification rate by qHRP2/3-del assay[c]
CHMI[a]	CHMI in TZ with PfNF54 strain (no deletion)	49	51.1 (1.5–152.5)	78%
PE	Peruvian samples around Iquitos city. High proportion of *pfhrp2/3* deletions	68	592.4 (186.7–1982.0)	99%
EG	Blood donors with asymptomatic malaria infection living on Bioko Island, Equatorial Guinea	47	4.8 (1.0–45.3)	51%
TZ	Sampling of symptomatic volunteers at two health facilities in Southern Tanzania	90	38.8 (0.7–808.6)	62%
Combined		**254**	**75.7 (2.2–571.6)**	**73%**

Table 2. Field samples used for evaluation of qHRP2/3-del assay. [a]Controlled Human Malaria Infection. [b]All confirmed by diagnostic qPCR assays. [c]Positive for internal control of assay (*pfrnr2e2*).

Analysis of *P. falciparum* field strains with qHRP2/3-del assay. The qHRP2/3-del assay was next tested using a collection of 254 *P. falciparum* isolates originating from East Africa, Central-West Africa and Latin America (Table 2). The infection status and parasitemia levels were well established in these samples by using published diagnostic qPCR assays routinely used in the laboratories in Tanzania[17], Equatorial Guinea[18] and Peru[19]. The overall median parasitemia in these samples was 75.7 parasites/µL (IQR: 2.2–571.6), which is below the LOD of 100 parasites/µL for PfHRP2-based RDTs[20,21]. First, the ability of the *pfrnr2e2* singly copy gene to serve as internal assay control and to quantify parasitemia levels was assessed. Out of the 254 samples, 186 (73.2%) amplified the *pfrnr2e2* singly copy gene. Failure in amplification of *pfrnr2e2* was associated with low parasitemia levels (Fig. 2A). In samples with parasitemia levels of 3 parasites/µL and above, more than 95% of all samples were amplified successfully. In samples with parasitemia >100 parasites/µL, the lower limit of detection for PfHRP2-based RDTs, all qPCR reactions were positive for *pfrnr2e2*. Parasitemia levels determined by using the amplification of *pfrnr2e2* correlated closely with parasite densities obtained from *P. falciparum* diagnostic qPCR assays (Fig. 2B), this is supported by the findings of the Bland-Altman plot which demonstrates a high order of agreement (Fig. 2C). The average ratio of parasite quantification based on diagnostic qPCR assays and qHRP2/3-del assay is 0.8 (95% CI: −1.7–3.3). In summary, the qHRP2/3-del assay amplifies 95% of samples with parasitemia levels of 3 parasites/µL and above and can be used to reliably quantify parasite levels in field samples.

Identification of *pfhrp2* and *pfhrp3* gene deletions using qHRP2/3-del assay. Next, we wanted to establish the performance of the qHRP2/3-del assay in comparison with nested PCR. Samples with known *pfhrp2/3* deletion status obtained from four different sources were included. Serial dilutions of DNA purified from PfDD2 (*pfhrp2* deletion), PfHB3 (*pfhrp3* deletion) and PfIS (no deletion) served as controls. Samples from CHMI using PfNF54 (no deletion) were added to test the specificity of the qHRP2/3-del assay. Two sample sets genotyped by nested PCR, one from Tanzania (TZ) dominated by *P. falciparum* strains without deletions and one from Peru (PE), with a high proportion of *pfhrp2/3* deletions were analysed. The Peruvian sample set consisted of 54 samples with both genes deleted and 7 samples with only one gene deleted. The qHRP2/3-del assay defines a deletion as failure of amplification of the *pfhrp2/3* genes (Fig. 3A, y axis, Cq set to 45) in samples which are positive for the internal control, *pfrnr2e2* (Fig. 3A, x axis). Sensitivity is defined as the proportion of correctly identified *pfhrp2/3* deletions, while specificity is the proportion of correctly identified strains without *pfhrp2/3* deletions. All control samples with known deletion status were identified as expected (Fig. 3A, first panel). Importantly, the qHRP2/3-del assay correctly identified samples with parasitemia levels ranging from 1–10'0000 parasites/µL, demonstrating the dynamic range of at least 5 logs of this assay. In samples collected from volunteers that have undergone CHMI with PfNF54 (CHMI, n = 38), one sample that is positive for *pfhrp2/3* genes was wrongly detected as a double deleted parasite, resulting in a reduced specificity (Fig. 3A, second panel). A high sensitivity was achieved with the Peruvian samples (PE, n = 67), *pfhrp2* and *pfhrp3* deletions were detected with sensitivity of 94.4% and 94.9%, respectively (Fig. 3A, third panel). The low specificity of 76.9% and 87.5% for *pfhrp2* and *pfhrp3*, respectively, is based on the incorrect detection of deletions in three samples. Among the samples from Tanzania (TZ, n = 56), no *pfhrp2/3* deletions were detected by the nested PCR. In contrast, the qHRP2/3-del assay identified three deletions, resulting in a specificity of 93.8% (Fig. 3A, fourth panel).

Grouping the samples with missed deletions (reducing the sensitivity) and the false deletions (reducing the specificity) by parasitemia levels revealed a high proportion of false deletions among the samples with the lowest parasitemia levels (Fig. 3B). Based on these findings, the inclusion criteria for samples to be analysed by qHRP2/3-del assay was changed. The threshold for the *pfrnr2e2* gene amplification was reduced from Cq < 40 to Cq < 37.5, corresponding to parasitemia levels of 5 parasites/µL. Based on these new inclusion criteria, the qHRP2/3-del assay obtained results from 106 samples out of 127 samples (inclusion rate of 83.5%) (Fig. 3C). 12 samples were not amplified by the qHRP2/3-del assay and an additional 9 samples excluded based on the new inclusion criteria. Samples which were not amplified by the qHRP2/3-del assay were mainly ultra-low parasite density samples from Tanzania (11 out of 12).

In 99 out of 106 samples (93.4%), the *pfhrp2/3* deletion status was identical when compared between qHRP2/3-del assay and nested PCR. This is reflected in the near perfect agreement between these two PCR based diagnostic methods for each of the amplified targets. Cohen's kappa was calculated as 0.89 and 0.91 for *pfhrp2* and *pfhrp3*, respectively. Out of the seven samples which were misidentified in four samples both *pfhrp* genes were affected, while in two samples the *pfhrp2* and in one sample the *pfhrp3* status was misclassified. For four misidentified samples with higher parasitemia levels the possibility of sample mix-up or cross-contamination

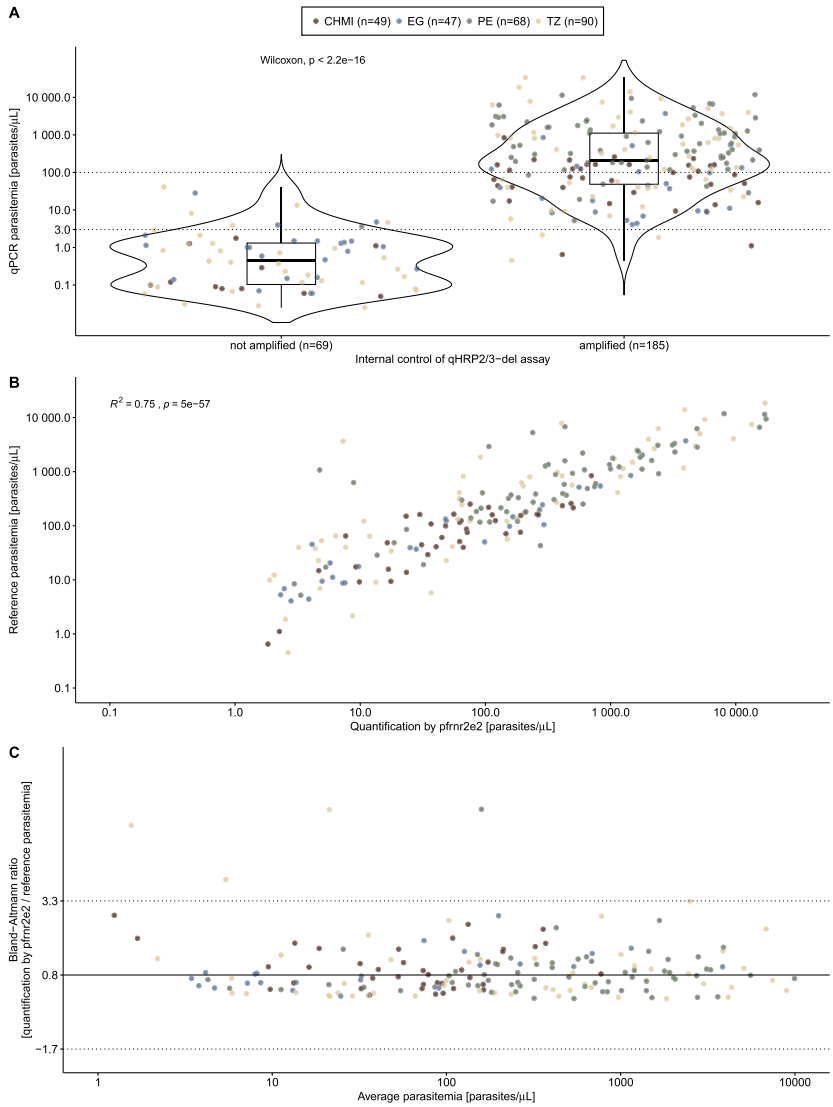

Figure 2. Detection and quantification of field samples using qHRP2/3-del assay. (**A**) Amplification rate of *pfrnr2e2* target, the internal control of qHRP2/3-del assay and association with parasitemia levels. Wilcoxon-Mann-Whitney test was used for comparison of parasitemia between groups. The dashed line at 3 parasites/µL represents the parasitemia at which more than 95% of the samples were amplified, while the dashed line at 100 parasites/µL represents the LOD of RDTs at which all samples are successfully amplified. (**B**) Correlation of parasitemia levels, obtained from diagnostic qPCR assays, and parasitemia, derived from the internal control of qHRP2/3-del assay, is shown. The color represents the different sample sets and R^2 the Spearman's rank correlation coefficient. (**C**) Bland-Altman plot of average parasitemia (x-axis) and ratio of parasitemia levels calculated between internal control of qHRP2/3-del assay and diagnostic qPCR assays (y-axis). Average ratio (black line) and 95% limits of agreement (dashed line) are depicted.

cannot be excluded, since these samples were located next to each other on the DNA plate which was shipped. In summary, the qHRP2/3-del assay specificity (94.4% and 96.0% for *pfhrp2* and *pfhrp3*, respectively) and sensitivity (94.2% and 94.6% for *pfhrp2* and *pfhrp3*, respectively) were above 90%. The negative predictive value (NPV) was calculated as 94.5% and 94.1% and the positive predictive value (PPV) as 94.2% and 96.4%, for *pfhrp2* and *pfhrp3*, respectively (Fig. 3D).

Multiple strain *P. falciparum* infections are masking *pfhrp2* and *pfhrp3* deletions. In many malaria endemic regions, particularly in sub-Saharan Africa, infections with multiple strains of *P. falciparum* are common[22]. A blood sample carrying multiple *P. falciparum* strains with and without *pfhrp2/3* deletions will result in failure to detect the deletion by nested PCR if the parasitemia level of the strain without deletion is sufficiently high for amplification. This limitation leads most likely to an underestimation of the prevalence of *pfhrp2/3* gene deletions in regions with high prevalence of multiple strain infections. We reasoned that the qHRP2/3-del assay could offer a solution by calculating the difference between the Cq values obtained for amplification of *pfhrp2* or *pfhrp3* and *pfrnr2e2*. To demonstrate the ability of the qHRP2/3-del assay to correctly identify and quantify

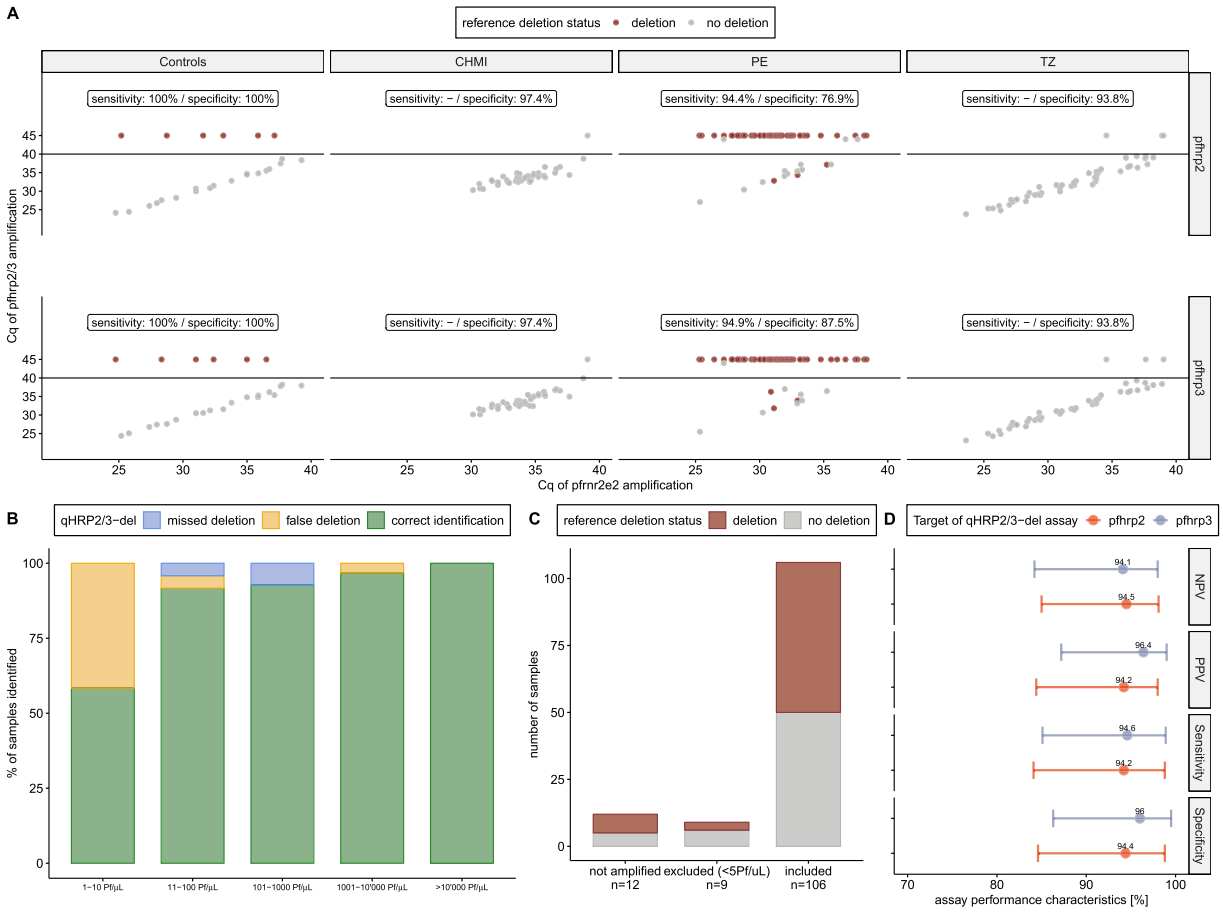

Figure 3. Diagnostic performance of qHRP2/3-del assay. (**A**) Samples with Cq values for *pfhrp2* and *pfhrp3* amplification >40 (shown on y-axis, black line indicates cut-off) are considered to carry a *pfhrp2/3* deletion. Reference deletion status, based on nested PCR, is color coded (red = deletion, grey = no deletion). (**B**) Proportion of correctly and incorrectly identified *pfhrp2/3* deletion status grouped by parasitemia. (**C**) Number of samples included for analysis by qHRP2/3-del assay (n = 106), excluded due to ultra-low parasitemia (n = 9) and not amplified (n = 12). (**D**) Analytical validation of qHRP2/3-del assay performance was assessed by comparing it to nested PCR. Standard parameters such as sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) including their 95% confidence intervals are shown.

"hidden" or "masked" *pfhrp2/3* gene deletions in mixed infections, we first tested defined mixtures of DNA from PfNF54 (no *pfhrp2/3* deletions) and PfDD2 (*pfhrp2* deletion) or PfHB3 (*pfhrp3* deletion) in a range of different ratios. For each combination of strain mixtures, PfDD2/PfNF54 or PfHB3/PfNF54, 10 mixtures were prepared containing varying ratios of strains with and without a *pfhrp2/3* deletion (Fig. 4A). The contribution from PfDD2 and PfHB3 strains to these mixtures ranged from 0.1% to 88% and 0.1% to 86%, respectively. In seven mixtures, the strain with a deletion constituted the minority (with less than 50% abundance) and in three mixtures the majority (with more than 50% abundance). None of these mixtures failed to amplify the *pfhrp2/3* genes, even if the strain carrying the deletion constituted the majority in the mixture. A positive correlation between abundance of isolate carrying a deletion and an increase of ΔCq (Cq of *pfhrp2* or *pfhrp3* minus Cq of *pfrnr2e2*) is observed (Fig. 4B). The qHRP2/3-del assay does not only successfully identify "masked" *pfhrp2/3* deletions but can also discriminates between mixtures where the strain with the deletion constitutes the majority or minority (Fig. 4C). A ΔCq cut-off value of 2.0 was chosen to identify "masked" *pfhrp* gene deletions. Applying this cut-off to our sample collections revealed that two isolates each from Tanzania and Peru have high ΔCq values for both *pfhrp* genes indicative of the presence of "masked" *pfhrp2/3* deletions (Fig. 4D). Three additional samples from the Peruvian collection had a ΔCq value > 2 for the *pfhrp2* gene only. No ΔCq values above 2 were found in Equatorial Guinean isolates and among samples collected from volunteers undergoing CHMI (Fig. 4D). These experiments demonstrate that by calculating the ΔCq values between Cq for *pfrnr2e2* and *pfhrp2* or *pfhrp3*, "masked" deletions can be identified.

Discussion

P. falciparum strains carrying *pfhrp2/3* deletions are an emerging threat to malaria control and elimination programs around the world. Novel analysis tools enabling high-throughput screening of *P. falciparum* populations from the field are needed. The currently published methods, mostly based on nested PCR, have clear limitations in that these methods are extremely time consuming, prone to detection of incorrect deletions at low parasitemia levels and unable to identify "masked" deletions in multiple strain co-infections.

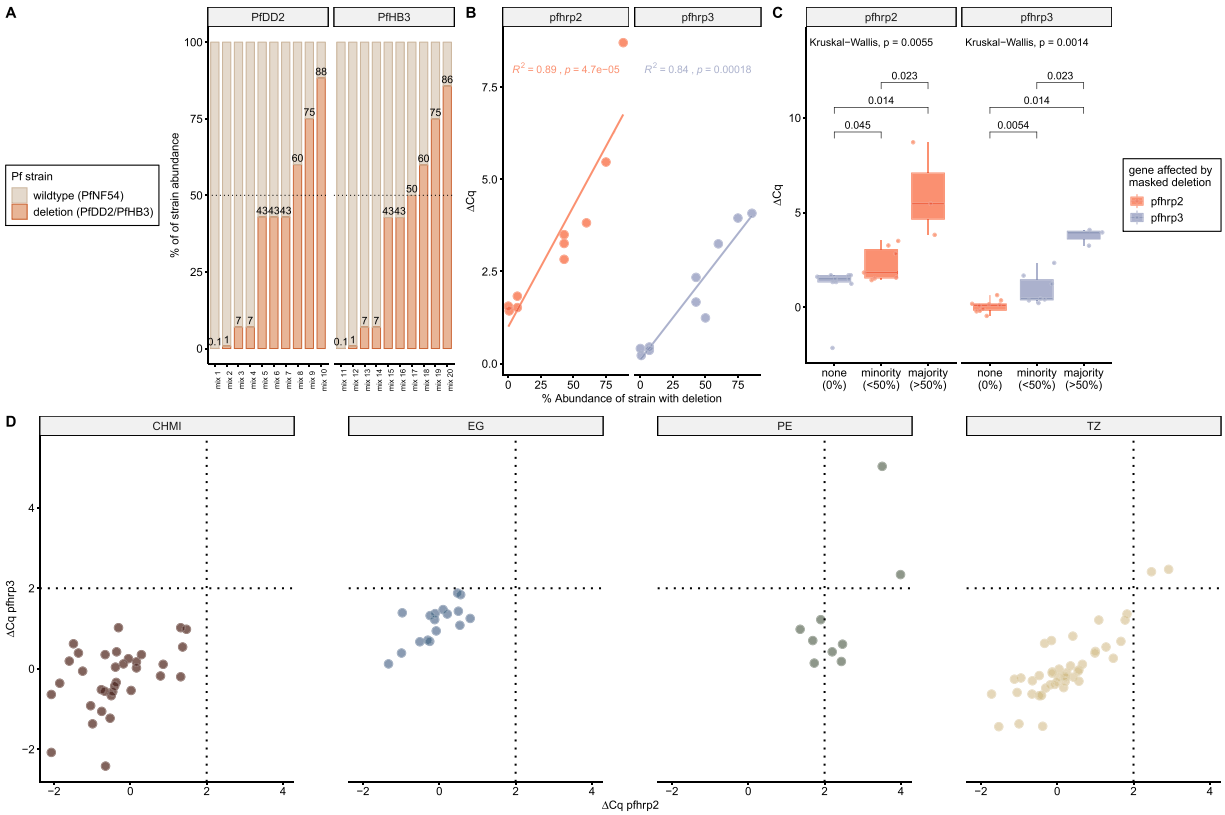

Figure 4. Identification of masked *pfhrp2/3* deletions in multiple strain infections. (**A**) Mixtures containing two strains, one with a *pfhrp* deletion (PfDD2 or PfHB3) and no deletion (PfNF54), were generated. (**B**) Correlation between abundance of strain carrying deletion and ΔCq is shown for both targets, *pfhrp2* (red) and *pfhrp3* (blue). (**C**) The ΔCq approach distinguishes between strain mixtures not carrying deletions, mixtures with minority abundance as well as majority abundance of strains with deletions. Statistical comparison was performed using the Kruskal-Wallis test followed by Wilcoxon-Mann-Whitney for pairwise comparisons. (**D**) The ΔCq approach of the qHRP2/3-del assay was applied to four sample collections to identify "masked" *pfhrp2/3* deletions. The control group, based on samples from CHMI, did not reveal isolates with increased ΔCq values. The dashed lines represent the ΔCq cut-off values for *pfhrp2* (x-axis) and *pfhrp3* (y-axis).

The sensitivity and specificity of the PfHRP2/3-del assay is comparable to the widely used nested PCR. However, the novel qHRP2/3-del assay is well suited for high throughput screening of *P. falciparum* isolates with approximately 30 samples analyzed in less than two hours - including DNA extraction and data analysis. Two additional major advantages of the qHRP2/3-del assay are obvious: firstly, the ability to quantify parasitemia levels and therefore include samples based on parasitemia and secondly, to identify "masked" deletions in multiple strain infections.

The identification of *pfhrp2/3* deletions in samples with low parasitemia levels is difficult since the absence of amplification could be due to lack of sufficient template leading to incorrect reports of deletions. The conventional method depends on the successful amplification of at least two single copy reference genes to ensure sufficient template in the PCR reaction. This procedure is time-consuming and labour-intensive. The qHRP2/3-del assay uses an alternative inclusion criterion, based on the Cq value of its internal control. The pre-defined exclusion criteria of all samples that have parasitemia below 5 parasites/µL will improve the quality, reproducibility and comparability of malaria parasite survey data obtained with the qHRP2/3-del assay.

The ability to detect "masked" *pfhrp2/3* deletions is probably the most interesting feature of the qHRP2/3-del assay, because it will allow studying the epidemiology of *pfhrp2/3* deletions in malaria endemic regions with a high proportion of infections caused by multiplicity of infections, particular sub-Saharan Africa[22]. The qHRP2/3-del assay correctly identified infections that contain two strains, one with a deletion and the other one without a deletion, based on a difference in the Cq values derived from the amplification of the *pfhrp2/3* gene targets and the *pfrnr2e2* control. However, currently we cannot exclude that nucleotide sequence variations located in the binding sites of the oligonucleotides used in the PfHRP2/3-del assay could potentially also lead to variation in ΔCq values. The ΔCq application of our novel PfHRP2/3-del assay in additional studies including a larger sample size will improve our understanding of the relevance of "masked" *pfhrp2* and *pfhrp3* gene deletions and their impact on reliability of malaria RDT test results.

Two Tanzanian isolates had an increased ΔCq value for both *pfhrp* genes, indicating the presence of *pfhrp2/3* deletions in the East African nation. This was recently confirmed when *pfhrp2* and *pfhrp3* deletions were

identified in Tanzania and Uganda[23].Together with findings from Kenya[24], the Democratic Republic of Congo[25], Rwanda[26] and Mozambique[27] there is strong evidence for the existence of *pfhrp2/3* deletions in this region. Therefore, establishing programs which systematically monitor *pfhrp2/3* deletions and their impact on the performance of RDTs is advised.

Conclusion

The qHRP2/3-del assay presented here is suitable for high-throughput screening of *P. falciparum* strains to identify *pfhrp2/3* gene deletions in different malaria endemic settings, including areas with high a proportion of multiple strain co-infections. With growing availability of qPCR instruments in reference laboratories in sub-Saharan countries, this assay could be used as surveillance method to monitor over time the potential expansion of *P. falciparum* strains carrying *pfhrp2* and *pfhrp3* deletions.

Methods

P. falciparum isolates from tanzania, equatorial guinea and peru. In this study a total of 205 *P. falciparum* isolates collected from three different malaria endemic regions, East Africa, West-Central Africa and South America were included. The samples from East Africa (n = 90) were collected in rural southern Tanzania (TZ) as part of a malaria baseline survey[28]. The West-Central African isolates (n = 47) were identified among blood donors living in Malabo, Equatorial-Guinea (EG)[18]. Both samples sets were analyzed locally, at the Bagamoyo branch of the Ifakara Health Institute and the laboratory of the Equatorial Guinea Malaria Vaccine Initiative using harmonized protocols. Briefly, genomic DNA was isolated either from 6 circles with 2 mm diameter of dried blood spots (Tanzania) or 180 µL whole blood (Equatorial Guinea) using the Quick-DNA Miniprep kits (Zymo Research, Irvine, USA). *P. falciparum* was identified and quantified using published qPCR protocols based on varATS[29]. Extracted DNA (n = 68) from Peruvian isolates (PE), collected between 2008–2009 and 2015–2016 around Iquitos city, was shipped to the Swiss Tropical and Public Health Institute for *pfhrp2/3* characterization by qHRP2/3-del assay.

Additional parasite isolates and laboratory strains. Forty-nine PfNF54 isolates from Controlled Human Malaria Infections (CHMI) conducted in Bagamoyo, Tanzania (ClinicalTrials.gov: NCT02613520[30]) as well as genomic DNA isolated from 8 laboratory strains with known *pfhrp2/3* deletion status (Pf3D7, Pf7G8, PfDD2, PfHB3, PfNF135.C10, PfNF166.C8, PfNF54 and PfFC27) were used as controls. The 1st WHO International Standard *for Plasmodium falciparum* DNA Nucleic Acid Amplification Techniques (NIBSC code: 04/176, herein referred to as PfIS) was used to assess the performance of the qHRP2/3-del assay. Non-falciparum *Plasmodium* species, including *P. malariae (Pm)*, *P. ovale curtisi (Poc)*, *P. ovale wallikeri (Pow)*, *P. vivax (Pv)* and *P. knowelsi (Pk)* and an additional 28 samples from malaria negative individuals living in Tanzania were used to assess specificity of the assay.

Detection of the _pfhrp2_ and _pfhrp3_ genes by conventional nested PCR. *P. falciparum* positive samples collected in Tanzania were selected for detection of *pfhrp2* and *pfhrp3* genes by nested PCR. As a reference gene, the *msp2* gene was amplified using a previously described protocol[31]. All isolates with successful *msp2* amplification were analyzed for the presence of *pfhrp2* and *pfhrp3* genes using primers spanning exon 1, the intron, and exon 2[32]. All PCR products were separated and visualized on a 2% agarose gel. Cultured parasite isolate PfDD2 (*pfhrp2* deletion) was used as a control for all nested PCR experiments on *pfhrp2* while PfHB3 (*pfhrp3* deletion) was used as a control for all nested PCR experiments on *pfhrp3*. PfNF54 (no *pfhrp2/3* deletion) was used as a positive control for both *pfhrp* genes. *Pfhrp2/3* deletion status of the Peruvian *P. falciparum* isolates was analyzed previously following the procedures described elsewhere[9]. Results were shared to be used for the evaluation of the qHRP2/3-del assay.

Design of qHRP2/3-del assay. Published *pfhrp2/3* primer sequences for conventional PCR were adapted to the qPCR platform using EvaGreen® qPCR Mix (Solis BioDyne, Tartu, Estonia). The primers were tested with different DNA concentrations extracted from PfNF54, PfDD2 and PfHB3 strains, corresponding to parasitemia levels of 1 and 100 parasites/µL. The best performing primer pairs, in terms of specificity and sensitivity, were then used in combination with newly designed TaqMan® hydrolysis probes. The *pfhrp2/3* oligo sequences were systematically optimized using the trial-and-error approach. As the internal control of the qHRP2/3-del assay we amplify a *P. falciparum* specific 107 bp long sequence of the ribonucleotide reductase R2_e2 (*pfrnr2e2*), a distantly related paralog of the canonical eukaryotic small subunit ribonucleotide reductase R2, that is unique to apicomplexan species[14]. The performance of *pfrnr2e2* as a biomarker for detection and quantification of *P. falciparum* was tested by direct comparison with parasitemia levels obtained from a 18 S rDNA based qPCR assay[33]. A sensitivity of 89.1% for samples with parasitemia >1 parasite/µL and a Bland-Altman ratio of 0.99 (95% CI: −0.012–2.5) demonstrate its robustness and accuracy as internal control (Supplementary File 4). Genomic sequences for *pfrnr2e2* (PF3D7_1015800), *pfhrp2* (PF3D7_0831800) and *pfhrp3* (PF3D7_1372200) of Pf3D7 strain were obtained from PlasmoDB. A *pfhrp2/3* sequence alignment including five reference strains from West-Africa (Pf3D7, PfNF54), Guinea (PfNF166.C8), Brazil (Pf7G8) and Cambodia (PfNF135.C10) revealed no SNPs in oligo binding regions suggesting a high degree of conservation within the target region of the *pfhrp2/3* genes (Supplementary Files 2 and 3). The *pfhrp2*, *pfhrp3* and *pfrnr2e2* sequences for Pf7G8, PfNF135.C10, PfNF166.C8 and PfNF54 were obtained from whole genome sequencing[34]. The Geneious version 8.1.9 software (Biomatters Ltd, Auckland, New Zealand) was used for sequence alignments and oligo designs. Relevant information concerning the oligos used in the qHRP2/3-del assay is summarized in Table 1.

Sample analysis with qHRP2/3-del assay. Amplification and qPCR measurements were performed using the Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA). The thermal profile used for qHRP2/3-del assay is as follows: Taq polymerase activation for 5 min at 95 °C, followed by 45 cycles of

15 s at 95 °C and 35 s at 57.5 °C. 2 µL DNA was added to 8 µL reaction master mix containing 1x Luna Universal Probe qPCR Master Mix (New England Biolabs, Ipswich, USA) and 1x qHRP2/3-del Primer Mix (Table 1). All qPCR assays were run in triplicates with appropriate controls including Non-Template Control and DNA from PfDD2, PfHB3 and PfNF54 as controls for the *pfhrp2/3* deletion status.

Data management and statistical analysis. *Preliminary analysis of qPCR data.* Cq values were obtained from the Bio-Rad CFX96 Manager 3.1 software (Bio-Rad Laboratories, California, USA) after setting the threshold manually. Cq values were transferred and linked to the samples' metadata using a custom-designed database for storage and analysis of qPCR data. Only samples with a Cq $\leq$ 40.0 for the internal control, *pfrnr2e2*, were considered eligible for analysis of *pfhrp2/3* deletion status. ΔCq were calculated by subtraction of *pfrnr2e2* Cq values from *pfhrp2* or *pfhrp3* Cq values.

Analytical performance of qHRP2/3-del assay and quantification of parasitemia. Based on the PfIS a serial dilution ranging from 0.01–10'000 parasites/µL was prepared and used to assess the performance of the qHRP2/3-del assay. The slope, y-intercept, qPCR efficiency and R^2 was established for each target. The Limit of Detection (LOD) was defined as the lowest PfIS parasitemia with a positive amplification in 4 out of 6 replicates. Parasitemia was estimated using linear regression derived from serial dilution of the PfIS and the *pfrnr2e2* target which serves as the internal control of the qHRP2/3-del assay.

Graphical representation and statistical analysis. We used R version 3.5.1 for creating ggplot2-based graphs using the packages *ggpubr*, *gridextra* and *scales*. The Diagnostic test evaluation calculator (freely available at https://www.medcalc.org/calc/diagnostic_test.php) was used for analytical validation of qHRP2/3-del assay performance. Cohen's kappa including 95% confidence intervals, providing a measure of agreement, was calculated using STATA version 12.0 software (Stata Corp LP; College Station, Texas, USA). P values < 0.05 were considered as significant for all statistical analysis.

Ethical approval and informed consent. The samples analyzed in this study were collected in different studies. All studies were approved by the appropriate institutions and informed consent was obtained from all participants. The CHMI samples were collected during a clinical study, registered at Clinical Trials.gov (NCT02613520), and conducted under a U.S. FDA IND application. The study was performed in accordance with Good Clinical Practices. All samples analyzed in this publication were obtained according to the approved study protocol. The protocol was approved by the institutional review boards of the Ifakara Health Institute (IHI/IRB/No: 32–2015), and the National Institute for Medical Research Tanzania (NIMR) (NIMR/HQ/R.8a/Vol.IX/2049), by the Ethikkommission Nordwest- und Zentralschweiz, Basel, Switzerland (Ref. No. 15/104), and by the Tanzania Food and Drug Authority (Auth. No. TZ15CT013). For the Tanzanian sample collection ethics approval for the study was granted by the institutional review boards of Ifakara Health Institute (IHI/IRB/No: 18–2015) and by NIMR (NIMR/HQ/R.8a/Vol.IX/2015). For the sample collection from Equatorial Guinea approval was given by the Ministry of Health and Social Welfare. The collection, transport and storage of the blood samples from Peru was approved by the Human Ethics Committee from Universidad Peruana Cayetano Heredia (UPCH 52707 & 59751).

Data Availability

All data generated or analyzed during this study are included in this published article and its Supplementary Information Files.

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Author Contributions

Study concept and design: T.S., A.C.D., C.D. Acquisition of data: T.S., A.C.D., M.F., E.G., S.M.M. Analyses and interpretation of data: T.S., A.C.D., D.G., P.M.V., C.D. Drafting the manuscript: T.S., A.C.D., C.D. Technical or material support: K.M., J.C.S., D.G., P.M.V., K.T., S.A., S.L.H., M.T. Sample collection and enrollment of patients: M.G.M., S.A.J., P.P.C., J.R.B., P.M., K.T. Study supervision: C.D. All authors read and approved the final manuscript.

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Early whole blood transcriptional responses to radiation-attenuated *Plasmodium falciparum* sporozoite vaccination in malaria naïve and malaria pre-exposed adult volunteers

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Abstract

Background: Vaccination with radiation-attenuated *Plasmodium falciparum* sporozoites is known to induce protective immunity. However, the mechanisms underlying this protection remain unclear. In this work, two recent radiation-attenuated sporozoite vaccination studies were used to identify potential transcriptional correlates of vaccination-induced protection.

Methods: Longitudinal whole blood RNAseq transcriptome responses to immunization with radiation-attenuated *P. falciparum* sporozoites were analysed and compared across malaria-naïve adult participants (IMRAS) and malaria-experienced adult participants (BSPZV1). Parasite dose and method of delivery differed between trials, and immunization regimens were designed to achieve incomplete protective efficacy. Observed protective efficacy was 55% in IMRAS and 20% in BSPZV1. Study vaccine dosings were chosen to elicit both protected and non-protected subjects, so that protection-associated responses could be identified.

Results: Analysis of comparable time points up to 1 week after the first vaccination revealed a shared cross-study transcriptional response programme, despite large differences in number and magnitude of differentially expressed genes between trials. A time-dependent regulatory programme of coherent blood transcriptional modular responses was observed, involving induction of inflammatory responses 1–3 days post-vaccination, with cell cycle responses apparent by day 7 in protected individuals from both trials. Additionally, strongly increased induction of inflammation and interferon-associated responses was seen in non-protected IMRAS participants. All individuals, except for non-protected BSPZV1 participants, showed robust upregulation of cell-cycle associated transcriptional responses post vaccination.

Conclusions: In summary, despite stark differences between the two studies, including route of vaccination and status of malaria exposure, responses were identified that were associated with protection after PfRAS vaccination. These comprised a moderate early interferon response peaking 2 days post vaccination, followed by a later proliferative cell cycle response steadily increasing over the first 7 days post vaccination. Non-protection is associated with deviations

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from this model, observed in this study with over-induction of early interferon responses in IMRAS and failure to mount a cell cycle response in BSPZV1.

Background

Despite the existence of effective anti-parasitic drugs, malaria remains a critical global health problem, estimated at causing 409,000 deaths and 229 million cases in 2019. Some 94% of cases were in Africa, where almost all infections were caused by *Plasmodium falciparum* [1]. Currently, the most advanced malaria vaccine, RTS,S, exhibits 28–36% efficacy in infants and children observed over an average time period of 4 years [2]. A more effective malaria vaccine would be a valuable tool for curbing malaria, especially given the emergence of resistance to frontline artemisinin combination therapy and development of insecticide-resistant mosquito vectors [3, 4]. Repeated natural malaria infections can result in acquisition of semi-protective immunity with persistent low level parasitaemia and primarily asymptomatic cases [5]. Serious malaria-related complications and death occur primarily in infants and children, prior to the development of partially protective immune responses [6]. However, acquisition of sterilizing immunity targeting the pre-erythrocytic stage of the parasite, resulting from immunization with radiation-attenuated malaria sporozoites, has been experimentally demonstrated in animal models and in humans [7–10].

Malaria sporozoites develop in the mosquito and are injected into the skin during a female mosquito blood meal from where they make their way to the liver and infect hepatocytes. There they multiply and over the course of 5–9 days, asymptomatically develop into thousands of merozoites which emerge from the liver and serially infect erythrocytes, resulting in blood-stage infection and disease. The pre-erythrocytic stage initiated by sporozoites is a population bottleneck in the parasite lifecycle, and is an attractive target for vaccine development strategies. It was first demonstrated in a mouse model in 1967 that immunization with radiation-attenuated sporozoites (RAS) results in effective protective immunity against challenge with infectious sporozoites [10], and demonstrated subsequently for *P. falciparum* RAS (PfRAS) in human cohorts in multiple clinical trials [7, 11].

The immune mechanisms of human protection resulting from immunization with whole-sporozoite vaccines remain poorly understood but available evidence indicates that the development of this immunity requires liver infection. Work in animal models shows important roles associated with protection for antibodies, liver resident CD8+ memory T cells (Trm) and type I interferon responses [12–14]. However, results in animal models may not directly translate to humans, and the ability to directly monitor responses in human liver during vaccination and after controlled human malaria infection (CHMI) is very limited. Blood represents an accessible and immunologically important tissue which is reflective of systemic immune responses and its analysis can aid investigation of immune protection against malaria.

Two human RAS vaccination trials that resulted in a portion of the trial participants being protected from infection following CHMI have been performed, allowing comparisons between protected and non-protected subjects. Immunization by mosquito bite with radiation attenuated sporozoites (IMRAS) [15], [NCT01994525] and Bagamoyo sporozoite vaccine 1 study; immunizations with Sanaria® PfSPZ Vaccine (BSPZV1) [16] trials, both included immunization of volunteers with five consecutive PfRAS vaccinations followed by homologous CHMI using *P. falciparum* strain NF54. Whole blood was sampled repeatedly from participants and analysed by RNAseq to provide longitudinal data on the immune responses. These trials differed with respect to malaria experience of the volunteers and route of administration for both immunization and CHMI, as described in "Methods". This study analysed comparable time points in both trials up to 1 week after the first RAS vaccination. Shared transcriptional responses were identified in volunteers participating in both studies after initial RAS vaccination, indicating the existence of an initial core transcriptional response programme to RAS vaccination across diverse populations. Deviations from this transcriptional programme were associated with lack of protection.

Methods

Challenge trials

The IMRAS trial was performed in Bethesda, MD, USA with malaria-naive participants immunized by four rounds of ~190 bites of PfRAS-infected mosquitoes at 4-week intervals followed by a fifth immunization a further 5 weeks later. Protection was assessed 3 weeks after last vaccination by homologous CHMI via bites of five mosquitoes carrying non-irradiated *P. falciparum* sporozoites. This intentionally suboptimal immunization schedule resulted in 55% protection among the IMRAS cohort examined in this study.

The BSPZV1 trial was carried out in Bagamoyo, Tanzania with volunteers who potentially had prior malaria

infections, but were free of symptomatic malaria for the previous 2 years. They were immunized 5 times by direct venous injection (DVI) with either 1.35×10^5 or 2.7×10^5 PfSPZ of Sanaria® PfSPZ Vaccine (aseptic purified cryopreserved PfRAS) given at 4-week intervals. This was followed by CHMI at 3 weeks after the final immunization via DVI with PfSPZ Challenge (NF54) (purified cryopreserved non-irradiated SPZ), at a dose of 3200 PfSPZ. The resultant protective efficacy was 20%. Because not all non-protected individuals were included in RNAseq analysis, there was 36% protection among analysed individuals.

Total RNA was extracted from whole blood in PAXgene Blood RNA tubes that had been stored at − 80 °C, using the PAXgene Blood Kit (PreAnalytiX) following the manufacturer's protocols. RNA was quantified by spectrophotometry and ~2.3 µg of total RNA per sample was processed using the GLOBINclear Human kit (Ambion) in order to remove globin mRNA. RNAseq was performed by Beijing Genomics Institute using either the Illumina Hiseq2000 (75 bp read length, paired-end), or the BGI500 platform (100 bp read length, paired-end) to a depth of at least 30 million reads per sample. Reads were aligned to the human hg19 genome using STAR [17], and htseq-count [18] using the intersection-strict option was used to convert mapped reads into a gene count table. Genes were filtered to retain only genes with >10 read counts in at least 5% of samples. Counts were then normalized using the R limma [19] and voom [20] packages to account for sequencing depth and log2 transformed for all downstream analysis.

Linear mixed-model analysis

The R lme4 [21] package was used to fit nested mixed models to assess differential gene expression in both cohorts, using the lmer function. Random intercepts were fit per-subject to account for multiple samples being drawn from the same study subjects. Five mixed models were fit to each gene, separately for differential expression at each sample day relative to the previous sample day in each study. For IMRAS these time intervals were days 1 vs 0, 3 vs 1, 7 vs 3, and for BSPZV1 days 2 vs 0 and 7 vs 2. Time and protection status were encoded as binary variables.

Formulae were fit as follows:

$$geneExpr \sim 1 + Time * Protection + (1|Subject) \quad (1)$$

$$geneExpr \sim 1 + Time + (1|Subject) \quad (2)$$

$$geneExpr \sim 1 + Protection + (1|Subject) \quad (3)$$

This model structure assumes that individual transcriptional responses are a function of (vaccine induced) responses over a time interval, and individual protection status. In the full model (1), both time and protection status are included. Reduced models comprising only time (2) or protection status (3) were also fit and, to assess the statistical significance of these parameters of interest, p-values associated with time- or protection-associated gene responses were determined by contrasting full models with reduced models. Specifically, p-values for time-associated genes were calculated by ANOVA to determine whether Eq. (1) significantly improved fit to the data compared to Eq. (3) using the anova.merMod() function. Similarly, Protection p-values were obtained by comparing Eq. (1) to Eq. (2). Response gene p-values were then false-discovery rate adjusted for multiple testing.

Directionality (UP, DN or NC (no change)) of each gene response was assessed using confidence intervals (CIs). The 90% CIs were estimated for the coefficient of Eq. (2), cases where the lower CI > 0 were considered UP genes, upper CI < 0 were considered DN genes.

Significant response genes were identified as those that met an false discovery rate (FDR) threshold < 0.2 a nominal p-value < 0.05, and were classified as UP or DN in direction. Candidate protection-associated genes were filtered to only include significant response genes, then false discovery rate adjusted. Protection genes were selected that met an FDR threshold < 0.33 and nominal p-value < 0.05. A more permissive FDR threshold was selected for response genes vs protection genes to reflect reduced statistical power comparing protection status within a timepoint vs comparing gene expression for all samples over a time interval.

Gene-set enrichment analysis

Previously published coherent blood transcriptional modules (BTMs) published by Li et al. [22], Chaussabel et al. [23], and the MsigDB Hallmark collection [24] were used for gene set enrichment analysis (GSEA) [25] of whole blood RNAseq profiles. A total of 656 BTMs were tested. Samples were grouped by sample time interval (as above), study, and protection status. Genes were ranked by calculating the average change in normalized expression for each gene over each time interval. These rankings were used as input to calculate GSEA normalized enrichment scores (NES) with accompanying p-values, nominal and FDR adjusted. GSEA was performed using the R fgsea package [26], with 10,000 random permutations. GSEA NES that did not pass the significance cut-off FDR < 0.05 were set to 0 for all further analysis. Correlations between subgroup module NES profiles were assessed using Spearman's rho.

Spline curve fitting

Gene-averaged responses for each BTM were calculated using the 25% trimmed mean of BTM gene expression per sample. Responses were made relative to day 0 for each subject by subtracting the subject day 0 BTM expression from subsequent days. A spline curve was fit to all IMRAS and BSPZV1 samples at all time points for each BTM, using the R smooth.spline function with 3 degrees of freedom. The 99% CIs around each spline were calculated using 500 bootstrap replicates, taking approximately 2/3 of samples for each replicate. To calculate CI deviation from the 0 response line, lower and upper 99% CIs were assessed at each time point measured, i.e., days 1, 2, 3, and 7. The BTM response was considered significant if both lower and upper CIs were either above or below the 0 response line and the magnitude of the difference was defined as: if upCI < 0, upCI else lowCI.

Table 1 Study composition

	IMRAS	BSPZV1
Study location	Bethesda, MD, USA	Bagamoyo, Tanzania
RAS delivery	Mosquito bite	Venous injection
Prev. malaria exposure	Naïve	Malaria free for > 2 years
N. protected	6	8
N. non-protected	5	14
RNAseq time-points		
D000	X	X
D001	X	
D002		X
D003	X	
D007	X	X

Table lists participants for whom longitudinal whole blood RNAseq profiles are available

Results

Study design

Participants were recruited as part of two independent RAS-vaccination studies: IMRAS, [NCT01994525], [15] and BSPZV1 [16]. Both trials comprised 5 immunizations with identical strains of *P. falciparum*, with protection assessed by homologous CHMI. In the case of BSPZV1, immunizations and CHMI were delivered intravenously as cryopreserved purified sporozoites while for IMRAS, irradiated sporozoites and CHMI were administered by mosquito bite. The IMRAS trial was performed in malaria-naive adults in the Bethesda, MD, USA, while BSZPV1 was conducted in Tanzanian adults with previous malaria experience. Longitudinal whole-blood RNAseq transcriptional profiles were obtained from the studies (Table 1). Comparable samples cross-study comprised 33 RAS-vaccinated participants, with 3 (BSPZV1) or 4 (IMRAS) time points per person, measured immediately prior to and up to 7 days after the initial RAS vaccination.

IMRAS and BSPZV1 share a small but significant overlap of vaccine-induced genes

Mixed-effects linear modelling was used to identify differentially expressed genes (DEGs) that significantly respond over any time interval after PfRAS vaccination. For IMRAS, these time intervals were day 0 (immediately prior to vaccination) to day 1 (post vaccination), day 1 to day 3, and day 3 to day 7. For BSPZV1, time intervals were day 0 to day 2, and day 2 to day 7. Significant DEGs (FDR < 0.2) over a time interval were identified using a nested mixed modelling approach (see "Methods") and classified as increased or decreased based on 90% CIs of the model time coefficient. This accounted for dynamic expression changes between subsequent sampling times. Subsequently, the changes in the DEGs over time intervals were tested for significant associations with protection (FDR < 0.33).

The numbers of genes that significantly increased or decreased at each time interval for each study are shown in Fig. 1a. Overall, IMRAS showed 2–3× more time-interval associated DEGs (UP: 3133, DN: 2709) compared with BSPZV1 (UP: 1413, DN: 1302) (Fig. 1b). However, a larger proportion of BSPZV1 time-interval DEGs differed in expression between protected and non-protected subjects (UP: 174, DN: 218) compared with IMRAS (UP:174, DN:110) (Fig. 1a). A modest but significant overlap was observed (377 genes) in UP, but not DN responses and genes between the trials ($p = 2.02 \times 10^{-4}$, Table 2, Fig. 1c). Fourteen of these genes were associated with protection, also representing a significant increase in what would be expected to be shared by random overlap in the gene lists ($p = 7.24 \times 10^{-7}$, Table 2, Additional file 1: Table S1). While this overlap represented a small minority of genes responding in each study, it pointed to conserved upregulated responses early after PfRAS vaccination.

In order to identify more broadly shared transcriptional response pathways between studies, previously published sets of transcriptionally coherent BTMs [22–24] were used. The 377 shared cross-study DEGs (UP in both IMRAS and BSPZV1) were assessed for BTM enrichment using the hypergeometric test. 35 significantly enriched BTMs (FDR < 0.1, Table 3) were identified, from a variety of functional classes, including erythrocytes, cell cycle and inflammatory modules. Six of these BTMs were also specifically enriched in the 14 upregulated cross-study protection associated genes (Table 3). Notably, 8 of 14 protection-associated genes, SPAG5, EZH2, NCAPH, HJURP, NUSAP1, DTL, CKAP2L, and

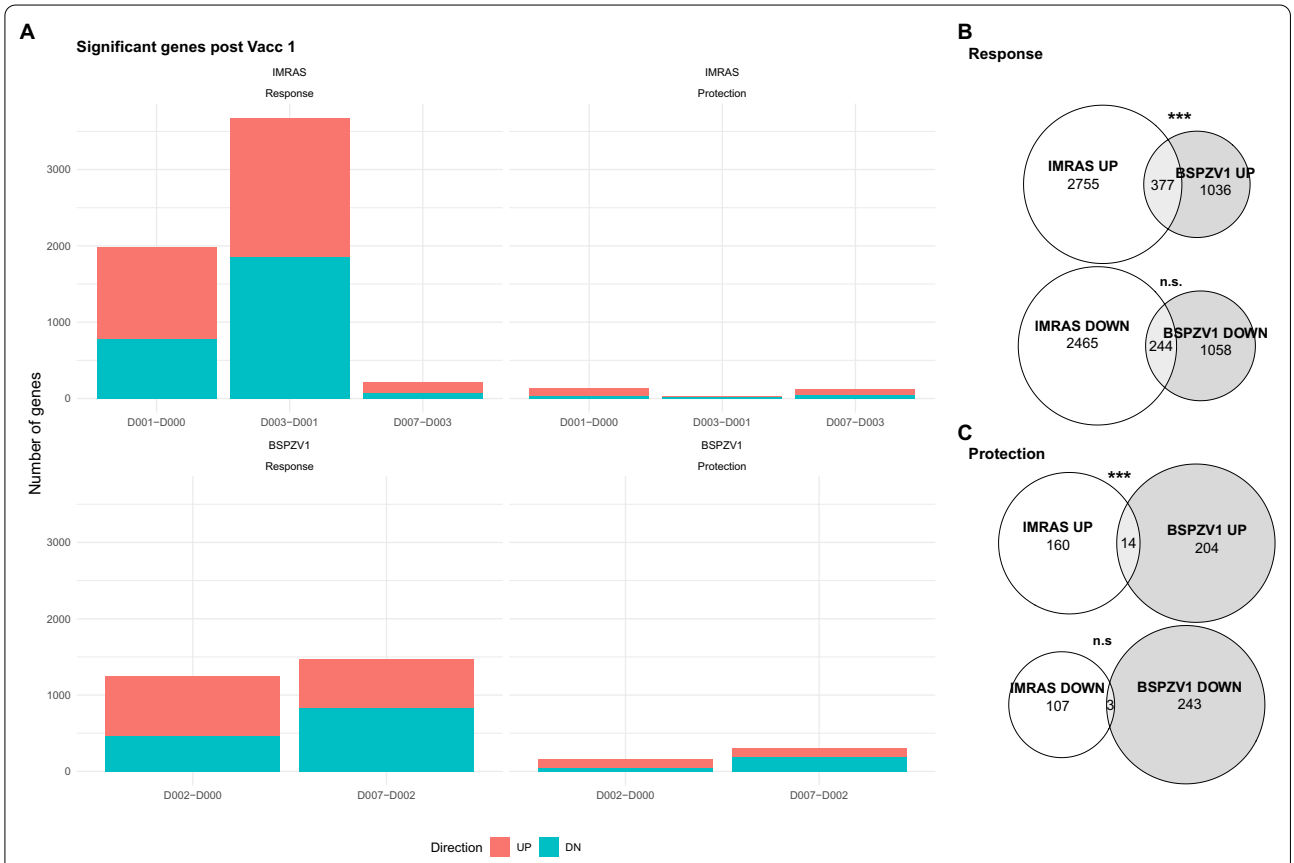

Fig. 1 Limited cross-study overlap of differentially expressed genes. **A** Counts of significantly up- and down-regulated genes for each time interval up to 1 week after first RAS vaccination for both IMRAS and BSPZV1, including both vaccine response and protection-associated genes. **B**, **C** Venn diagrams showing intersection of up and down-regulated response (**B**) and protection (**C**) associated genes between IMRAS and BSPZV1. Annotations indicate hypergeometric test p-value of gene overlap: '***' $p < 0.001$; 'n.s.' $p > 0.05$

Table 2 Limited cross-study overlap of differentially expressed genes

			N shared genes	N expected	p
Response	IMRAS UP	BSPZV1 UP	377	323	2.02×10^{-4}
Response	IMRAS DOWN	BSPZV1 DOWN	244	257	0.84
Protection	IMRAS UP	BSPZV1 UP	14	3	7.24×10^{-7}
Protection	IMRAS DOWN	BSPZV1 DOWN	3	2	0.32

Hypergeometric test p-values and overlap sizes for the up- and down-regulated genes identified by mixed modelling

RRM2 were members of a single BTM: LI.M4.0_cell cycle and transcription (Additional file 1: Table S1). A further two, HAGH and CARM1, were part of the DC.M3.1_Erythrocytes BTM. Several other protection-associated genes were part of immune-related BTMs: AGPAT3 in LI.S5_DC surface signature, LGALS3BP in DC.M3.4_Interferon, CHKA in DC.M7.1_Inflammation. CREBL2 was the sole protection-associated gene not found in a well-annotated BTM; it was only found in the DC.M8.8_Undetermined BTM.

Gene-set enrichment analysis reveals shared response pathways between studies

Module enrichment based on DEGs was limited by the statistical power to accurately identify differentially expressed genes, and did not take into account responses specific to either IMRAS or BSPZV1. To expand on the previous analysis and more broadly identify transcriptional response pathways, BTM responses were assessed using GSEA separately for BSPZV1 and IMRAS, and for protected: P and non-protected: NP

Table 3 Blood transcriptional modules enriched in overlapping differentially expressed genes

N genes	N BTM	N shared	N expected	p value	FDR	Blood transcriptional module (BTM) name	Protection associated
377	82	26	2	4.58E−21	3.01E−18	DC.M3.1_Erythrocytes	
377	145	32	4	2.55E−20	8.35E−18	LI.M4.1_cell cycle (I)	*
377	335	44	9	3.53E−18	7.72E−16	LI.M4.0_cell cycle and transcription	*
377	49	17	1	6.00E−15	9.84E−13	DC.M3.3_Cell Cycle	*
377	77	20	2	1.31E−14	1.71E−12	DC.M4.4_Undetermined	
377	34	14	1	9.42E−14	1.03E−11	LI.M4.2_PLK1 signaling events	*
377	200	29	6	1.89E−13	1.77E−11	HALLMARK_E2F_TARGETS	*
377	200	27	6	7.41E−12	6.08E−10	HALLMARK_HEME_METABOLISM	
377	71	16	2	6.30E−11	4.59E−09	DC.M2.3_Erythrocytes	
377	35	11	1	1.36E−09	8.91E−08	LI.M4.5_mitotic cell cycle in stimulated CD4 T cells	
377	200	23	6	6.65E−09	3.97E−07	HALLMARK_G2M_CHECKPOINT	
377	51	10	1	1.03E−06	5.61E−05	LI.M103_cell cycle (III)	
377	32	8	1	1.79E−06	9.05E−05	LI.M6_mitotic cell division	
377	47	9	1	4.42E−06	2.07E−04	LI.M49_transcription regulation in cell development	
377	12	5	0	1.03E−05	4.53E−04	LI.M4.12_C-MYC transcriptional network	
377	20	6	1	1.16E−05	4.77E−04	DC.M6.11_Cell Cycle	
377	21	6	1	1.59E−05	6.13E−04	LI.M4.7_mitotic cell cycle	
377	14	5	0	2.50E−05	9.11E−04	LI.M4.10_cell cycle (II)	*
377	97	11	3	7.04E−05	2.43E−03	DC.M5.3_Undetermined	
377	245	18	7	1.51E−04	4.97E−03	DC.M5.5_Undetermined	
377	59	8	2	1.98E−04	6.18E−03	DC.M4.14_Monocytes	
377	34	6	1	2.91E−04	8.68E−03	DC.M7.31_Undetermined	
377	14	4	0	4.53E−04	1.29E−02	LI.M173_erythrocyte differentiation	
377	26	5	1	6.26E−04	1.71E−02	DC.M8.26_Undetermined	
377	16	4	0	7.88E−04	2.07E−02	LI.M4.9_mitotic cell cycle in stimulated CD4 T cells	*
377	17	4	0	1.01E−03	2.45E−02	LI.M19_T cell differentiation (Th2)	
377	17	4	0	1.01E−03	2.45E−02	LI.M136_TBA	
377	33	5	1	1.93E−03	4.52E−02	LI.M10.0_E2F1 targets (Q3)	
377	10	3	0	2.14E−03	4.85E−02	LI.M4.14_Rho GTPase cycle	
377	11	3	0	2.89E−03	5.74E−02	LI.M4.15_enriched in monocytes (I)	
377	11	3	0	2.89E−03	5.74E−02	LI.M12_CD28 costimulation	
377	11	3	0	2.89E−03	5.74E−02	LI.M33_inflammatory response	
377	11	3	0	2.89E−03	5.74E−02	LI.M171_heme biosynthesis (I)	
377	12	3	0	3.77E−03	7.28E−02	LI.M4.11_mitotic cell cycle in stimulated CD4 T cells	
377	13	3	0	4.81E−03	9.01E−02	LI.M15_Ran mediated mitosis	

Hypergeometric p-values and overlap sizes for blood transcriptional modules enriched in shared IMRAS and BSPZV1 upregulated DEGs. BTMs enriched in the 14-gene protection associated subset are indicated by as asterisk (*)

individuals. In other words, GSEA was performed on sample sub-groups: one sub-group per study (BSPZV1 or IMRAS) per protection status per time interval (defined as for the DEG analysis), for a total of 10 sub-groups. This level of stratification was chosen to reveal all potential combinations of study- and protection-specific BTM responses. In contrast to DEG-based enrichment analysis, GSEA takes into account the rank expression level of all detectable genes in the transcriptome.

GSEA NES were calculated separately for each sub-group. Figure 2a shows GSEA NES for significant BTMs (FDR < 0.05) in at least 8 of the 10 total sub-groups. Hierarchical clustering revealed related BTM responses clustered closely together, showing similar time and protection-associated responses for functionally similar modules. Three common cross-study response groupings were apparent from hierarchical clustering, representing inflammatory/interferon responses: erythrocytes/myogenesis; cell cycle responses. Interferon-associated BTM

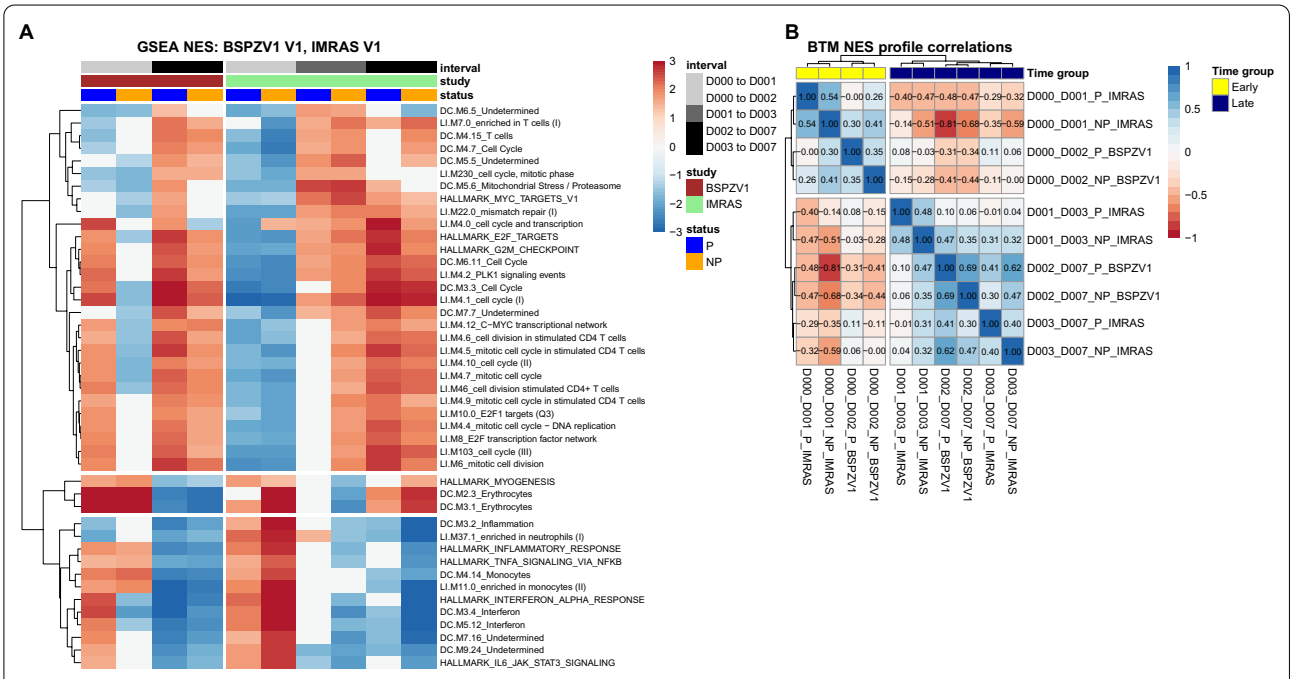

Fig. 2 GSEA reveals shared co-ordinated cross-study responses up to 1 week after 1st RAS vaccination. **A** Heatmap shows GSEA normalized enrichment scores (NES) for modules significantly enriched across at least 8 of 10 total time interval/protection groups. **B** Hierarchically clustered correlation matrix indicating Spearman correlations between GSEA NES scores for each time interval/protection group. Black rectangles indicate two largest hierarchical subclusters, corresponding to early (day 1 or 2) and late (day 3 or 7) responses

responses were increased in the day 0–1 (IMRAS) or day 0–2 (BSPZV1) time intervals after vaccination, while cell cycle responses were most increased in the day 2/3–7 time intervals. Protection-associated and trial-cohort specific differences were also apparent, with IMRAS NP subjects showing increased interferon responses relative to P in the day 0–1 interval, while these differences were not observed in BSPZV1 participants. In addition, BSPZV1 P subjects exhibited upregulated cell cycle responses in the day 0–2 interval that was not evident in BSPZV1 NP subjects. Induction of cell-cycle responses in IMRAS over the day 0–1 interval was not observed, and cell cycle responses between days 1 and 3 in IMRAS were primarily apparent in NP individuals.

Figure 2b shows correlations between BTM NES values per sub-group. The sub-groups have been arranged by hierarchical clustering, revealing two positively correlated cross-study response sub-groups, consisting of 'early' responses in the day 1 or 2 intervals post vaccination for P and NP subjects in both BSPZV1 and IMRAS; and 'late' responses in the day 7 intervals post vaccination. Importantly, response profiles from both IMRAS and BSPZV1, and from P and NP subjects clustered together by time, suggesting a shared temporal response program after RAS vaccination for both IMRAS and BSPZV1.

Temporal modelling reveals time dynamics of cross study BTM responses

Given the observed overlap in gene and BTM responses between IMRAS and BSPZV1, it was hypothesized that a shared transcriptional response may be elicited by PfRAS in both trials in the days after PfRAS vaccination. However, the differing timepoints measured in IMRAS and BSPZV1 complicated direct day-by-day comparisons. Therefore, to directly explore time dynamics underlying cross-study BTM responses in IMRAS and BSPZV1, continuous spline curves were fit to averaged BTM responses. Samples from P and NP subjects from both IMRAS and BSPZV1 were combined, and BTM responses were calculated as the average of BTM gene expression, relative to the day of vaccination. The 99% CIs were calculated, and deviation of the CI from the zero-response line was used to identify significantly responsive BTMs. For significant BTMs, the time associated with the maximum response was used to classify modules into those whose response peaks at day 7 or those with a peak response reached by day 3. 42 BTMs showed significant responses (Fig. 3a), with the 9 most strongly changed BTMs also shown in Fig. 3b of which 39 increased and 3 decreased in expression post vaccination. The majority of BTMs reached their maximum response by 2 or 3 days post vaccination, with the exception being BTMs associated with cell-cycle processes, such as 'LI.

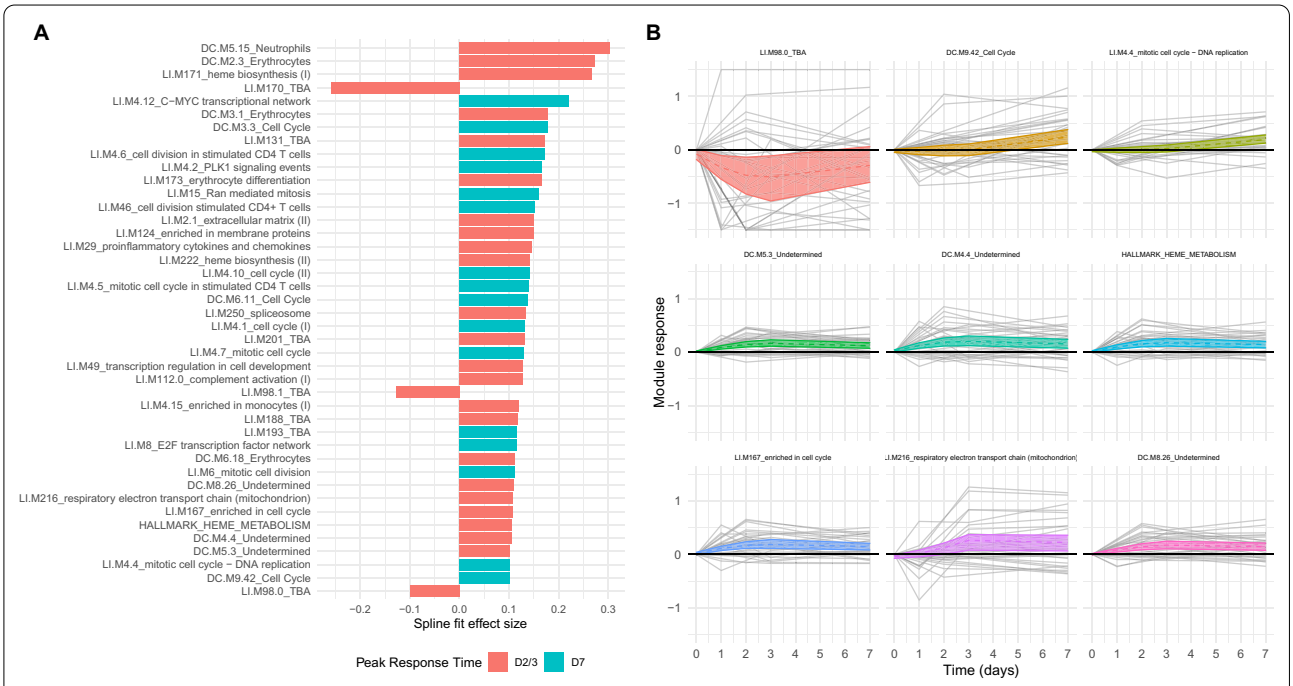

Fig. 3 Temporal modelling reveals time dynamics of cross study module response. **A** Bar plot of module response effect sizes, i.e. maximum separation of spline fit 99% CI range from average day 0 level. Colors indicate sample day closest to maximum module response. All modules with absolute effect sizes > 0.1 shown. **B** Top 9 module response spline fits (dashed line) with 99% CIs (ribbon). Individual subject module responses from IMRAS and BSPZV1 are shown as light grey lines

M4.12_C-MYC transcriptional network' and 'DC.M3.3_Cell Cycle.'

Overall, there was very strong overlap between BTMs which were identified as significantly responsive by three orthogonal approaches, i.e., enrichment in DEGs, GSEA and temporal modelling. Additional file 2: Fig. S1 shows a proportional Venn diagram indicating the overlap in BTMs identified by each approach. For each of the three approaches: Mixed Model DEG enrichment, GSEA, and Curve fitting, at least half the modules revealed by any one approach were also identified by one or more alternate approaches, e.g. of the 44 BTMs identified by GSEA, 22 were also identified by DEG enrichment or curve fitting approaches, or both. A core set of 11 BTMs were identified by all three approaches (Additional file 2: Fig. S1), principally consisting of cell cycle related BTMs, one of which was specifically associated with mitotic cell cycle in stimulated CD4 T cells.

Study specific responses correlate with protection and include baseline expression differences

GSEA analysis (Fig. 2) revealed gene sets responsive to vaccination in both studies and study-specific protection-associated differences between IMRAS and BSPZV1. Interferon and inflammatory responses were increased in IMRAS NP vs IMRAS P in the day 0 to 1 interval, while cell cycle responses were stronger in BSPZV1 P compared with BSPZV1 NP in the day 2 to 7 interval. Since both the shared DEG and curve fitting analysis were aimed at identifying shared cross-study responses, they could not have detected any study-specific differences. Six interferon, inflammatory and cell-cycle modules were selected from Fig. 2, and the average BTM expression was calculated (Fig. 4), showing distinct study-specific patterns. BTMs associated with inflammation and neutrophil signalling (Fig. 4a, b), were consistently more highly expressed in IMRAS compared with BSPZV1 over the entire study period. In contrast, interferon response BTMs (Fig. 4c, d) did not show stark differences at day 0; however, responses were specifically increased in IMRAS NP vs IMRAS P and BSPZV1 P and NP subjects. For cell-cycle associated modules (Fig. 4e, f), both BSPZV1 P and NP subjects showed higher expression at baseline relative to IMRAS; however, BSPZV1 NP individuals, uniquely, did not exhibit further increased cell cycle responses at any point after vaccination.

Overall, synthesizing information from each approach suggests a model for responses consistent with protection after PfRAS vaccination, incorporating a moderate early interferon response peaking 2 days post vaccination followed by a later proliferative cell cycle response steadily increasing over the first 7 days post vaccination (Fig. 5). Non-protection is associated with deviations

from this model, observed in this study with over-induction of early interferon responses in IMRAS and failure to mount a cell cycle response in BSPZV1.

Discussion

Human malaria correlates of protection/response to PfRAS vaccination have been difficult to identify. This may be due to the mechanisms of PfRAS-mediated protection because PfRAS vaccination results in a truncated liver stage parasite development, with no subsequent blood stage. Presumably, key protective immune processes happen at the site of infection, in the liver, where direct measures of responses are limited. This study represents the first time, to the authors' knowledge, that common transcriptional correlates of RAS vaccination have been identified in multiple cohorts. This was done via analysis of whole blood RNAseq after vaccination, which reflects systemic immune responses.

The time-points examined here, up to 1 week post vaccination, were too early to capture the adaptive immune response to vaccination. Therefore, it is likely that the shared response identified here represents an effective innate immune response capable of presenting antigen and kickstarting the adaptive response. Whole blood RNAseq can detect systemic responses to PfRAS vaccination but is limited by the fact that sporozoite antigen specific cells represent a small minority of circulating blood cells. Additionally, whole blood RNAseq reflects the transcriptional state of every circulating leukocyte, and it is not possible to definitively link changes in expression to immune cell populations. Neither can it be determined whether changes in transcription reflect expansions or decreases in numbers of immune cells, or changes in transcriptional state of specific cell types. Despite these limitations, modules identified suggest an initial innate immune response followed by upregulation of cell cycle BTMs which may reflect rapid innate detection of PfRAS, followed by immune cell activation and proliferation. That the majority of the 14 shared protection associated genes identified were also associated with innate immunity or cell cycle suggests that deviations from the coordinated cross-study response programme are associated with non-protection. Recent work by Tran et al. [27] on whole sporozoite vaccination by administration of infectious sporozoites under chloroquine prophylaxis observed changes in T-cell associated BTMs in protected individuals 3 weeks after initial vaccination. This suggests that an extended series of cross-study comparable time-points may reveal adaptive immune associated responses. Unlike this study, they did not see significant changes in BTM expression within 1 week, however their study design did not include any sampling within 1–5 days post vaccination. They did observe increase of inflammatory/interferon and DC activation processes specifically in non-protected individuals 9 days after the third CPS immunization. Despite the difference in timescale, this shares some similarity with the IMRAS study, where over-induction of interferon is associated with non-protection. Recent work in a mouse model also supports the hypothesis that excessive type-I interferon inhibits the production of malaria-specific IFNγ producing CD8+ T cells [13]. In contrast, transcriptional profiling of RTS,S vaccinees revealed multiple BTMs correlated with immunogenicity and protection within 1 week of vaccination, including cell cycle and inflammatory response BTMs [28]. In contrast to the results seen after whole-sporozoite immunization, expression of interferon-associated and inflammatory-associated genes was positively associated with protection after RTS,S vaccination, and this is especially evident during the week after the second immunization. This may reflect differing pathways to protection induced by whole-sporozoite and subunit vaccines.

Stark differences in protective efficacy were observed between IMRAS, where 55% of study subjects were protected after intentional suboptimal immunization, and BSPZV1 where only 20% of the subjects were protected from CHMI. Although 20% of BSPZV1 subjects overall were protected, the RNAseq analysis was performed on a subset of BSPZV1 participants in which 36% were protected (Table 1). Unlike vaccination with PfRAS, it has long been observed that repeated natural malaria infection does not lead to sterilizing immunity [29–34]. Reasons for this are unclear; however, it may be associated with low doses of sporozoites delivered in natural infections or active immune evasion strategies mounted by the parasite. Indeed, it has been observed that blood stage malaria inhibits or actively dysregulates the development of effective CD8+ T-cell and antibody-mediated liver stage immunity [35, 36]. Previous immune exposure may be a factor in the observed reduction in protective efficacy of PfRAS vaccination in BSPZV1 vs IMRAS. Thus, it is plausible that the functional pathways leading to antigen presentation and adaptive immune priming may operate using different mechanisms in both trials.

(See figure on next page.)

Fig. 4 Protection associated responses differ between studies after vaccination and at baseline. **A**–**F** Average BTM responses for modules differentially expressed between studies at day 0 (**A**, **B**), associated with protection in IMRAS (**C**, **D**) or BSPZV1 (**E**, **F**)

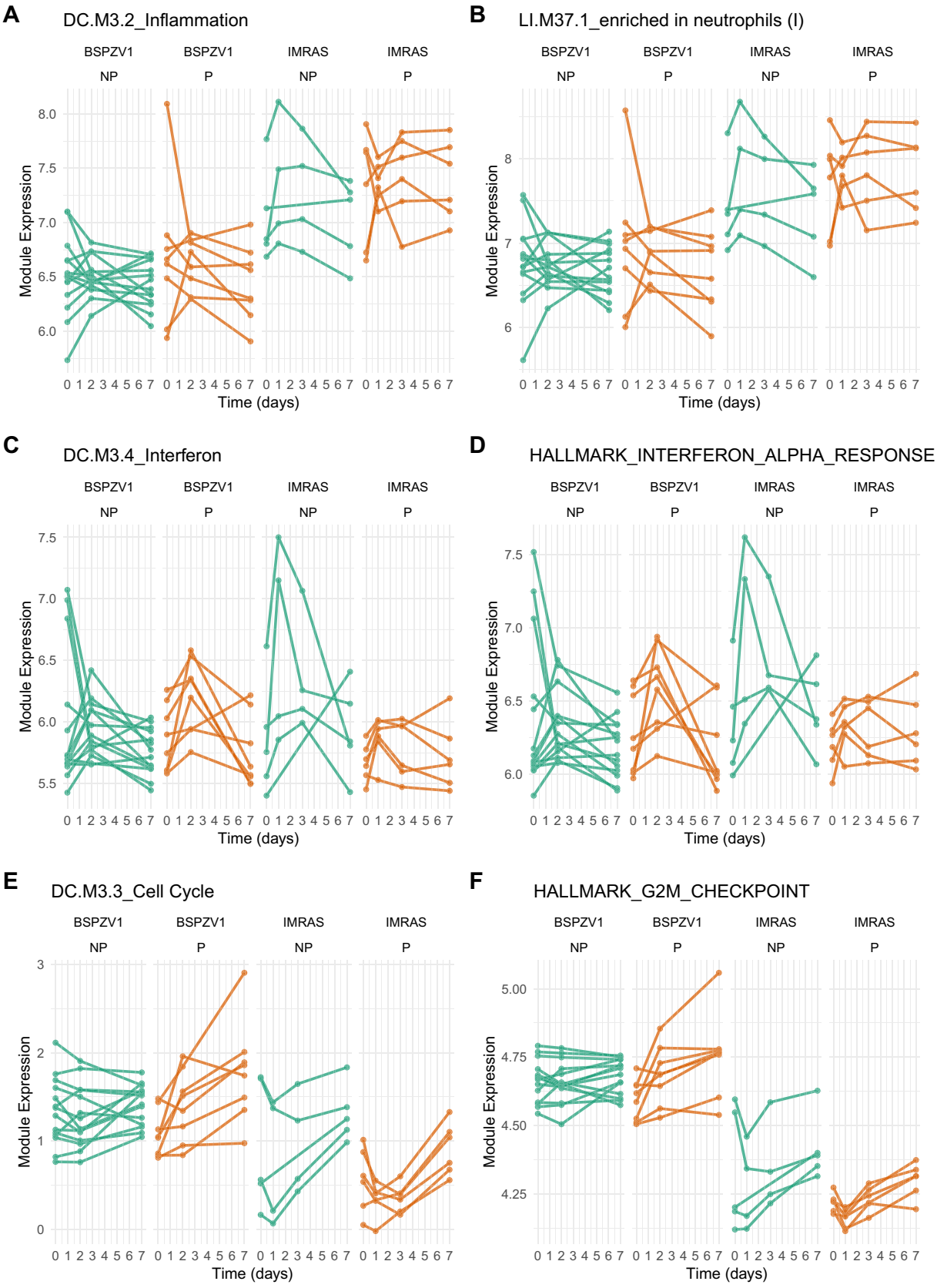

Fig. 4 (See legend on previous page.)

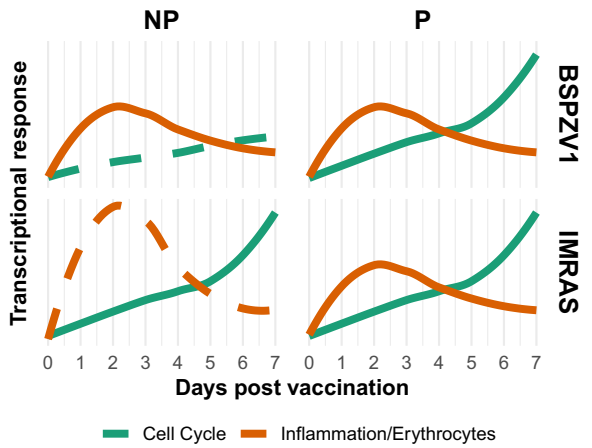

Fig. 5 Proposed model for cross-study RAS responses. Line plots illustrate responses common to protected individuals in IMRAS and BSPZV1, along with study-specific deviations from these responses in non-protected individuals

In other words, the different delivery method, dose, and previous malaria experience of BSPZV1 participants may have led to a qualitatively different, or non-naive, immune response to PfRAS in BSPZV1 compared to that observed in IMRAS. Therefore, true correlates of protection may exist that are distinct to both studies and would not be captured by this joint analysis.

Host intrinsic factors, e.g., genetic differences, and extrinsic factors, e.g., co-morbidities, microbiome, and general immune status, could also have contributed to the differences between IMRAS and BSPZV1. However, reduced levels of PfRAS-induced protection have been previously observed in malaria-experienced vs malaria-naive adults [9] suggesting that this issue is not specific to these two study cohorts. Intriguingly, IMRAS showed increased expression of inflammation associated BTMs pre-immunization, compared with BSPZV1. This may influence the induction of inflammatory responses post RAS vaccination, and a more effective initial innate response may partially explain better protection in IMRAS.

However, other differences between the two trials are likely to be associated with protection status. IMRAS participants received PfRAS vaccination and PfSPZ infection via mosquito bite, while BSPZV1 used cryopreserved parasites administered intravenously. A comparison of protection vs non-protection does not capture any potential immune response to mosquito bites, independent from PfRAS, in the IMRAS cohort. Previous work suggest that mosquito saliva can affect T cell and NK cell populations potentially up to 7 days post-bite [37]. The doses of PfRAS are very difficult to compare between trials, as IMRAS PfRAS doses were measured in terms of numbers of bites from infected irradiated mosquitos (~200 per vaccination), while BSPZV1 injected precise numbers of cryopreserved PfRAS. Another potential consequence of cryopreservation may be reduced PfRAS viability. Additionally, cryopreserved parasites were injected directly into the circulation while mosquito bites deliver PfRAS into the skin. Altogether, these differences would have resulted in differences in the number of hepatocytes that were infected by PfRAS during vaccination, and the number of PfRAS cleared by the innate immune system without reaching the liver.

While this study revealed common and distinct differences in whole blood gene expression patterns that correlate with protection and non-protection between these two studies, there are limitations to this analysis. This work relied on two study cohorts, with small numbers of study participants, comprising a total of 33 individuals to draw our conclusions. In addition, this analysis is limited to systemic responses the time period shortly following prime RAS vaccination. While consistent cross-study responses were identified, differing mechanisms of non-protection in each study were observed that would ideally be validated by further RAS cohorts. To validate these observations, it would be expected that malaria-naive RAS cohorts behave similarly to IMRAS and malaria experienced cohorts behave similarly to BSPZV1.

Conclusion

This work has produced a conceptual model of an innate immune response programme consistent with PfRAS-induced protection, based on cross-study responses in two diverse cohorts. These responses are evident early after PfRAS primary vaccination and may serve as correlates of efficacy for future attenuated sporozoite vaccine candidates. Future work will comprise characterization of cell phenotypic changes over the course of vaccination, identifying the cell types responsible for the transcriptional changes seen here and explore adaptive immune responses to identify antibody and T cell responses that mediate sterilizing immunity.

Abbreviations

RAS: Radiation-attenuated sporozoites; PfRAS: *Plasmodium falciparum* Radiation attenuated sporozoites; CHMI: Controlled human malaria infection; BTM: Blood transcriptional module; P: Protected; NP: Non-protected; DEG: Differentially expressed gene; NES: Normalized enrichment score; GSEA: Gene-set enrichment analysis; CI: Confidence interval; DVI: Direct venous injection; IMRAS: IMmunization by mosquito bite with Radiation Attenuated Sporozoites study; BSPZVI: Bagamoyo Sporozoite Vaccine 1 Study; DEG: Differentially Expressed Gene; UP: Up; DN: Down; NC: No change.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12936-021-03839-3.

Additional file 1: Table S1. Shared protection genes in IMRAS and BSPZV1, and associated BTMs.

Additional file 2: Figure S1. Intersection of modular approaches reveals core responsive modules. Proportional venn diagram shows the overlap of significant response modules identified by spline-curve fitting, GSEA, and hypergeometric tests for BTM enrichment in individual response genes identified by mixed modelling.

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JEE was an active duty military personnel at the time she contributed to this work. The work was prepared as part of official government duties. Title 17 U.S.C. 1105 provides that 'Copyright protection under this title is not available for any work of the United States Government.' Title 17 U.S.C. 1101 defines a U.S. Government work as a work prepared by a military service member or employee of the U.S. Government as part of that person's official duties.

Authors' contributions
Conceptualization and funding: KS, CD, JEE, JA. Data analysis: FD, YD. Sequencing: JC. PfRAS Cohort studies: JEE, SA, SJ, MM, CD, SH. Writing original draft: FD, KS. Writing final manuscript: All authors. All authors read and approved the final manuscript.

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Availability of data and materials
All sequencing data analysed in this paper will be made available through the ImmPort portal (immport.org).

Declarations

Ethics approval and consent to participate
The IMRAS study was conducted at the Naval Medical Research Center (NMRC) Clinical Trials Center from 2014 to 2016; the CHMIs were conducted at the Walter Reed Army Institute of Research (WRAIR) secure insectary. The study protocol was reviewed and approved by the NMRC Institutional Review Board in compliance with all federal regulations governing the protection ofhuman subjects. WRAIR holds a Federalwide Assurance from the Office of Human Research Protections (OHRP) under the Department of Health and Human Services as does NMRC. NMRC also holds a Department of Defense/Department of the Navy Federal- wide Assurance for human subject protections. All key personnel were certified as having completed mandatory human subjects' protection curricula and training under the direction of the WRAIR Institutional Review Board or the NMRC Office of Research Administration (ORA) and Human Subjects Protections Branch (HSPB). All potential study subjects provided written, informed consent before screening and enrollment and had to pass an assessment of understanding. This study was conducted according to the Declaration of Helsinki as well as principles of Good Clinical Practices under the United States Food and Drug Administration Investigational New Drug (IND) application BB-15767. This trial was performed under an IND allowance by the Food and Drug Administration (FDA) and was registered on Clinical-Trials.gov (NCT01994525).The BSPZV1 trial was conducted in Bagamoyo, Tanzania between April 2014 and August 2015 in accordance with Good Clinical Practices. All study participants had to complete a 20-question assessment of trial understanding with a 100% correct response rate on the first or second attempt to be eligible. The protocol was approved by institutional review boards (IRBs) of the Ifakara Health Institute (IHI) (Ref. No. IHI/IRB/No:02-2014), the National Institute for Medical Research Tanzania (NIMR/HQ/R.8a/ Vol.IX/1691), the Ethikkommission Nordwest-und Zentral- schweiz, Basel, Switzerland (Reference number 261/13), and by the Tanzania Food and Drug Authority (Ref. No. TFDA 13/CTR/ 0003); registered at Clinical Trials.gov (NCT02132299); and conducted under U.S. FDA IND application.

Consent for publication
All authors have seen and approved the content of this manuscript.

Competing interests
The authors declare that no competing interests exist.

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Publisher's Note

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Activation of TCR Vδ1$^+$ and Vδ1$^-$Vδ2$^-$ γδ T Cells upon Controlled Infection with *Plasmodium falciparum* in Tanzanian Volunteers

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Our understanding of the human immune response to malaria remains incomplete. Clinical trials using whole-sporozoite-based vaccination approaches such as the Sanaria PfSPZ Vaccine, followed by controlled human malaria infection (CHMI) to assess vaccine efficacy offer a unique opportunity to study the immune response during *Plasmodium falciparum* infection. Diverse populations of T cells that are not restricted to classical HLA (unconventional T cells) participate in the host response during *Plasmodium* infection. Although several populations of unconventional T cells exist, the majority of studies focused on TCR Vγ9Vδ2 cells, the most abundant TCR γδ cell population in peripheral blood. In this study, we dissected the response of three TCR γδ cell subsets and mucosal-associated invariant T cells in healthy volunteers immunized with PfSPZ Vaccine and challenged by CHMI using Sanaria PfSPZ Challenge. Using a flow cytometry-based unbiased analysis followed by T cell cloning, several findings were made. Whereas major ex vivo alterations were not detectable after immunization with PfSPZ Vaccine, TCR Vδ2, and mucosal-associated invariant T cells expanded after asexual blood-stage parasitemia induced by CHMI. CHMI, but not vaccination, also induced the activation of TCR Vδ1 and Vδ1$^-$Vδ2$^-$ γδ T cells. The activated TCR Vδ1 cells were oligoclonal, suggesting clonal expansion, and upon repeated CHMI, showed diminished response, indicating long-term alterations induced by blood-stage parasitemia. Some TCR Vδ1 clones recognized target cells in the absence of parasite-derived Ags, thus suggesting recognition of self-molecules. These findings reveal the articulate participation of different populations of unconventional T cells to *P. falciparum* infection. *The Journal of Immunology*, 2020, 204: 180–191.

Compared with the year 2010, global malaria disease burden has decreased significantly. In recent years, however, this reduction has stalled and, in some regions, even reversed. In 2017, 219 million cases of malaria were reported, leading to 435,000 deaths worldwide (1).

A valuable tool to fight malaria would be an effective vaccine that leads to long-lasting protection. RTS,S, the most advanced malaria vaccine, received a positive scientific opinion under Article 58 from the European Medicines Agency and is being further assessed in pilot implementation programs in Ghana, Kenya, and Malawi, starting in 2019 (2). However, RTS,S/AS01 provides only partial protection against clinical malaria episodes in African children and infants (3). Recently, Sanaria PfSPZ Vaccine, which is composed of aseptic, purified, cryopreserved, metabolically active, whole, live, irradiation-attenuated *Plasmodium falciparum* sporozoites (PfSPZ), has led to more promising results in European and United States volunteers (4, 5). Results from clinical trials in malaria pre-exposed volunteers from sub-Saharan Africa indicate significantly lower immunogenicity than achieved in European or United States volunteers using similar vaccination regimen (6–9). The reasons for the differences in immunogenicity and protective efficacy remain obscure, demonstrating our incomplete understanding of the interactions between malaria pre-exposure, malaria vaccination, and vaccination outcome.

The human immune response to *Plasmodium* spp. is parasite-stage dependent and involves myeloid cells, NK cells, B cells, and T cells (10). Many studies of T cell biology in human malaria have focused on conventional CD4$^+$ and CD8$^+$ T cells bearing a TCR

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Abbreviations used in this article: CHMI, controlled human malaria infection; DBSCAN, density-based spatial clustering of applications with noise; MAIT, mucosal-associated invariant T; Mo-DC, monocyte-derived dendritic cell; PfRBC, *P. falciparum*–infected erythrocyte; PfSPZ, metabolically active, whole, live, irradiation-attenuated *Plasmodium falciparum* sporozoite; T_{EE}, early effector T; *t*-SNE, *t*–distributed stochastic neighbor embedding; uRBC, uninfected erythrocyte.

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αβ (10). However, apart from Ab responses (11), no clear correlate of protection either under natural conditions or upon vaccination has been identified (4).

Expansion of TCR γδ T cells upon asexual erythrocytic stage *P. falciparum* infection has been observed in several settings (12–17). In a population of adult malaria patients in Thailand, both Vδ1$^+$ and Vδ1$^-$ γδ T cells were found to be expanded after treatment, compared with the day of admission (15). In contrast, an increase in TCR γδ cells observed in Ethiopian malaria patients was mainly attributed to an increase in Vδ1 T cells, but not Vγ9Vδ2 T cells (16). Similar findings were made in a study in Ghanaian children, where an increase of γδ T cells after treatment for malaria was found, an effect that was mainly attributed to expansion of TCR Vδ1 cells. However, length spectratyping of the CDR3δ of these TCR Vδ1 cells did not reveal a dominant public clonotype to be expanded across all patients in a cross-sectional analysis (17).

TCR Vγ9Vδ2 cells are the most abundant TCR γδ cell population in human peripheral blood and are activated in the presence of small, phosphorylated compounds, including *P. falciparum*–derived Ags, leading to expansion of TCR Vγ9Vδ2 cells in response to *P. falciparum* both in vitro and in vivo (18–21). In addition, T cells expressing the TCR Vγ9Vδ2 present at the time of immunization and after immunization have been suggested to be a possible correlate of protection after PfSPZ Vaccine administration (4, 22).

Mucosal-associated invariant T (MAIT) cells, a second population of unconventional T cells, expand after treatment for asexual erythrocytic stage infection induced by controlled human malaria infection (CHMI) (23). MAIT cells recognize microbial-derived riboflavin metabolites presented by the MHC class I–like molecule MR1 (24) and express semi-invariant TCRs αβ and high levels of CD161.

Taken together, the involvement of unconventional T cell subsets in human malaria infection is evident. However, the diversity of study designs and experimental approaches taken as well as the differences in the examined study populations make it difficult to reach a clear understanding of their functions and contributions to malaria immunity.

To overcome these gaps, we made use of the highly controlled setting of the PfSPZ Vaccine studies BSPZV1 (8) (ClinicalTrials.gov identifier: NCT02132299) and BSPZV2 (25) (ClinicalTrials.gov identifier: NCT02613520) in Tanzania, which included CHMI to assess vaccine efficacy. PBMC collected longitudinally during the study were analyzed by two multicolor flow cytometry panels in combination with an unbiased analysis pipeline to dissect the responses of three major TCR γδ cell subsets and MAIT cells to immunization with PfSPZ Vaccine and CHMI. Our results showed that these T cells are distinctly activated after erythrocytic stage parasitemia in malaria pre-exposed volunteers. We also found that TCR Vδ1 cells show hallmarks of adaptive-like T cells associated with expansion of distinct TCR clonotypes in a donor-dependent manner.

Materials and Methods

Cells and cell culture

The HC-04 human hepatocyte cell line was obtained from BEI Resources. The human cell lines THP-1 (monocytic leukemia) and HeLa (cervical cancer) were obtained from American Type Culture Collection.

PBMC were isolated from participants in the BSPZV1 clinical trial (ClinicalTrials.gov identifier: NCT02132299). Details are published in Jongo et al. (8). All participants were healthy, male Tanzanians between 18 and 35 y of age. Samples analyzed in this study were derived from the high-dose group that received 2.7×10^5 PfSPZ of PfSPZ Vaccine per injection. CHMI consisted of 3200 PfSPZ of PfSPZ Challenge administered by direct venous inoculation. The second sample set that was analyzed was from the BSPZV2 clinical trial (25) (ClinicalTrials.gov identifier: NCT02613520). From this study, analyzed samples were derived from vaccinated individuals from groups 1a and 1b, who received three times 9×10^5 or three times 1.8×10^6 PfSPZ Vaccine, respectively. Details about the donors are published in Jongo et al. (25).

PBMC were isolated by density gradient centrifugation and were cryopreserved in 90% FCS with 10% DMSO. The cells were stored and transported in liquid nitrogen vapor phase until their usage in the assays.

Monocytes were isolated using a Human CD14 Positive Selection Kit (STEMCELL Technologies) according to the manufacturer's instructions. Monocyte-derived dendritic cells (Mo-DC) were generated by culturing CD14-positively selected monocytes in the presence of human rIL-4 and GM-CSF (BioLegend) for 5 d. Differentiation was controlled by cell surface staining of CD209 (DC-SIGN).

T cell lines and clones were established as previously described (26).

Flow cytometry and Abs

Flow cytometry staining, analyses, and cell sorting were performed using standard protocols. Data were acquired using an LSR II Fortessa (BD Biosciences). The following Abs were obtained from BioLegend: CCR6 (fluorochrome BV421, clone G034E3), CCR7 (APC/Cy7, G043H7), CD3 (BV650, OKT3), CD4 (Alexa 700, OKT4), CD27 (BV785, O323), CD28 (Alexa 700, CD28.2), CD38 (APC/Cy7, HB-7), CD57 (PE/dazzle, HNK-1), CD69 (PE/Dazzle, HNK-1), CD94 (PerCP/Cy5.5, DX22), CD161 (BV605, HP-3G10), CD209 (PE, 9E9A8), CD294 (BV421, BM16), HLA-DR (Alexy700, L243), KLRG1 (APC, SA231A2), NKp80 (APC, 5D12), pan-γδ (PE, B1), PD-1 (BV785, EH12.2H7), TCR Vα7.2 (BV510, 3C10), and TCR Vδ2 (BV711, B6). Anti–LAG-3 (PE/Cy7, 3DS223H) and LILRB1 (PE/Cy7, GHI/75) were obtained from eBioscience, TCR Vδ1 (FITC, TS8.2) from GeneTex, and CD8 (BUV496, RPA-T8) from BD Biosciences. Cell proliferation was assessed in vitro using the CellTrace Violet Proliferation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Data analysis and software

Standard flow cytometry analyses were performed using FlowJo (Tree Star). Dimensionality reduction and unsupervised clustering was performed using a custom R script. Briefly, flow cytometry data were gated on live, single cells, and the subsets of interest (MAIT, TCR Vδ1, TCR Vδ2, or TCR Vδx cells) were exported separately from FlowJo and imported into R. Fluorescent parameters were transformed using an individually centered inverse hyperbolic sine function for each fluorochrome. The *t*–distributed stochastic neighbor embedding (*t*-SNE) dimensionality reduction (27) was performed using the Rtsne package (28). Clustering was performed by multiple rounds of density-based spatial clustering of applications with noise (DBSCAN) with custom parameters using the DBSCAN package (29) in R. Then, cell frequencies for each cluster were calculated for each sample and statistical analyses performed. Cluster frequencies between visits were compared using Wilcoxon signed-rank test adjusted for multiple comparisons using the Benjamini–Hochberg method. Additionally, only clusters with a fold change of the mean >2.5 were considered to be significant. To assess vaccination-induced changes, only the vaccinated volunteers were analyzed ($n = 20$), and to assess CHMI-induced changes, only the volunteers who developed blood-stage parasitemia upon CHMI were included ($n = 20$).

For the clonality analysis, the D50 value was defined as the number of distinct clonotypes necessary to account for 50% of the total cells within one T cell population. This value was then normalized by the total number of cells per population.

Tree maps were constructed using the R treemapify package (30).

T cell activation assays

In T cell activation assays with cell lines, 5×10^4 T cells were cocultured with 5×10^4 stimulatory cells in a final volume of 200 μl. Adherent cells were plated and allowed to adhere for 2 h before addition of T cells. T cells and stimulatory cells were cocultured for 16 h.

For proliferation assays, 2×10^5 Mo-DC were cocultured with 1×10^6 PBMC and 2×10^5 uninfected erythrocytes (uRBC) or *P. falciparum*–infected erythrocytes (PfRBC) in a final volume of 2 ml, and proliferation was assessed after 6 d. Activation of T cell clones was performed by coculture of 5×10^4 Mo-DC and 5×10^4 uRBC or PfRBC in a final volume of 200 μl. For the screening of the T cell clones, T cells were not counted.

Single-cell sorting and Vδ1 CDR3 sequencing

Sorting and CDR3δ sequencing were performed as previously described (31). CD38$^+$PD-1$^+$ or CD38$^-$PD-1$^-$ TCR Vδ1 cells were single-cell sorted

into 96-well plates containing 2 μl of SuperScript IV VILO Master Mix (Invitrogen) using a FACSAria (BD Biosciences). The CDR3δ was amplified by nested PCR with the primers as previously described (31) and sequenced using Sanger sequencing (Microsynth).

P. falciparum *blood-stage parasite enrichment*

P. falciparum parasites strain NF54 from fresh cultures were enriched using MACS magnetic columns as published (32). Briefly, fresh parasite cultures were passed on top of the MACS magnetic column and eluted using RMPI 1640 after removing the column from the magnetic stand. Enrichment of infected erythrocytes was assessed by Giemsa staining. Typical parasitemia after enrichment was ~90%.

Results

TCR Vδ2 cells and MAIT cells expand significantly upon asexual blood-stage parasitemia

We assessed ex vivo frequencies of four groups of unconventional T cells before and after immunization with PfSPZ Vaccine and after CHMI with PfSPZ Challenge that consists of the same strain of *P. falciparum* (NF54) as PfSPZ Vaccine (Fig. 1A). Cell frequencies were analyzed by multicolor flow cytometry, focusing on a group of 24 adult, male, healthy Tanzanian volunteers who received five doses of 2.7×10^5 PfSPZ Vaccine during the BSPZV1 study. We grouped the TCR γδ cells according to expression of the TCRδ chain into TCR Vδ1, TCR Vδ2 T cells, and TCR $Vδ1^-Vδ2^-$ (Vδx) cells. MAIT cells were identified by the expression of TCR Vα7.2 and high levels of CD161. Between the baseline visit and 2 wk after the fifth vaccination, there were no significant changes in the frequencies of unconventional T cells in peripheral blood (Supplemental Fig. 1A). However, in PBMC collected 28 d after initiation of CHMI, TCR Vδ2 cells and MAIT cells were markedly expanded in the volunteers who developed asexual blood-stage parasitemia (infected individuals), but not in the participants who were protected from blood-stage parasitemia (protected individuals). In contrast, the frequencies of TCR Vδ1 cells and TCR Vδx cells were not significantly altered after CHMI in any group of volunteers (Supplemental Fig. 1B).

TCR Vδ1 and Vδx cells are distinctly activated upon asexual blood-stage parasitemia

Next, we examined the activation state of each T cell subset after immunization with PfSPZ Vaccine and after CHMI by PfSPZ Challenge ex vivo. To this purpose, we designed a multicolor flow cytometry panel (Supplemental Table I, panel 1) that, in addition to Abs identifying TCR γδ cells and MAIT cells, included Abs targeting NK receptors as well as markers for lymphocyte activation and exhaustion (CD38, CD69, CD94, CD161, HLA-DR, LAG-3, NKp80, and PD-1). We used an unbiased approach to identify significant phenotypic changes upon vaccination and CHMI. First, to reduce the high-dimensional flow cytometry data to two dimensions, we used the *t*-SNE algorithm (27). Then, clusters of phenotypically similar T cells were identified using the clustering algorithm DBSCAN (33), and the cell frequency within each cluster was calculated. Finally, statistically significant alterations in cell frequency upon vaccination and CHMI were identified. Consistent with the absence of expansion upon immunization with PfSPZ Vaccine (Supplemental Fig. 1), we did not observe significant vaccination-induced phenotypic changes in any T cell subset (Fig. 1B, Supplemental Figs. 2A, 3A, 4A). However, in infected individuals post-CHMI, several clusters of T cells were significantly increased in all T cell subsets. These alterations were not observed in the protected volunteers. The most prominent common feature of all TCR γδ cells and MAIT cells was an increase of CD38 expression in infected volunteers after CHMI, an effect that was not observed in non-γδ, non-MAIT cells (Supplemental Fig. 5). A more detailed analysis revealed distinct patterns of surface marker expression in the different T cell subsets. Within TCR Vδ1 cells, we identified five clusters that were increased after CHMI (Fig. 1D). Notably, these clusters expanded across all 20 infected volunteers after CHMI, but not in the four protected volunteers (Fig. 1E). All clusters were negative for CD161, showed expression of CD38 and PD-1, and, in some cases, coexpressed LAG-3 or HLA-DR, indicative of strong in vivo activation upon erythrocytic stage parasitemia (Fig. 1F). In addition, the increase of $CD38^+PD\text{-}1^+$ expression on TCR Vδ1 cells after blood-stage parasitemia was positively correlated with the peak parasite density (Supplemental Fig. 6).

Among TCR Vδx cells, we identified two main groups of cells that were expanded in infected volunteers (Supplemental Fig. 2). One group was phenotypically similar to the expanded TCR Vδ1 cells, coexpressing CD38 and PD-1, but not CD161 (Supplemental Fig. 2E). The other group of cells expressed CD161 and CD38, but was negative for PD-1. Thus, the mutually exclusive expression of PD-1 and CD161 divided the Vδx subset into two main groups. The upregulation of $CD38^+PD\text{-}1^+$ in the Vδ1 and Vδx T cell subsets was in marked contrast with the phenotype of expanded TCR Vδ2 cells and MAIT cells, which were positive for CD38, but negative for PD-1 (Supplemental Figs. 3, 4).

We aimed to confirm the differences in frequency of $CD38^+PD\text{-}1^+$ T cells using conventional flow cytometry analysis based on the same dataset. Although there was considerable interdonor variability, TCR Vδ1 cells clearly showed the strongest expansion of $CD38^+PD\text{-}1^+$ cells upon blood-stage parasitemia (Fig. 2). Consistent with the *t*-SNE analysis, the Vδx T cell subset showed a lower, but still significant, increase in $CD38^+PD\text{-}1^+$ cells. In contrast, no significant increase of this phenotype was detectable in TCR Vδ2 cells and MAIT cells.

TCR Vδ1 and Vδx early effector cells expand after asexual erythrocytic stage parasitemia

To investigate the differentiation state of TCR γδ cells and MAIT cells during immunization with PfSPZ Vaccine and CHMI, we designed a second flow cytometry panel that included Abs specific for CD27, CD28, CD57, LILRB1, KLRG1, CCR6, and CCR7 (Supplemental Table I, panel 2). Using the same detailed analysis including dimensionality reduction and clustering, we confirmed that there are no significant ex vivo phenotypic alterations upon immunization with PfSPZ Vaccine (Fig. 3A, Supplemental Fig. 7). In contrast, in infected individuals post-CHMI, TCR Vδ1 cells showed an increased frequency of four clusters, defined by the lack of surface expression of CD27, CD28, CCR6, and CCR7, indicative of an effector phenotype (Fig. 3). Interestingly, the expanded cells were also negative for CD57, an Ag that is often present on late effector cells (34), in particular on chronically stimulated T cells that have reached replicative senescence (35, 36). The $CD27^-CD57^-$ TCR Vδ1 cells that expanded after blood-stage parasitemia could be further subdivided by the expression of KLRG1, a marker that is upregulated on effector cells differentiating into senescent cells (Fig. 3E). When KLRG1 and CD57 were measured on total $CD27^-$ TCR Vδ1 cells, both markers were found to be expressed on the large majority of cells (Supplemental Fig. 8A), confirming increased expression on Ag-experienced, late effector cells. Thus, the absence of CD27 and CD57 expression on the expanded T cells might identify a population of recently differentiated early effector T (T_{EE}) cells in contrast to chronically stimulated $CD27^-CD57^+$ terminal effector T cells. The expansion upon blood-stage parasitemia was specific to the $CD27^-CD57^-$ subset, as the frequency of $CD27^-CD57^+$ TCR

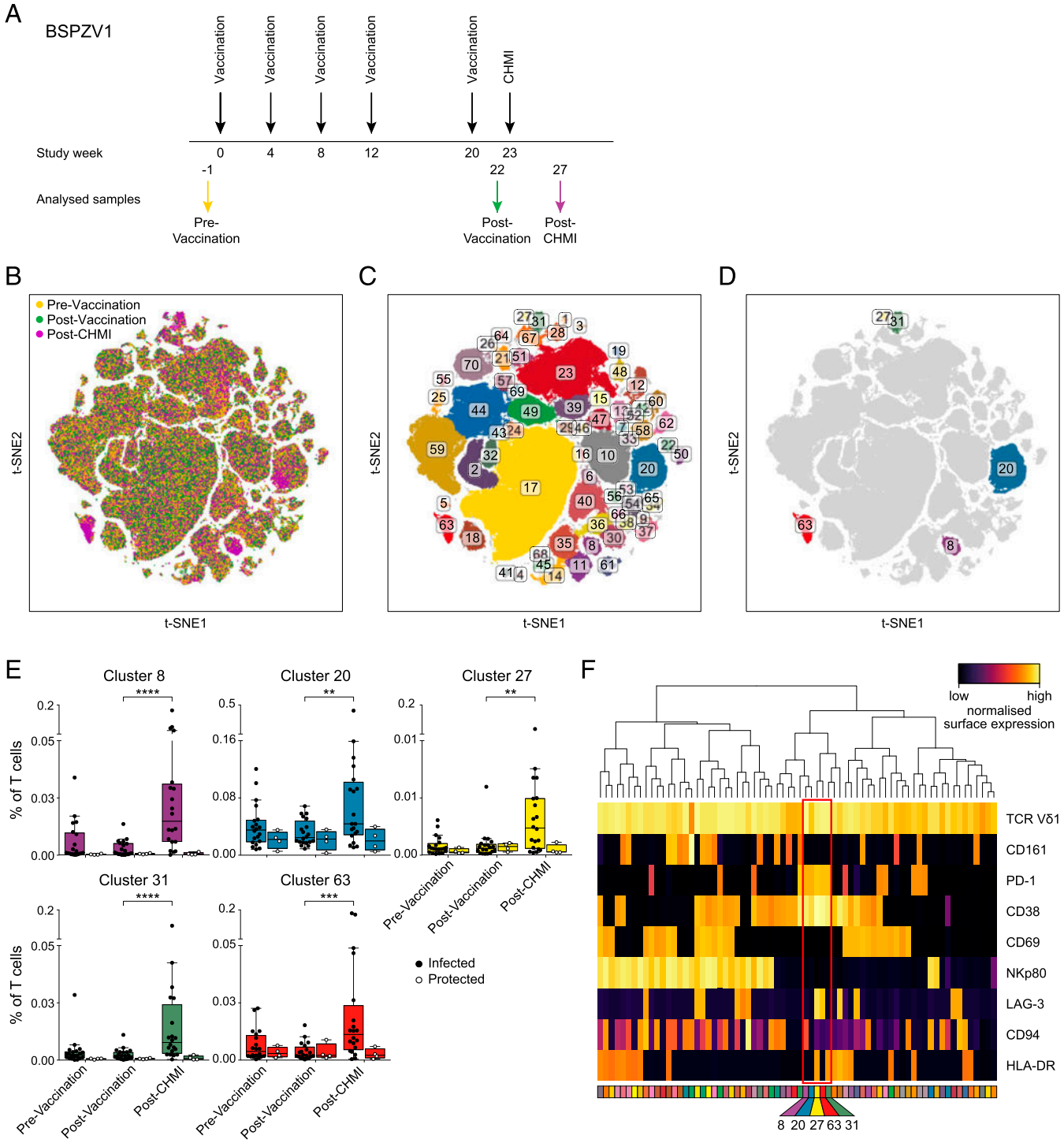

FIGURE 1. Subsets of Vδ1 T cells are activated after asexual blood-stage infection induced by PfSPZ Challenge. (**A**) Timeline of the clinical trial BSPZV1. (**B**) *t*-SNE plot of TCR Vδ1 cells from all volunteers color coded by visit: prevaccination (yellow), postvaccination (green), and post-CHMI (magenta). Regions with increased cell frequency after CHMI by PfSPZ Challenge appear in magenta ($n = 24$). (**C**) *t*-SNE plot after clustering by DBSCAN. Clusters are color coded and assigned numbers. (**D**) Clusters with significantly altered frequency in infected volunteers after CHMI are highlighted. Clusters are considered significant with $p < 0.01$ (Wilcoxon signed-rank test adjusted with the Benjamini–Hochberg method), and at least 2.5-fold change of the mean frequency across the 20 infected volunteers. (**E**) Frequency of the five clusters that are significantly increased in infected volunteers after CHMI. Infected volunteers (black dots) are separated from protected volunteers (white dots). Horizontal lines represent the median value, boxes span the interquartile range, and whiskers extend to the furthest point still within 1.5 interquartile ranges from the box. Infected, $n = 20$; protected, $n = 4$. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, Wilcoxon signed-rank test adjusted with the Benjamini–Hochberg method. (**F**) Heat map showing the median normalized surface expression of all clusters. The five significantly increased clusters are highlighted with a red frame and labeled with their cluster identifications.

Vδ1 cells remained unchanged during the course of the CHMI (Supplemental Fig. 8B).

An increase in the frequency of T_{EE} cells in infected volunteers was detected in TCR Vδ1 and Vδx cells, but not in Vδ2 or MAIT cells (Fig. 4). This finding is reminiscent of the increased expression of $CD38^{+}PD\text{-}1^{+}$ that occurred only on TCR Vδ1 and Vδx cells (see Fig. 2). Indeed, the increase in frequency of these two phenotypes upon CHMI correlated significantly (Supplemental Fig.

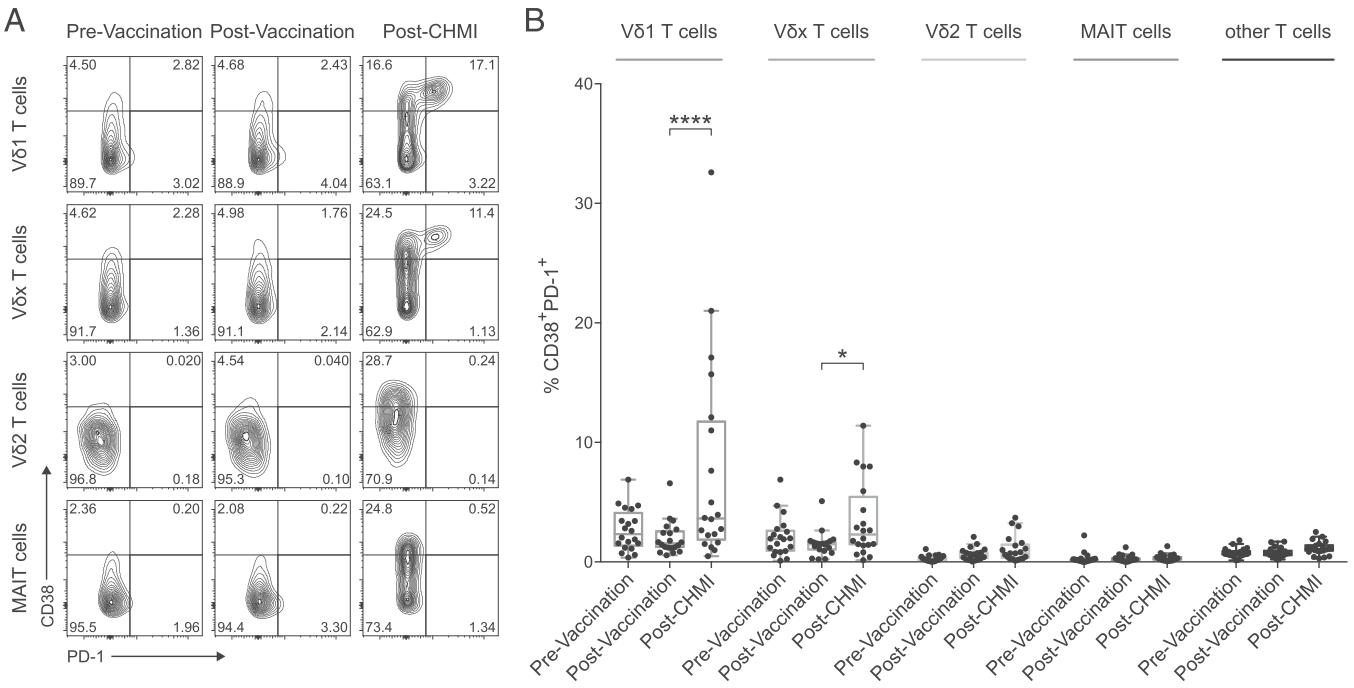

FIGURE 2. TCR Vδ1 cells and TCR Vδx cells show an increased frequency of $CD38^+PD\text{-}1^+$ cells after blood-stage infection induced by PfSPZ Challenge. (**A**) Contour plot of TCR γδ cells and MAIT cells at baseline, postvaccination, and post-CHMI from an infected volunteer with a strong increase of $CD38^+PD\text{-}1^+$ TCR Vδ1 cells after CHMI. (**B**) Frequency of $CD38^+PD\text{-}1^+$ T cells across all infected volunteers ($n = 20$). Lines represent the median value, boxes span the interquartile range, and whiskers extend to the furthest point still within 1.5 interquartile ranges from the box. Data were analyzed by two-way ANOVA corrected for multiple comparisons using Dunnett test. $^{****}p < 0.0001$, $^{*}p = 0.011$.

9B, 9D), whereas no association was detectable upon immunization with PfSPZ Vaccine (Supplemental Fig. 9A, 9C).

Specific expansion of $CD38^+PD\text{-}1^+$ Vδ1 and Vδx T_{EE} cells in a second clinical cohort

To confirm that $CD38^+PD\text{-}1^+$ and $CD27^-CD57^-$ T cells represented the same subset, Abs against those markers were combined in a single flow cytometry panel (Supplemental Table I, panel 3) and used to analyze PBMC from adult volunteers enrolled into the clinical trial BSPZV2. In this study, volunteers underwent three immunizations with PfSPZ Vaccine, followed by two consecutive homologous CHMIs to assess vaccine efficacy (Fig. 5A). The first CHMI (CHMI no. 1) was conducted 3–11 wk after last immunization with PfSPZ Vaccine, and the second CHMI (CHMI no. 2) was conducted 37–41 wk after last vaccination.

We first assessed whether asexual blood-stage infection was associated with specific activation and expansion of Vδ1 T_{EE} cells. Indeed, $CD38^+PD\text{-}1^+$ Vδ1 T_{EE} cells expanded selectively in the volunteers who developed blood-stage parasitemia after CHMI no. 1, whereas the frequency of other (non-T_{EE}) $CD38^+PD\text{-}1^+$ TCR Vδ1 cells remained unchanged (Fig. 5B–D). Consistent with these findings, in the volunteers who developed blood-stage parasitemia only after CHMI no. 2, an increase of $CD38^+PD\text{-}1^+$ Vδ1 T_{EE} cells was detectable only after CHMI no. 2 (Supplemental Fig. 10). Again, the effect was specific for $CD38^+PD\text{-}1^+$ T_{EE} cells.

Strikingly, these alterations were long-lasting, as the frequency of $CD38^+PD\text{-}1^+$ Vδ1 T_{EE} cells was still elevated before initiation of CHMI no. 2, 6 mo after CHMI no. 1 (Fig. 5C, left panel). Furthermore, fold expansions of $CD38^+PD\text{-}1^+$ Vδ1 T_{EE} cells upon CHMI no. 1 and CHMI no. 2 in the volunteers who became infected during both CHMIs were compared. Whereas a strong expansion was observed during CHMI no. 1, no significant increase occurred upon CHMI no. 2 in the same volunteers (Fig. 5E). Taken together, these findings indicate an altered immune response upon parasite rechallenge and suggested long-term functional alterations of TCR Vδ1 cells upon malaria infection. A similar analysis of TCR Vδx cells in the same cohort led to comparable results (Supplemental Fig. 11).

Interestingly, in this cohort, we detected a slight, but significant, decrease in the frequency of TCR Vδ1 and Vδx cells upon immunization with PfSPZ Vaccine. However, this observation was not associated with protection (Supplemental Fig. 12).

Activation of TCR Vδ1 cells upon CHMI occurs in an oligoclonal manner

Loss of CD27 expression on TCR Vδ1 cells has been linked with oligoclonal expansion, accompanied by functional changes such as acquisition of cytotoxicity (31). We hypothesized that the observed expansion of $CD38^+PD\text{-}1^+$ Vδ1 T_{EE} cells after blood-stage parasitemia was also associated with oligoclonal expansion, possibly following Ag-dependent stimulation. Therefore, we assessed whether expansion of $CD38^+PD\text{-}1^+$ TCR Vδ1 cells upon blood-stage parasitemia was accompanied by expansion of distinct Vδ1 clonotypes. We selected the three volunteers from the BSPZV1 cohort with the highest increase of $CD38^+PD\text{-}1^+$ expression in TCR Vδ1 cells upon asexual blood-stage parasitemia. From each of the three volunteers, we sorted 32 single TCR Vδ1 cells from both the $CD38^+PD\text{-}1^+$ and $CD38^-PD\text{-}1^-$ subsets of PBMC-collected post-CHMI. The CDR3 of the TCRδ chains was determined, and the clonotype frequencies were analyzed (Supplemental Table II). In all three examined volunteers, the $CD38^+PD\text{-}1^+$ TCR Vδ1 cells were more oligoclonal than the $CD38^-PD\text{-}1^-$ population, probably as a consequence of clonotype-dependent activation of TCR Vδ1 cells after asexual erythrocytic stage parasitemia (Fig. 6).

TCR Vδ1 cells proliferate extensively in response to asexual blood-stage parasites in vitro

Our data suggested that the expansion of $CD38^+PD\text{-}1^+$ TCR Vδ1 cells after asexual blood-stage parasitemia could be due to Ag-dependent activation of these cells. Thus, we aimed to assess

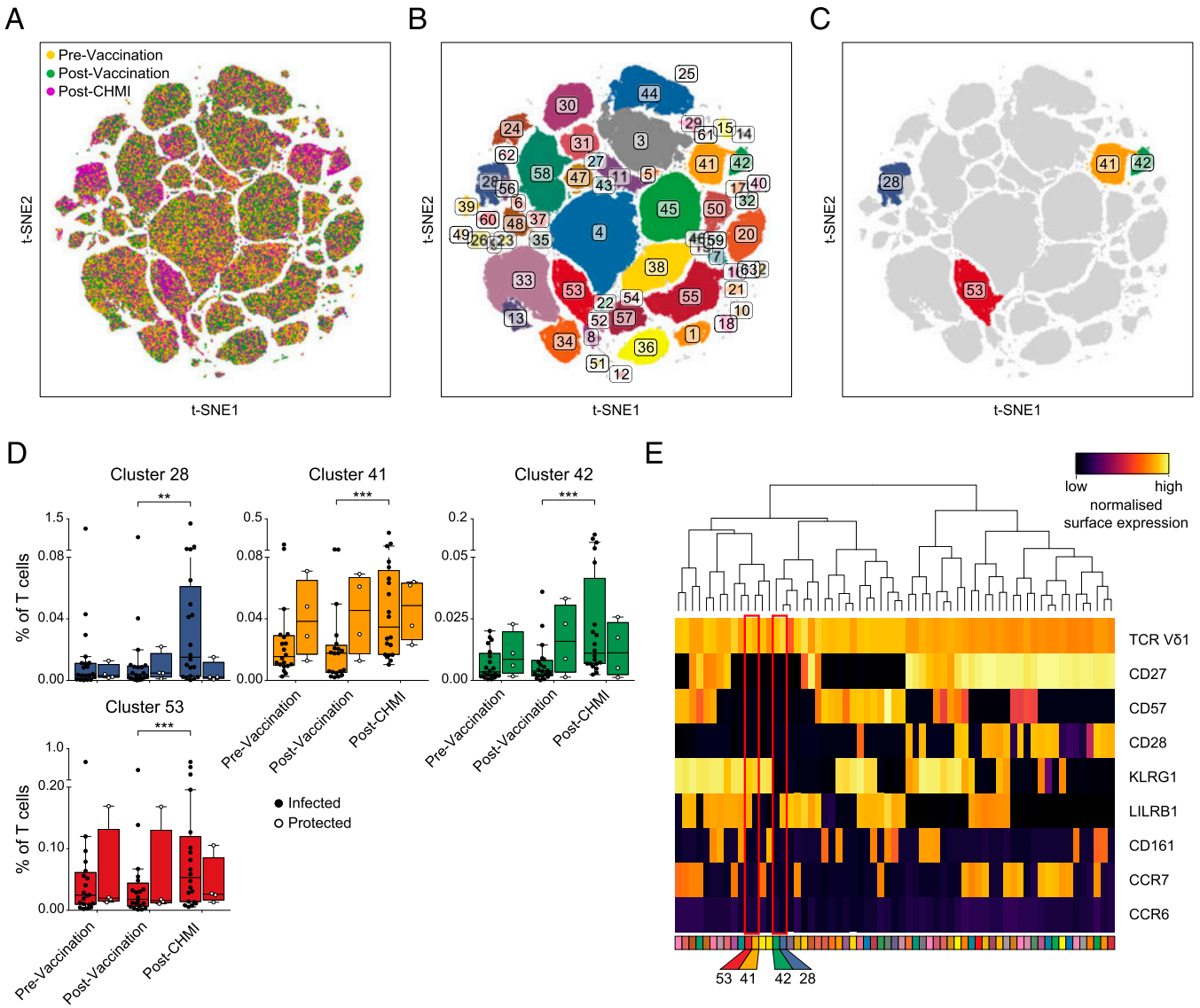

FIGURE 3. Vδ1 T_{EE} cells are expanded after erythrocytic stage infection induced by CHMI. (**A**) *t*-SNE plot of TCR Vδ1 cells from all volunteers color coded by visit: prevaccination (yellow), postvaccination (green), and post-CHMI (magenta). Regions with increased cell frequency after CHMI appear in magenta ($n = 24$). (**B**) *t*-SNE plot after clustering by DBSCAN. Clusters are color coded and assigned numbers. (**C**) Clusters with significantly altered frequency after CHMI are highlighted. Clusters are considered significant with $p < 0.01$ (Wilcoxon signed-rank test adjusted with the Benjamini–Hochberg method) and at least 2.5-fold change of the mean. (**D**) Frequency of the four clusters that are significantly increased in infected volunteers after CHMI. Infected volunteers (black dots) are separated from protected volunteers (white dots). Horizontal lines represent the median value, boxes span the interquartile range, and whiskers extend to the furthest point still within 1.5 interquartile ranges from the box. Infected, $n = 20$; protected, $n = 4$. $^{***}p < 0.001$, $^{**}p < 0.01$, Wilcoxon signed-rank test adjusted with the Benjamini–Hochberg method. (**E**) Heat map showing the median normalized surface expression of all clusters. The four significantly increased clusters are highlighted with a red frame and labeled with their cluster identification.

T cell expansion upon parasite exposure using an in vitro model. Mo-DC from four BSPZV1 donors were used to stimulate autologous PBMC in the presence of either uRBC or PfRBC. PfRBC were infected with the *P. falciparum* strain NF54 that is also used in PfSPZ Vaccine and PfSPZ Challenge. Robust proliferation of $CD4^+$ T cells and TCR Vδ2 T cells was observed after stimulation with Mo-DC and PfRBC for 6 d (Fig. 7A). MAIT cells also proliferated, albeit to a lesser extent (Fig. 7A). Mo-DC and uRBC failed to induce comparable responses. Interestingly, a marked PfRBC-dependent proliferation was observed in TCR Vδ1 and Vδx cells (Fig. 7A). Reminiscent of the ex vivo phenotype observed after CHMI, in vitro proliferating TCR Vδ1 and Vδx cells showed increased expression of CD38 and PD-1 (Fig. 7B).

To further characterize the proliferating TCR Vδ1 cells and investigate their responsiveness to *P. falciparum* Ags, we interrogated T cell clones derived from sorted cells that proliferated after stimulation with Mo-DC and PfRBC. One group of cells was sorted as TCR $\gamma\delta^+$ Vδ2 negative and a control group as $CD4^+$ T cells. Both Vδ1 and $CD4^+$ TCR$\alpha\beta^+$ clones were established and screened for activation in response to autologous Mo-DC and PfRBC. The activation marker that was selected for this screening is CD137, whose upregulation allows for detection of Ag-specific T cells (37). The $CD4^+$ T cell clones showed a consistent increase of CD137 expression only upon stimulation with PfRBC, indicating Ag-dependent activation and proving the validity of our approach to isolate *P. falciparum*–responsive T cell clones (Fig. 7C, upper panel). In contrast, the Vδ1 T cell clones showed marked expression of CD137 when cocultured with autologous Mo-DC, independently of the presence of blood-stage parasites (Fig. 7C, upper panel). Similar reactivity was observed when TCR Vδ1 clones were investigated for IFN-γ release (Fig. 7C,

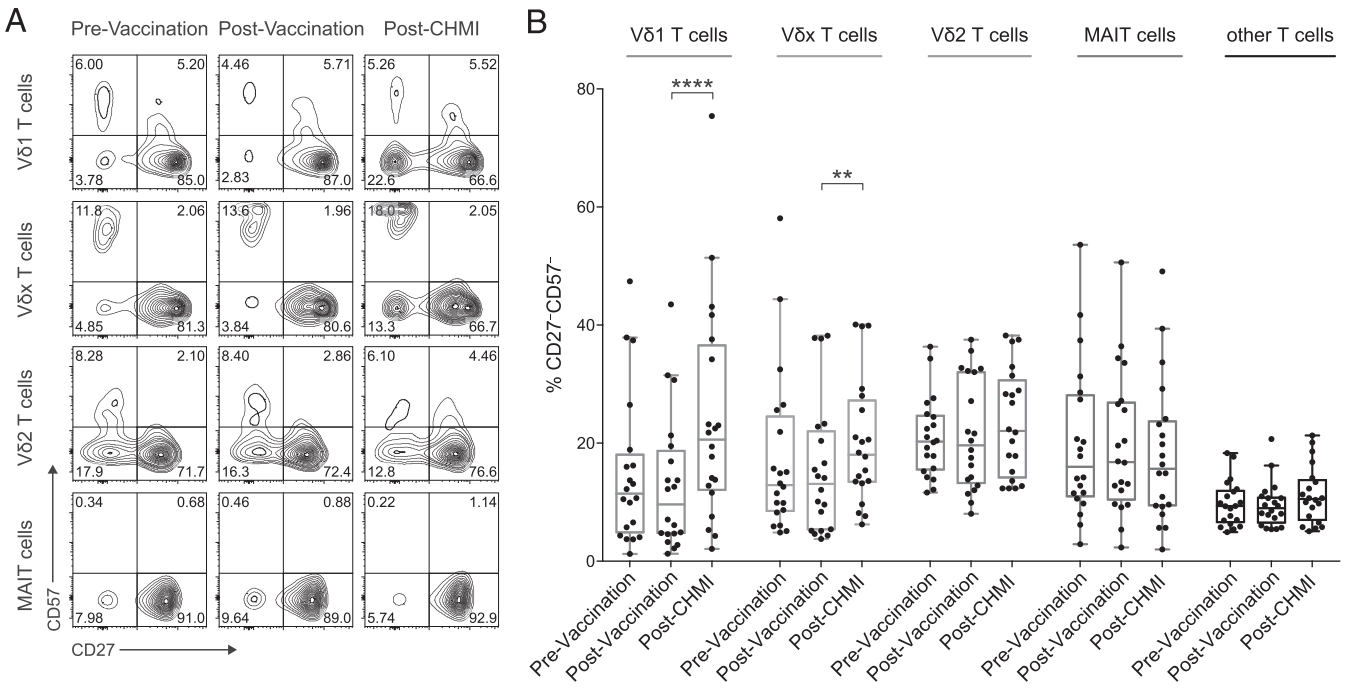

FIGURE 4. TCR Vδ1 cells and TCR Vδx cells show an increased frequency of early effector cells after erythrocytic stage infection induced by CHMI. (**A**) Contour plot of TCR γδ cells and MAIT cells at baseline, postvaccination, and post-CHMI from a representative, infected donor. (**B**) Frequency of $CD27^{-}CD57^{-}$ cells across all infected volunteers ($n = 20$). Horizontal lines represent the median value, boxes span the interquartile range, and whiskers extend to the furthest points still within 1.5 interquartile ranges from the box. Data were analyzed by two-way ANOVA corrected for multiple comparisons using Dunnett test. $^{****}p < 0.0001$, $^{**}p = 0.0026$.

lower panel). These unexpected findings indicate that at least some TCR Vδ1 cells that expanded upon coculture with Mo-DC and PfRBC are activated by self-encoded ligands independently of parasite-derived Ags. The PfRBC-dependent proliferation observed with freshly isolated PBMC might have been facilitated by soluble factors or additive signals provided by $CD4^{+}$ and TCR Vδ2 cells responding to *P. falciparum* Ags.

To further investigate the possible self-reactivity of TCR Vδ1 cells, we assessed the ex vivo response of TCR Vδ1 cells to THP-1, HC-04 and HeLa cell lines, which are derived from different human tissues. PBMC collected at baseline, postvaccination, and post-CHMI during the BSPZV1 study were cocultured for 6 d with each cell line. Analysis by flow cytometry showed that TCR Vδ1 cells proliferated and upregulated CD38 and PD-1 (Fig. 7D, left panels), an effect that was almost absent in the TCR Vδ2 cell subset (Fig. 7D, right panels). Whereas all cell lines induced some degree of proliferation and activation, the strongest response was induced by the hepatocyte-derived cell line HC-04. Furthermore, activation and proliferation of TCR Vδ1 cells was remarkably pronounced in PBMC derived from the post-CHMI visit of donor A, who displayed the highest frequency of $CD38^{+}PD\text{-}1^{+}$ TCR Vδ1 cells and profound oligoclonal expansion of TCR Vδ1 cells upon CHMI (see Fig. 6).

Discussion

CHMI conducted in malaria pre-exposed populations is an excellent tool to dissect malaria parasite–host interactions under highly defined conditions (38). The role of T cells during malaria remains poorly understood. In particular, the function of unconventional T cells, comprising CD1- and MR1-restricted T cells (39), TCR Vγ9Vδ2 cells stimulated by butyrophilin 3A1 (40), and other subsets of TCR γδ cells remains unclear. We analyzed these populations in depth and found important changes for some of them. During CHMI in *P. falciparum* pre-exposed adult Tanzanian volunteers, we confirmed previous findings that T cells expressing the TCR Vγ9Vδ2 heterodimer as well as MAIT cells expand upon asexual blood-stage parasitemia (12, 23).

We also investigated TCR γδ cells expressing a TCR Vδ1 chain and the TCR γδ populations expressing other Vδ chains (in this study indicated as Vδx), which represent a minority of TCR γδ cells in most individuals (41). Very little is known regarding the Ag specificity and the restriction molecules of TCR Vδ1 and Vδx cells (42). In addition, their function in infectious diseases, including malaria, remains to be determined (17). TCR Vδ1 cells may recognize different types of Ags and expand clonally after Ag recognition. When activated, these T cells release a variety of cytokines, may exert helper functions, and may also kill target cells, thus resembling MHC-restricted adaptive T cells (43). Activated TCR γδ cells may also express surface markers identical to those expressed by MHC-restricted T cells. We found that only TCR Vδ1 and Vδx cells showed expansion of the $CD38^{+}PD\text{-}1^{+}$ population, a phenotype not observed on expanded TCR Vδ2 and MAIT cells. Both CD38 and PD-1 are markers of cell activation, and although they exert different functions, they both contribute to regulation of expressing T cells. The unique coexpression of CD38 and PD-1 implies a qualitatively distinct activation of TCR Vδ1 and Vδx cells upon CHMI compared with other expanded T cells such as MAIT and TCR Vγ9Vδ2 cells.

Both CD38 and PD-1 can be upregulated by soluble factors (44, 45), and their expression is more efficiently induced on Ag-stimulated T cells either within the tumor microenvironment or during chronic infectious diseases (46, 47). The presence of both CD38 and PD-1 might result from a continuous Ag stimulation in donors who developed blood-stage parasitemia, possibly following recognition of either parasite-derived Ags or self-antigens induced by *P. falciparum* infection. Alternatively, the expansion of unique T cells might be initially Ag driven and then facilitated by cytokines present in the microenvironment, thus representing a bystander effect. For this reason, we investigated the TCR repertoire of the expanded TCR Vδ1 $CD38^{+}PD\text{-}1^{+}$ cells.

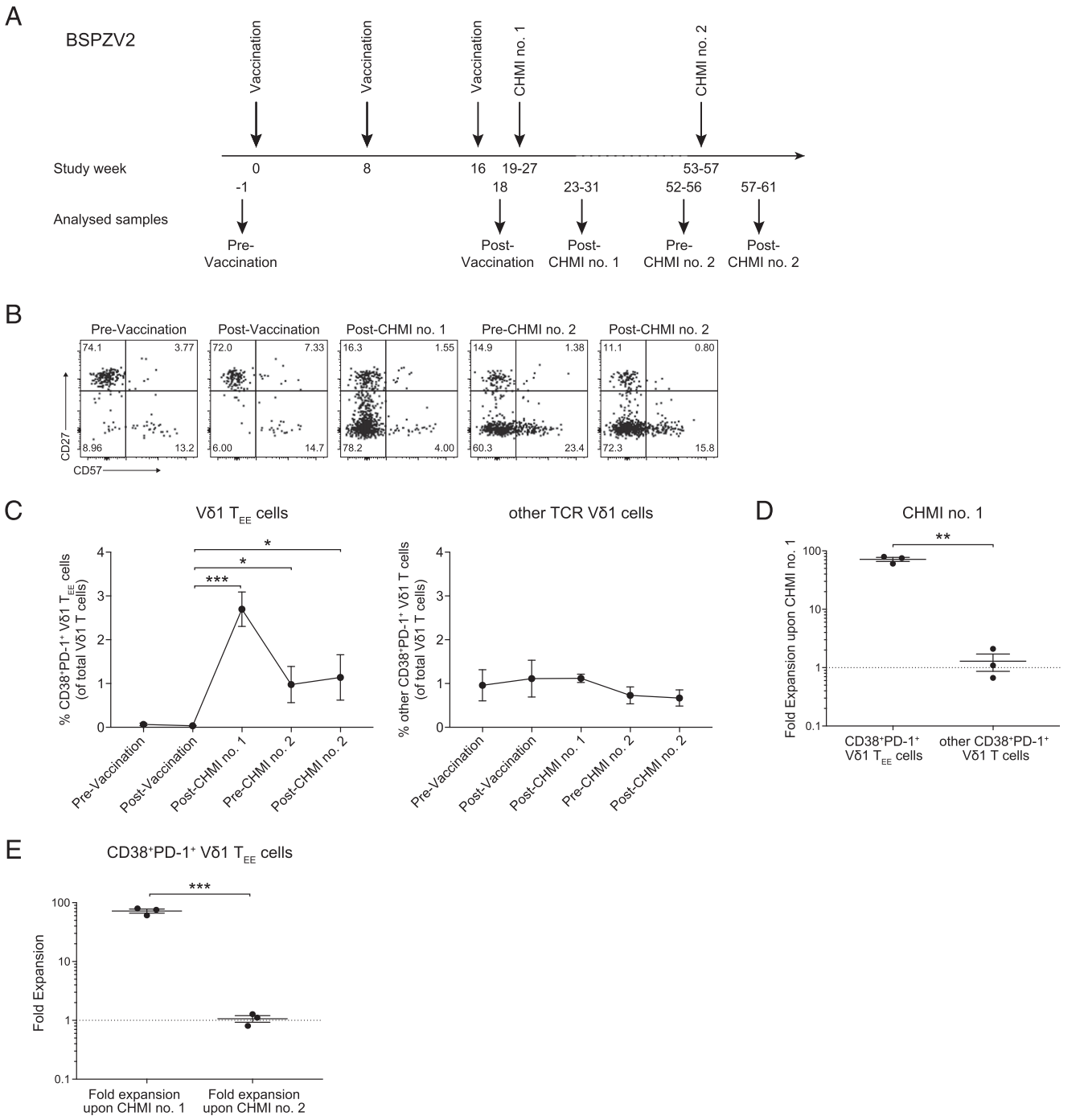

FIGURE 5. TCR Vδ1 T_{EE} cells are specifically activated after asexual erythrocytic stage infection and show long-lasting alterations. (**A**) Timeline of the BSPZV2 cohort. (**B**) Dot plot of $CD38^+PD\text{-}1^+$ TCR Vδ1 cells of a volunteer who was infected after both CHMIs. (**C**) Frequency of $CD38^+PD\text{-}1^+$ Vδ1 T_{EE} cells and $CD38^+PD\text{-}1^+$ Vδ1 non-T_{EE} cells in volunteers who were infected after both CHMIs ($n = 3$). Plotted is the mean ± SEM. $^{***}p = 0.002$, $^{*}p < 0.05$. (**D**) Fold expansion of $CD38^+PD\text{-}1^+$ Vδ1 T_{EE} cells and other $CD38^+PD\text{-}1^+$ Vδ1 T cells upon CHMI no. 1. $^{**}p = 0.0099$. (**E**) Fold expansion of $CD38^+PD\text{-}1^+$ Vδ1 T_{EE} cells during CHMI no. 1 and CHMI no. 2. Fold changes were compared by ratio paired *t* test. Shown are the three volunteers who were infected after CHMI no. 1 as determined by thick blood smear. $^{***}p = 0.0003$. Frequencies between visits were compared by one-way ANOVA, adjusted for multiple comparisons using the Holm–Sidak method.

Our analysis was performed on single cells to reduce the bias introduced by the variable quantities of TCR gene mRNAs present in different cells. This approach forced us to limit the study to a relatively small number of cells. We investigated ~30 individual T cells isolated from both TCR Vδ1 $CD38^+PD\text{-}1^+$ and TCR Vδ1 $CD38^-PD\text{-}1^-$ populations of three donors. The data clearly indicated a significantly reduced breadth of TCR repertoire in the $CD38^+PD\text{-}1^+$ cells in comparison with TCR Vδ1 $CD38^-PD\text{-}1^-$ cells in all examined donors.

The possibility that the expansion of distinct clonotypes is Ag-driven is also indicated by the noted alterations of the T cell differentiation state upon erythrocytic stage parasitemia. Naive T cells generally express CD27 and CD28 but are negative for CD57 (34, 48). Upon repeated antigenic stimulation, expression of CD28 and CD27 is lost, and concomitantly, CD57 expression is gained. Late effector or senescent T cells are mostly $CD27^-CD28^-CD57^+$ (49). We found that expanded TCR Vδ1 and Vδx cells are $CD27^-CD28^-CD57^-$, thus representing a

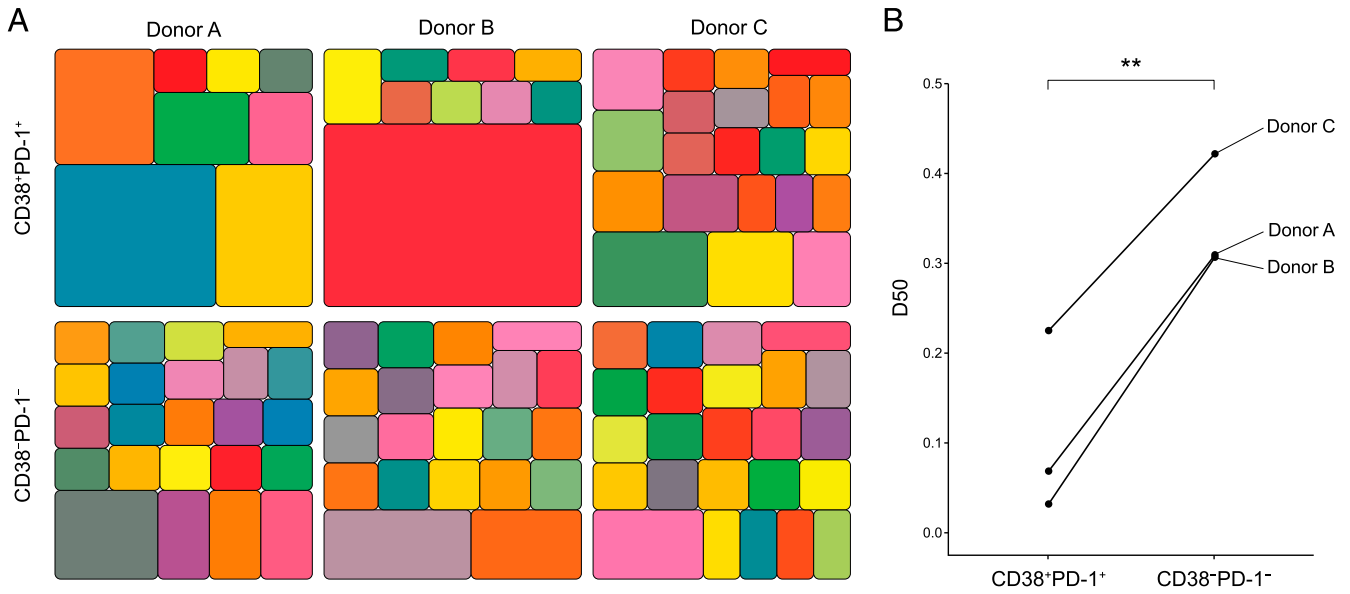

FIGURE 6. TCR Vδ1 cells are oligoclonally expanded after asexual blood-stage infection. (**A**) Tree maps indicating CDR3δ clonotype usage of $CD38^{+}PD\text{-}1^{+}$ and $CD38^{-}PD\text{-}1^{-}$ TCR Vδ1 cells after CHMI. Three infected BSPZV1 donors were chosen because of their strong expansion of $CD38^{+}PD\text{-}1^{+}$ TCR Vδ1 cells post-CHMI. (**B**) Clonality is quantified by the D50 value, the number of clonotypes required to cover 50% of analyzed cells, normalized by the total number of analyzed cells per condition. Data were analyzed by paired, two-tailed Student *t* test. $^{**}p = 0.009$.

relatively rare phenotype of Ag-experienced cells that are in a transition stage and are not yet terminally differentiated and have not reached replicative senescence (49). $CD28^{-}CD57^{-}$ T cells have been described in the context of HIV infection, retain proliferative capacity, and have limited replicative history (35). This phenotype of intermediate differentiation fits well with Ag-dependent activation of TCR Vδ1 and Vδx cells in volunteers who develop blood-stage parasitemia after CHMI.

Within TCR Vδx cells, we found a second cell cluster that was significantly expanded after erythrocytic stage infection and was characterized by the absence of PD-1 and expression of CD161 (Supplemental Fig. 2). The nature of this cell population is difficult to assign. CD161 is usually expressed on both TCR αβ and γδ cells with high responsiveness to IL-12 and IL-18 stimulation (50). During asexual blood-stage malaria, both these cytokines are produced in large amounts (51), and it is tempting to speculate that this second population of TCR Vδx $CD161^{+}$ cells expands during this stage. Also MAIT cells are prone to cytokine-mediated activation (52), and a similar cytokine-dependent mechanism of activation and proliferation might explain our finding of increased MAIT cells in volunteers who develop blood-stage parasitemia during CHMI. This possibility is supported by the fact that *P. falciparum* does not encode the enzymes of the riboflavin biosynthesis pathway (53) necessary for generation of the canonical MAIT cell Ags (54).

In the BSPZV2 cohort, we detected a slight, but significant, decrease in the frequency of Vδ1 and Vδx T cells postvaccination (Supplemental Fig. 12A, 12B). This might imply that these T cells get recruited to the liver during early liver stage, when the development of sporozoites from PfSPZ Vaccine is thought to arrest (55). Their decrease in peripheral blood was not associated with the protection status of the volunteers (Supplemental Fig. 12C, 12D). The accumulation of $CD38^{+}PD\text{-}1^{+}$ Vδ1 and Vδx T cells post-CHMI might indicate that Ag-stimulation occurs between mid–liver stage and blood stage. Analysis of samples from volunteers who received PfSPZ Challenge during antimalarial chemoprophylaxis (56), in which *P. falciparum* is allowed to develop through the entire liver stage, might help to narrow down the time point of Vδ1 T cell stimulation and/or their migration to peripheral blood.

Of note, the phenotypic alterations that we detected were exclusive to volunteers who experienced a recent asexual blood-stage infection, whereas volunteers who remained blood-stage negative after CHMI did not show any detectable changes. These findings indicate that the stimulatory Ags that induce the expansion of TCR Vδ1 and Vδx $CD38^{+}PD\text{-}1^{+}$ cells might be of microbial origin. Alternatively, other classes of Ags of self-origin might be expressed during the blood-stage infection and stimulate these cells. Consistent with both of these hypotheses, the volunteers with the strongest increase of $CD38^{+}PD\text{-}1^{+}$ TCR Vδ1 cells after CHMI were the ones with the highest peak parasitemia (Supplemental Fig. 6). Our in vitro experiments showed that TCR Vδ1 and Vδx cell expansion depended on the presence of *P. falciparum*–infected RBC. Thus, *P. falciparum* was important for the proliferation of these cells, possibly through an indirect effect, such as the induction of the release of cytokines by *P. falciparum*–specific non-Vδ1 T cells. However, TCR Vδ1 clones established from CHMI donors also reacted to Mo-DC in the absence of *P. falciparum*, suggesting a self-reactive capacity. In addition, fresh TCR Vδ1, but not Vδ2, cells from CHMI donors responded to the hepatocyte HC-04 cell line and, in a reduced manner, to the myelomonocytic cell line THP-1 in the absence of *P. falciparum*, thus confirming the ability of these cells to recognize self-molecules. These findings may indicate that, during the liver stage, some mechanisms already operate to activate the immune response. Our unpublished, preliminary results of vaccination by PfSPZ Challenge during chemoprophylaxis of malaria pre-exposed volunteers in Equatorial Guinea during the EGSPZV2 trial indicate that liver-resident, malaria-specific immunity might be induced under natural malaria exposure (Olotu et al., submitted for publication; ClinicalTrials.gov identifier: NCT02859350). Furthermore, data from mouse models suggest that the pre-erythrocytic stage of malaria is not immunologically silent but that liver cells may sense *Plasmodium* infection through recognition of *Plasmodium* RNA and respond with release of type I IFNs (57). Thus, liver-resident TCR Vδ1 cells might also be alerted by innate immune mechanisms during *P. falciparum* liver stage.

Our findings raised multiple questions regarding the nature of the Ags recognized by self-reactive TCR Vδ1 cells, the elements that

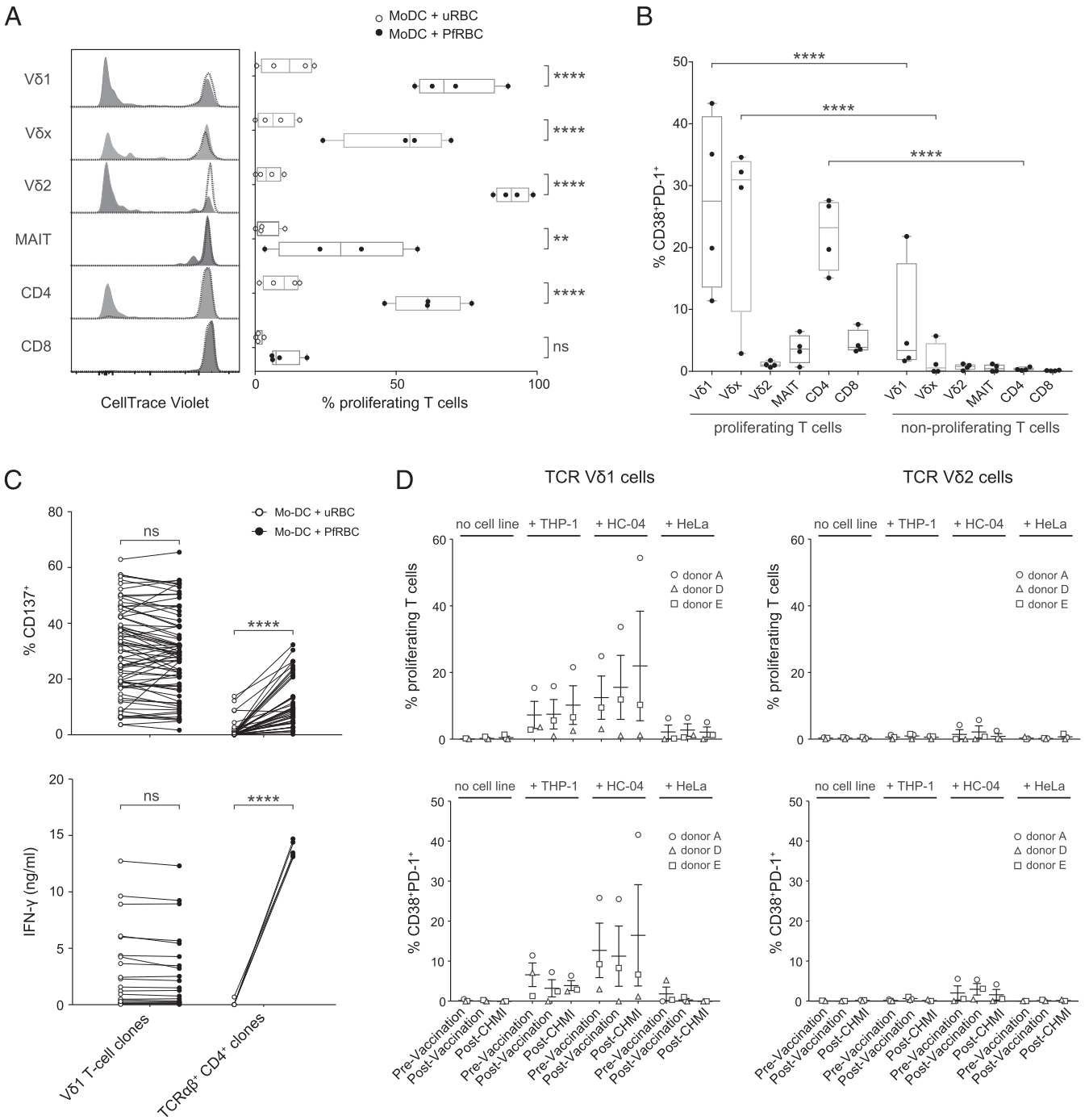

FIGURE 7. TCR Vδ1 cells proliferate and upregulate CD38 and PD-1 in response to Mo-DC and PfRBC as well as distinct human cell lines. (**A**) Proliferation of PBMC from BSPZV1 volunteers in response to autologous Mo-DC and PfRBC or uRBC. Proliferation was assessed after 6 d of coculture ($n = 4$). Histograms show CellTrace Violet staining of T cells from one representative volunteer. Filled histograms, coculture with Mo-DC and PfRBC; dashed lines, coculture with Mo-DC and uRBC. ****$p < 0.0001$, **$p = 0.0037$. (**B**) Frequency of $CD38^+PD\text{-}1^+$ cells in proliferating and nonproliferating cells after coculture with Mo-DC and PfRBC. ****$p < 0.0001$. (**C**) Percentage of $CD137^+$ T cells upon coculture of Vδ1 T cell clones or $TCR\alpha\beta^+$ $CD4^+$ T cell clones with autologous Mo-DC and uRBC or PfRBC (upper panel). Rescreen of selected candidate clones using IFN-γ ELISA (lower panel). ****$p < 0.0001$. (**D**) Frequency of proliferating TCR Vδ1 cells and $CD38^+PD\text{-}1^+$ TCR Vδ1 cells (left panels) as well as TCR Vδ2 cells (right panels) upon coculture of ex vivo PBMC with indicated cell lines. PBMC from three BSPZV1 donors were derived from prevaccination, postvaccination, and post-CHMI visits and cocultured for 6 d with the cell lines. Shown is the mean ± SEM. Data were analyzed by two-way ANOVA, adjusted for multiple comparisons using the Holm–Sidak methods.

restrict their response, and the physiological role of these T cells. All these aspects warrant further investigations.

The expansion of TCR Vδ1 cells in nonprotected patients argues in favor of an indirect role for these T cells during malaria immunity. An intriguing possibility is that they are involved in the enhancement of the immune response within the organs where they become activated. In a normal human liver, TCR γδ cells are very abundant (10–15% of total $CD3^+$ cells), and TCR Vδ1 cells represent the largest population of TCR γδ cells (58). These cells release a variety of effector molecules, including perforin and granzyme B, and are oligoclonal. Whether they recognize Ags derived from gut microbiota or self-antigens was not investigated in these studies.

The expansion of TCR Vδ1 cells in peripheral blood of malaria patients and their release of a variety of cytokines, including IFN-γ, TNF, and IL-10, has been observed (14, 15, 17, 59). TCR Vδ1 and Vδx cells that become activated after blood-stage parasitemia might participate in recruiting other effector cells, as recently suggested (60). For example, they might release chemokines and M-CSF that attract and enhance the microbicidal activity of macrophages, as found in mice infected with *P. chabaudi* (61). Finally, TCR Vδ1 cells might directly inhibit the in vitro replication of *P. falciparum*, as previously shown (62), even not recognizing parasite-derived Ags.

In conclusion, we found that populations of TCR Vδ1 and Vδx cells become activated and show long-term phenotypic changes in volunteers who develop blood-stage parasitemia. These TCR γδ cells show features similar to classical adaptive T cells, and at least a fraction of them are activated in the absence of parasite-derived molecules. It will be important to identify the mechanisms of activation to shed light on the biological functions of these T cell populations.

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Disclosures

S.L.H. and B.K.L. are salaried and full-time employees of Sanaria, Inc., the developer and sponsor of Sanaria PfSPZ Vaccine. The other authors have no financial conflicts of interest.

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eLife

RESEARCH ARTICLE

Proteome-wide analysis of a malaria vaccine study reveals personalized humoral immune profiles in Tanzanian adults

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Abstract Tanzanian adult male volunteers were immunized by direct venous inoculation with radiation-attenuated, aseptic, purified, cryopreserved *Plasmodium falciparum* (Pf) sporozoites (PfSPZ Vaccine) and protective efficacy assessed by homologous controlled human malaria infection (CHMI). Serum immunoglobulin G (IgG) responses were analyzed longitudinally using a Pf protein microarray covering 91% of the proteome, providing first insights into naturally acquired and PfSPZ Vaccine-induced whole parasite antibody profiles in malaria pre-exposed Africans. Immunoreactivity was identified against 2239 functionally diverse Pf proteins, showing a wide breadth of humoral response. Antibody-based immune 'fingerprints' in these individuals indicated a strong person-specific immune response at baseline, with little changes in the overall humoral immunoreactivity pattern measured after immunization. The moderate increase in immunogenicity following immunization and the extensive and variable breadth of humoral immune response observed in the volunteers at baseline suggest that pre-exposure reduces vaccine-induced antigen reactivity in unanticipated ways.

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Introduction

Malaria control and elimination remain a significant public health challenge, and an effective malaria vaccine targeting *Plasmodium falciparum* (Pf) would be an important tool to accelerate burden reduction, curb the spread of drug resistant strains and facilitate focal Pf malaria elimination (***Greenwood, 2008***). While the most advanced vaccine, RTS,S/AS01A, an adjuvanted subunit vaccine for pediatric indications based on the major Pf sporozoite surface protein (***Alonso, 2006***), is currently being assessed in a large pilot study for safety, impact, and feasibility of routine immunization of children (***World Health Organization, 2018***), other vaccines are in development or currently tested. The Malaria Vaccine Roadmap provides guidance for next-generation vaccine development targeting all age groups with improved efficacy and extended duration of protection (***malERA Refresh Consultative Panel on Tools for Malaria Elimination, 2017***). However, although extensive work has been undertaken to understand potential immune mechanisms of vaccine-induced protection against malaria infection and disease, much remains unknown.

An alternative to the subunit vaccine approach is immunization with attenuated whole Pf sporozoite vaccines (***Richie et al., 2015***). Malaria sporozoites have been studied in the context of inducing sterile immunity first in mouse models (***Nussenzweig et al., 1967***), and later in humans via Pf-

infected mosquitoes (**Clyde et al., 1973**; **Rieckmann et al., 1974**; **Hoffman et al., 2002**). Whole sporozoite malaria vaccine development is currently based on aseptic, purified, metabolically active Pf sporozoites (NF54 strain), either radiation-attenuated (PfSPZ Vaccine), chemo-attenuated through concurrent antimalarial administration (PfSPZ-CVac), or genetically modified (PfSPZ-GA2) (**Richie et al., 2015**). Clinical trials have evaluated the efficacy of PfSPZ Vaccine (e.g. **Epstein et al., 2011**; **Seder et al., 2013**; **Ishizuka et al., 2016**; **Epstein et al., 2017**; **Lyke et al., 2017**) resulting in evidence that intravenous injections provide higher protection than intradermal applications against homologous and heterologous CHMI. Specifically, all US adults (n = 6) volunteers were protected against homologous CHMI in a 5-dose schedule (**Seder et al., 2013**), of whom 5 of 6 remained protected following CHMI at 59 weeks after last immunization (**Ishizuka et al., 2016**). Protection following heterologous CHMI (7G8 strain) has been achieved for up to 33 weeks after last vaccine dose (**Epstein et al., 2017**; **Lyke et al., 2017**). These results enabled the PfSPZ Vaccine to receive an FDA Fast Track designation (**Sanaria Inc, 2016**).

To evaluate the protective efficacy against CHMI of PfSPZ Vaccine in malaria pre-exposed volunteers, PfSPZ Vaccine was evaluated in a dose-escalation study in a cohort of adult, male Tanzanian volunteers (acronym: BSPZV1) (**Jongo et al., 2018**). In this study, vaccine-induced protection against homologous CHMI was assessed for the first time in Sub-Saharan Africa by direct venous inoculation (DVI) of 3200 fully infectious sporozoites (PfSPZ Challenge) three and/or 24 weeks after last immunization. 1/18 volunteers who received 5 doses of 1.35×10^5 PfSPZ (group 2,) and 4/20 volunteers who received 5 doses of 2.7×10^5 PfSPZ (group 3) were sterilely protected 3 weeks after last vaccine dose. The four individuals from the higher dose group protected in the first CHMI remained protected at a second CHMI conducted 24 weeks after final PfSPZ immunization (**Jongo et al., 2018**).

Our understanding of naturally or vaccine induced cellular immune dynamics has considerably improved over the years, however much remains unknown. The immune mechanisms conferring protection associated with PfSPZ Vaccine have been studied in mouse and non-human primate models, and in human biological specimens (**Lefebvre and Harty, 2020**). In mice, the role of liver resident CD8+, IFNγ producing T cells, and γδT Cells have been considered paramount for conferring protection (**Lefebvre and Harty, 2020**), but their role in humans remain more difficult to assess. Pf-specific T cells found in peripheral blood might not represent the more abundant and stable tissue resident effector T cells (**Ishizuka et al., 2016**), and thus might not represent a good proxy for cellular immunity in the liver. CD4 and CD8 T cells producing IFNg, interleukin 2 (IL2) and tumor necrosis factor alpha (TNFa) were detected in peripheral blood after immunization of malaria naïve volunteers, however, their levels rapidly declined over time and were not correlated with protection (**Ishizuka et al., 2016**; **Lyke et al., 2017**). No increase in CD8 T cell responses was observed following immunization of Tanzanian volunteers, and CD4 levels increased to lower magnitudes compared to malaria naïve individuals, with no association with protection (**Jongo et al., 2019**).

There has been significant work to assess the quality and quantity of antibody responses following PfSPZ Vaccine application. IgG specific to the Pf circumsporozoite protein (CSP) measured by ELISA or detected against PfSPZ by automated immunofluorescence assay (aIFA) correlated with protection in malaria naïve volunteers at 3 week and 21–25 week post-vaccination homologous CHMI in one study (**Ishizuka et al., 2016**). In contrast, the PfCSP ELISA and PfSPZ aIFA trend was not significant when the sporozoite dose was increased to 9×10^5 PfSPZ administered three times (**Lyke et al., 2017**), and these assays, along with an inhibition of sporozoite invasion (ISI) assay, did not correlate with protection in follow up studies (**Epstein et al., 2017**).

A functional role of antibodies in protection after PfSPZ Vaccination was proposed after passive transfer of the IgG fraction of immune sera from protected volunteers into humanized FRG-huHep mice that led to an 88% and 65% reduction in parasite liver burden with immune sera collected 2–3 weeks and 59 weeks after last PfSPZ immunization, respectively (**Ishizuka et al., 2016**). Further evidence of functional activity of humoral immunity was shown with anti-PfCSP monoclonal antibodies (mAbs) isolated from Tanzanian volunteers (**Tan et al., 2018**) and from U.S. vaccinees (**Kisalu et al., 2018**). Additionally, IgM specific to PfCSP was detected in Tanzanian pre-exposed adult males after immunization with PfSPZ Vaccine, and these IgM inhibited liver cell sporozoite invasion in vitro and fixed complement on whole Pf sporozoite (**Zenklusen et al., 2018**). Antigen-specific humoral immune responses have been studied in clinical trials by ELISA for a selection of antigens, such as PfCSP, PfEXP1, PfEBA-175, PfLSA1, PfMSP1 and PfMSP3 (see **Epstein et al., 2017** for summary of

findings). However, the number of antigens that can be assessed in parallel with ELISA is limited and thus constrained to pre-selected of antigens.

In contrast, malaria protein microarrays enable a less biased approach to assess humoral immunity against a large proportion of Pf encoded proteins (**Liang and Felgner, 2015**). This technology has assessed potential boosting of natural immunity in RTS,S vaccinated individuals (**Campo et al., 2015**), and was used to better understand the natural history of malaria infection in the field (**Boudová et al., 2017**). Microarrays have also identified immune-reactive antigens associated with malaria exposure for immune-epidemiological studies, or to examine potential correlates of protection in children and adults from endemic areas (**Dent et al., 2015**). Serum samples from malaria-naïve individuals infected by radiation-attenuated sporozoites via mosquito bites have been probed with microarrays covering 23% of all Pf proteins, providing insight into humoral immunity induced by both whole sporozoite vaccination and its association with sterile protection following CHMI (**Trieu et al., 2011**). More recently, a whole proteome microarray produced by Antigen Discovery, Inc (ADI) that includes 91% of all predicted Pf 3D7 strain proteins was used to study humoral immunity after PfSPZ-CVac vaccination in European malaria-naive volunteers (**Mordmüller et al., 2017**). 22 proteins were identified, which were recognized by more than 50% of the volunteers in the highest dose group, all of whom were protected against homologous CHMI (**Mordmüller et al., 2017**).

The main *in-vitro*, animal, and human studies described above suggest that both cellular immune response and antibody mediated immune response plays a role in inducing protection. However, a complete understanding of the mechanisms of vaccine-induced protection against malaria infection, and its interplay with pre-built natural immune response in exposed populations, remains unknown. For a comprehensive and unbiased description of Pf-specific humoral immune responses in malaria pre-exposed volunteers, we analyzed serum samples using the Pf protein microarray featuring 7455 full-length or fragmented proteins of the Pf proteome (3D7) (**Mordmüller et al., 2017**). Serum samples were collected pre-vaccination and 14 days past last vaccination to understand the PfSPZ Vaccine-induced IgG profiles in Tanzanian male adults participating in the BSPZV1 study and potential correlations between humoral immune responses and PfSPZ vaccine induced protection against homologous CHMI (**Jongo et al., 2018**). We show for the first time that our study population displayed highly personalized immune profiles based on a broad range of antigens recognized before vaccination. Surprisingly, this humoral immune pattern remained largely unchanged following the whole organism based PfSPZ immunization, leading to the hypothesis of natural imprinting of humoral immune responses.

Results

Study volunteers and serum sampling

In total, 92 serum samples from 46 volunteers enrolled in the BSPZV1 study (all volunteers from group 2 and group 3 in the clinical trial [**Jongo et al., 2018**]) were probed on Pf whole proteome microarrays, including samples collected at baseline (before vaccination) and 2 weeks after last immunization (**Figure 1**). Eight non-vaccinated placebo controls, 18 volunteers who were immunized with the lower PfSPZ Vaccine dose (group 2) and 20 volunteers who received the higher PfSPZ Vaccine dose (group 3) were included (**Figure 1**; **Jongo et al., 2018**). All volunteers included in the study had no parasitemia at the start of the study (measured by malaria thick blood smears (TBS)) and no parasitemia before CHMI (measured by TBS and the more sensitive qPCR) (**Jongo et al., 2018**). Additional exclusion criteria included history of malaria in the previous 5 years or antibodies to PfEXP1 by ELISA above a threshold level (**Jongo et al., 2018**) associated with recent infection by CHMI (**Shekalaghe et al., 2014**).

Tanzanian male adults recognize a high diversity of pf proteins

Across the 7455 Pf full length or fragmented proteins, 2804 probes corresponding to 2239 Pf proteins were considered as reactive antigens in the 92 samples tested for having a seropositive response (normalized signal intensity $\geq$1) in at least 10% of volunteers at either or both time points.

First, we examined the antibody profiles for each volunteer individually and the results of the paired samples are presented in the heatmap (**Figure 2a**). Pattern of antigens recognized across all peptides was largely unchanged before and after immunization. This was further confirmed by

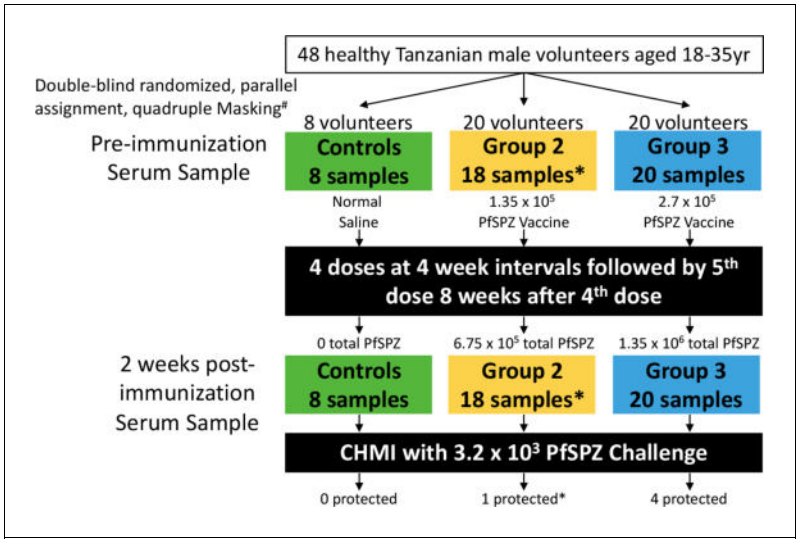

Figure 1. Sampling and volunteer information for proteome microarray studies. Three arms of a randomized, double-blind Phase 1 trial of PfSPZ Vaccine were selected for antibody profiling on Pf whole proteome microarrays: normal saline controls, a lower dose (group 2, 1.35 × 10^5 PfSPZ Vaccine/dose) and a higher dose (group 3, 2.7 × 10^5 PfSPZ Vaccine/dose). Serum samples were collected before immunization and 2 weeks after the final immunization. Information on the protection status of the volunteers after a 3 week post-immunization CHMI is provided. #Masking included participant, care provider, investigator and outcome assessor. *Samples were unavailable for protein array screening from two group 2 volunteers, one did not receive the 5th immunization dose and one left the country before CHMI (***Jongo et al., 2019***). All volunteers in the clinical trial who received 5 doses of immunization and who underwent CHMI 3 weeks after last immunization dose were included in the current analysis.

applying a dimensionality reduction algorithm (t-Distributed Stochastic Neighbor Embedding (t-SNE)) to represent the 2804 probes recognized in two dimensions and including time points assessed (***Figure 2b***). The t-SNE algorithm estimates the probability distribution of neighbors around each point, that is, it models the set of points which are closest to each point. A distinct clustering of observations for 43 out of the 46 volunteers was evident, with each pair of sample's nearest neighbor in the first two dimensions as the corresponding sampling time point for that volunteer (***Figure 2b***). Volunteer samples did not cluster according to treatment allocation with no difference identified by t-SNE between controls and vaccinees, but rather volunteers preserved their immunoreactive 'fingerprint' measured in their samples taken at baseline and two weeks following last PfSPZ Vaccination. It was not obvious why these three subjects did not maintain longitudinally consistent antibody profiles and we cannot rule out technical or sampling issues, or if they are due to unidentified biological factors associated with vaccination or temporal immune status. However, our results show these three individuals had slightly higher than average baseline antibody breadth and steeper decrease of breadth after immunization (***Figure 3c***).

To further investigate the breadth of humoral immune response against the 2804 peptides, the total number of peptides regarded as sero-reactive per sample were analyzed (***Figure 3***). Antigen recognition varied widely among individuals, with breadth of humoral immune response ranging from 187 to 2360 reactive antigens across all samples before immunization, and from 217 to 1535 and 187 to 1965 reactive antigens per volunteer in group 2 and group 3, respectively, after PfSPZ Vaccination (***Figure 3a–b***). Median antibody breadth from the PfSPZ-immunized volunteers across both immunization groups was 720 and 669 reactive peptide features recognized before and after immunization, respectively.

The effect of vaccination on breadth was analyzed by comparing breadth between the control and the PfSPZ Vaccine-immunized groups, and by quantifying the change in breadth after immunization compared to baseline levels. No significant differences in antibody breadth between the control and group 2 or group 3, nor between group 2 and group 3 after vaccination were detected (results of the negative binomial regression summarized in Table supplement 1). During the period before

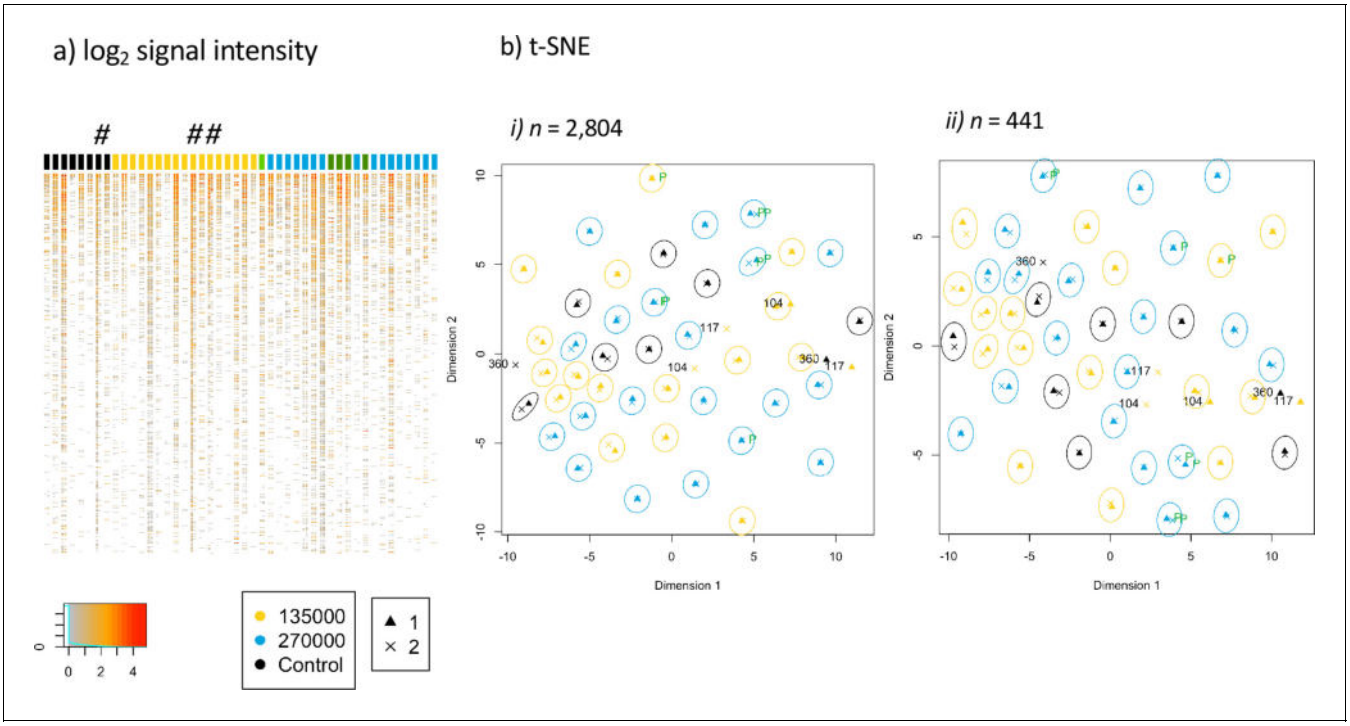

Figure 2. Antibody immune profile of Tanzanian healthy male adult volunteers is personalized. The heatmap of the normalized signal intensities of each sample per subject is shown in (**a**), with the signal intensity of each protein or fragment (rows) displayed for the samples before and after immunization of each volunteer (columns). The colored column headers represent volunteers ordered according to treatment allocation. 8 subjects of the control group (black), CHMI unprotected subjects of group 2 (n = 17, yellow), unprotected subjects of group 3 (n = 16, blue), CHMI protected subjects (n = 5, green). The first and second columns for each subject display the results obtained from baseline and after immunization samples, respectively. The # indicates volunteers BSPZV1-360, BSPZV1-104 and BSPZV1-117. (**b**) A t-SNE projected dimensionality reduction of normalized signal intensities across the microarray spots measured at baseline (triangles) and after PfSPZ vaccination (crosses) is shown. In (b-*i*) data are shown for the total 2804 reactive spots and in (b-*ii*) for the subset of 441 reactive proteins fragments predicted to be expressed at the sporozoite stage (***Florens et al., 2002***), with the signals obtained for each subject at the two bleeding time points grouped in circles. For 3 out of 46 subjects, namely BSPZV1-360, BSPZV1-104 and BSPZV1-117, the signals do not cluster in this t-SNE analysis.
The online version of this article includes the following source data for figure 2:

Source data 1. Data frame of the normalized signal intensities of the protein microarray.

and after vaccination, antibody breadth declined in many individuals in the control and immunized groups (note that samples were balanced for group and time point factors across technical microarray factors using a block randomization design, see Materials and methods), the relative differences in the medians between the two time points were of −4.8%, −13.6% and −4.7% seropositive signals in the control, group 2 and group 3, respectively (effect of vaccination on breadth tested with the inverted beta-binomial test for paired count data, resulting in an estimated fold change of −1.23,–1.16 and 1.03, and a p-value of 0.24, 0.03 and 0.43, respectively) (***Figure 3c***). In controls, 2/8 volunteers (25%) had higher antibody breadth after placebo inoculation compared to baseline (***Figure 3c***), and antibody breadth after PfSPZ Vaccine immunization increased in 6/18 (34%) and 12/20 (60%) individuals in group 2 and group 3, respectively (***Figure 2c***). Note that the volunteers for which samples that did not cluster in the t-SNE outputs appear to have higher than average baseline breadth and steeper decrease of breadth after immunization (***Figure 3c***). Overall, there was no dramatic change in breadths between both time points, which aligns with the immune fingerprint analysis in ***Figure 2***. There was a small decreased average breadth in group 2 driven by 2 of the three individuals whose samples did not cluster for immune-fingerprinting.

To assess the biological characteristics of this large number of reactive proteins, we used the DeepLoc method for *in silico* prediction of protein subcellular localization using the 3D7 protein amino acid sequences (***Almagro Armenteros et al., 2017***; ***Table 1***). Numerous reactive proteins predicted to be exported (*n* = 53) or cell membrane associated (*n* = 208) were identified. The

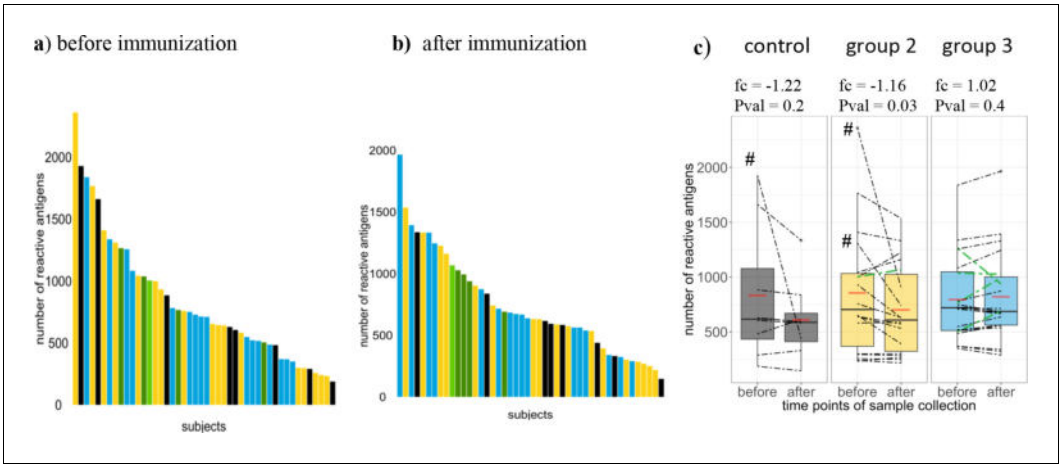

Figure 3. Breadth of Pf-specific humoral immunity upon PfSPZ vaccination. Breadth of Pf-specific antibody responses per volunteer (**a**) before and (**b**) after PfSPZ vaccination, stratified according to intervention and ordered according to their respective number of seropositive responses from highest to lowest. In (**c**) boxplots show median, interquartile range (IQR) and 1.5xIQR limits of the antibody breadth grouped by study arm and time point, means for each group are represented by red lines, and an estimated fold change with p-value from the inverted beta-binomial test are indicated for each group. Breadth of each volunteer are indicated by dashed lines. Controls, group 2 (1.35×10^5 PfSPZ Vaccine/dose) and group 3 (2.7×10^5 PfSPZ Vaccine/dose) volunteers are marked in black, yellow and blue, respectively. Results of the five CHMI protected individuals are highlighted in green (light green in group 2).

The online version of this article includes the following source data for figure 3:

Source data 1. Breadth of Pf-specific humoral immunity in each sample.
Source data 2. Summary statitistics on breadth per group and protection level.

majority of reactive antigens were predicted to be intracellular proteins (n = 1978). However, many of the well-known proteins present in the parasite organelles such as rhoptries and micronemes and proteins exported to the surface of infected erythrocytes such as PfEMP1 variants were predicted to be localized intracellularly, which shows that the currently available prediction algorithms remain limited by complex parasite biology. The full list of reactive antigens and DeepLoc subcellular localization predictions is shown in ***Table 1—source data 1***.

Moderate increase in antigen recognition following immunization with PfSPZ vaccine

To investigate potential vaccine induced increase in antigen immunogenicity, we compared mean reactivity of each antigen 2 weeks after immunization in relation to the baseline responses, grouped according to treatment intervention (***Figure 4a–c***). Reactivity against most antigens decreased after immunization in group 2 and in the placebo group during the ~24 week time interval between the two comparison time points, although not significantly when adjusted for the false discovery rate (FDR) with the Benjamini-Hochberg method (BH) (***Benjamini and Hochberg, 1995***; ***Figure 4a,b***), which aligns with the observed slight decrease in breadth of immune response following immunization. Antibodies binding to the Pf circumsporozoite protein (CSP) increased in group 2 and higher in group 3, although not significantly when adjusted for FDR (unadjusted p-value of $4.4 * 10^{-4}$ [adjusted: 0 .42] and $2.1 * 10^{-5}$ [adjusted: 0.06] for the t-test in group 2 and group 3, respectively) (***Figure 4c***). The mean negative value for PfCSP immunogenicity at baseline (before immunization, mean intensity = −0.73) suggests that very limited anti-PfCSP responses existed at baseline, with no significant differences between the study groups observed. Notably, the nonsignificant trend for reactivity decline observed in controls in almost all protein fragments of group 2 was not present in group 3.

'Deltas', the fold change in antibody reactivity (averaged across each individual group) for each individual peptide recognized before and after immunization, was higher for PfCSP in group 2 and group 3 when compared to controls, but not statistically significant after adjustment (***Figure 4—figure supplement 2a–b***). In addition, no significant difference was observed by comparing group 2

Table 1. Intracellular proteins are the most abundant reactive proteins.
The frequencies of reactive antigens allocated into the different subcellular localization categories (rows) for each group (columns), tested using 2-propotions Z-test and p-values adjusted using the Benjamini-Hochberg method (BH) (***Benjamini and Hochberg, 1995***), are shown (for all reactive proteins with p-values<0.05). Column two indicate the total number of reactive antigens, and columns 3–8 detail the number of significantly differentially reactive proteins localized in each compartment across samples before immunization, after immunization, in the protected group before and after immunization, in the unprotected group before and after immunization, respectively. The first row shows extracellular proteins, the second row is cell membrane associated proteins and the following rows are predicted intracellular proteins split according to subcellular localisation. The percentage of the reactive proteins found in each group compared to all samples (first column) are indicated in parenthesis.

	Subcellular localization	*N* reactive proteins	Baseline reactivity	Post-Immz reactivity	Baseline reactivity (protected)	Post-Immz reactivity (protected)	Baseline reactivity (unprotected)	Post-Immz reactivity (unprotected)
	Extracellular	53	3 (6%)	3 (6%)	10 (19%)	12 (23%)	3 (6%)	3 (6%)
	Cell membrane	208	11 (5%)	8 (4%)	63 (30%)	70 (34%)	10 (5%)	5 (2%)
Intracellular (N = 1978)	Cytoplasm	661	14 (2%)	16 (2%)	73 (11%)	79 (12%)	12 (2%)	14 (2%)
	Endoplasmic reticulum	429	12 (3%)	11 (3%)	53 (12%)	60 (14%)	11 (3%)	10 (2%)
	Golgi apparatus	76	2 (3%)	2 (3%)	13 (17%)	15 (20%)	2 (3%)	2 (3%)
	Lysosome/Vacuole	32	1 (3%)	1 (3%)	3 (9%)	2 (6%)	1 (3%)	1 (3%)
	Mitochondrion	150	3 (2%)	3 (2%)	11 (7%)	14 (9%)	3 (2%)	3 (2%)
	Nucleus	624	24 (4%)	20 (3%)	107 (17%)	115 (18%)	17 (3%)	18 (3%)
	Peroxisome	3	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	Plastid	3	2 (67%)	2 (67%)	2 (67%)	2 (67%)	2 (67%)	2 (67%)
	Total	2239	72 (3%)	66 (3%)	335 (15%)	369 (16%)	61 (3%)	58 (3%)

The online version of this article includes the following source data for Table 1:
Source data 1. The full list of reactive antigens and DeepLoc subcellular localization predictions.

deltas to group 3 deltas ***Figure 4—figure supplement 2c***). The nonsignificant trend of higher deltas in group 3 is consistent with the observations of less declining antibodies in the paired analysis.

To identify potential differences in vaccine induced immunogenicity between protected and unprotected individuals, we compared the difference in the mean immunoreactivity (i.e. signal intensity) for each antigen at baseline and 2 weeks after immunization between protected (n = 5) and unprotected (n = 33) volunteers, and the difference in the mean immunogenicity of each antigen between baseline and post immunization time points in the protected group (***Figure 5***). Four proteins were recognized as significantly higher in the protected volunteers after immunization compared to unprotected volunteers (***Figure 5b***). These proteins were the apical membrane antigen 1 (PfAMA1, gene ID PF3D7_1133400) and 3 fragments of the erythrocyte membrane protein 1 (PfEMP1, gene IDs PF3D7_0412900, PF3D7_1240400 and PF3D7_0711700). Interestingly, amino acid sequence alignment of the three identified PfEMP1 protein fragments with the predicted sporozoite encoded variant PF3D7_0809100, recently described to contribute to inhibition of hepatocyte invasion (***Zanghì et al., 2018***), demonstrated long stretches of linear protein sequence conservation (***Figure 5—figure supplement 3***). Nevertheless, the sample size of the protected group is low (n = 5), and considering the low effect size measured by Cohen's distance (***Figure 5—figure supplement 1***), no strong argument for association with protection of these four antigens can be made from this analysis. The mean immunogenicity of PfAMA1 and PfCSP levels across the protected group increased from baseline to after immunization, but not reaching the significance threshold (***Figure 5c***). Notably, a trend of higher reactivity levels to the 3 PfEMP1 fragments in the protected group was observed at baseline, albeit not significantly (***Figure 5a***). No antigen showed an increase in reactivity (delta) significantly higher in the protected group (***Figure 5—figure supplement 2***).

Breadth of humoral immune response in protected individuals

Analysis above indicates that breadth of humoral immune response was highly variable in samples across all groups and mostly conserved from baseline to post-immunization levels, irrespective of

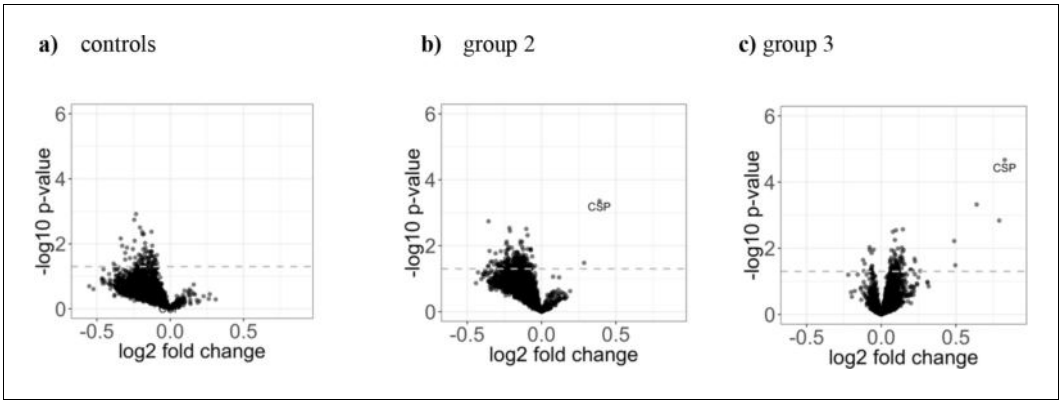

Figure 4. Increase in antigen recognition from baseline to after PfSPZ vaccination is moderate. The three volcano plots in the upper row show (**a**) the mean fold change in the control group (n = 8), (**b**) in group 2 (1.35×10^5 PfSPZ Vaccine/dose) (n = 18), and (**c**) in group 3 (2.7×10^5 PfSPZ Vaccine/dose) (n = 20). In all groups, the samples collected at baseline and two weeks past last vaccination were compared. The dashed line represents the threshold of statistical significance (p=0.05) not adjusted for the FDR (none of the antigens had a FDR adjusted p-value<0.05). For effect size estimates see ***Figure 4—figure supplement 1***.

The online version of this article includes the following source data and figure supplement(s) for figure 4:

Source data 1. Source data for plot **a**.
Source data 2. Source data for plot **b**.
Source data 3. Source data for plot **c**.
Figure supplement 1. Effect size for the increase in antigen recognition from baseline to after PfSPZ vaccination.
Figure supplement 2. Differential antigen reactivity between control and immunization groups is moderate.
Figure supplement 2—source data 1. Source data for plot **a**.
Figure supplement 2—source data 2. Source data for plot **b**.
Figure supplement 2—source data 3. Source data for plot **c**.
Figure supplement 3. Variance and mean of the $\log_2$ signal intensities.

protection status (***Figure 3***). We thus further compared breadth of humoral immune response in the protected versus unprotected individuals. Despite a mean breadth in the protected group 12% higher than in the unprotected individuals before immunization ($breadth_{protected}$ = 914, $breadth_{unprotected}$ = 810) and 28% higher after immunization ($breadth_{protected}$ = 943, $breadth_{unprotected}$ = 738), as antibody breadth was highly over-dispersed and sample size in the protected group small we found that differences in the means are not significant for either time points (logistic regression p-values of 0.6 and 0.3, respectively) (***Figure 6***). Furthermore, there was also limited discrimination by protection status in antibody breadth at both time points via receiver operating characteristics (ROC) analysis (area under the ROC curve: $AUC_{pre\text{-}immunization}$ = 0.64, Wilcoxon rank sum test W = 60, p-value=0.35; $AUC_{post\text{-}immunization}$ = 0.73, Wilcoxon rank sum test W = 44, p-value=0.1).

Finally, in order to identify antibodies consistently present in the samples of the protected individuals, we defined common antigens to a group as antigens which are reactive in at least 80% of the samples for each of the groups (i.e. considering the signal intensity of a given antigen as a binary outcome, either reactive or non-reactive). Common antigens in the protected group (reactive in at least 4 out of the five samples) were higher than in the unprotected groups (reactive in at least 27 out of the 33 samples) both 2 weeks after last immunization (383 common antigens in the protected group versus 58 in the unprotected group, 2-sample test for equality of proportions with continuity correction p-value<2.2E-16, N = 2804) (***Figure 7b***), and at baseline (350 reactive antigens in protected group versus 62 in unprotected group, p<2.2E-16) (***Figure 7a***). Both at baseline and 2 weeks after immunization, almost all reactive antigens in common in the unprotected group were also present in the protected group (60 out of 62 and 56 out of 58 antigens at baseline and after immunization, respectively) (***Figure 7***). The trend for higher common antigens in the protected group compared to the unprotected group was also noticeable for different thresholds, comparing antigens reactive in at least 60% or in 100% of the samples in a given group (***Figure 7—figure supplement 1***). Given the large difference in sample sizes between the protected (n = 5) and unprotected (n = 33) groups, bootstrap samples with n = 5 samples in each group were repeatedly drawn

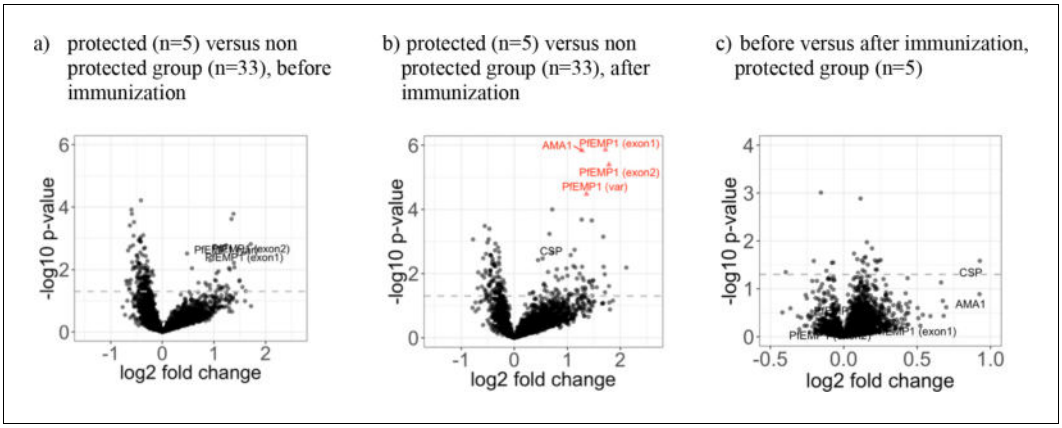

Figure 5. The five volunteers protected against homologous CHMI showed higher recognition of four distinct proteins after immunization. The mean fold change between antigen reactivity in the protected (n = 5) and the non-protected (n = 33) individuals from groups 2 and 3 are represented in volcano plots (**a**) for baseline and (**b**) after PfSPZ vaccination, plotted against the inverse $\log_{10}$ t-test p-value. In the protected group (n = 5), the samples collected at baseline and two weeks past last vaccination were compared and the mean fold change of the increased immunogenicity is showed in (**c**). Red triangles represent antigens with significant differences in antibody levels between protected and non-protected volunteers after BH adjustment of p-values, although size effect measured by Cohen's distance remains low (see ***Figure 5—figure supplement 1***). The dashed line represents the threshold of statistical significance for the unadjusted p=0.05.

The online version of this article includes the following source data and figure supplement(s) for figure 5:

Source data 1. Source data for plot a.
Source data 2. Source data for plot b.
Source data 3. Source data for plot c.
Figure supplement 1. The increased recognition of four distinct proteins in the protected group show small effect size.
Figure supplement 2. No antigens show a significant differential antigen reactivity between the protected and unprotected group.
Figure supplement 2—source data 1. Source data for plot.
Figure supplement 3. Multiple sequence alignment of four PfEMP1 protein fragments.

(repeated 1000 times), with replacement. Consistent with previous analyses above, we find a higher number of common antigens in the protected compared to the unprotected group, although uncertainty due to small sample size is inevitable (***Figure 7—figure supplement 1***). Taken together this analysis suggests a higher number of commonly recognized antigens in the protected individuals after immunization and also at baseline, but the findings are limited by a small sample size in the protected group. A list of the commonly reactive antigens and antigens with increased reactivity levels following immunization per groups can be found in the ***Figure 7—source data 1***.

To understand the biological function of proteins reactive in protected volunteers, we used DeepLoc subcellular localization prediction, Pfam protein family prediction (***El-Gebali et al., 2019***), and gene ontology prediction available on Plasmodb.org (***Huntley et al., 2015***) and identified protein characteristics and distinct functional categories with higher representation in the protected volunteers. Predicted cell membrane proteins were more broadly recognized in the five protected volunteers at baseline (63 vs. 10 proteins, 2-proportions Z-test p-value=2.53E-9) and post-immunization (70 vs. 5 proteins, p-value=4.11E-13), but so were intracellular proteins localized to the cytoplasm, endoplasmic reticulum, Golgi apparatus, mitochondrion and nucleus (all p-values<0.05) (***Table 1***). Gene and protein families present in both protected and non-protected groups at both time points included integral components of membranes, host cell plasma membranes and infected host cell surface knobs, signal receptor activity and cell adhesion molecule binding, pathogenesis, cell-cell adhesion, antigenic variation and cytoadherence to the microvasculature, and PfEMP1-related families (see ***Supplementary file 1***) . Gene and protein families uniquely reactive in the protected volunteer group at either or both time points included Mauer's cleft, host cell surface receptor binding, regulation of immune response, the Rifin protein family, a head domain of trimeric autotransporter adhesins (TAAs) family that acts as virulence factors for Gram-negative bacteria and have a head-stalk-anchor structure, a procyclic acidic repetitive protein family that was identified as abundant surface

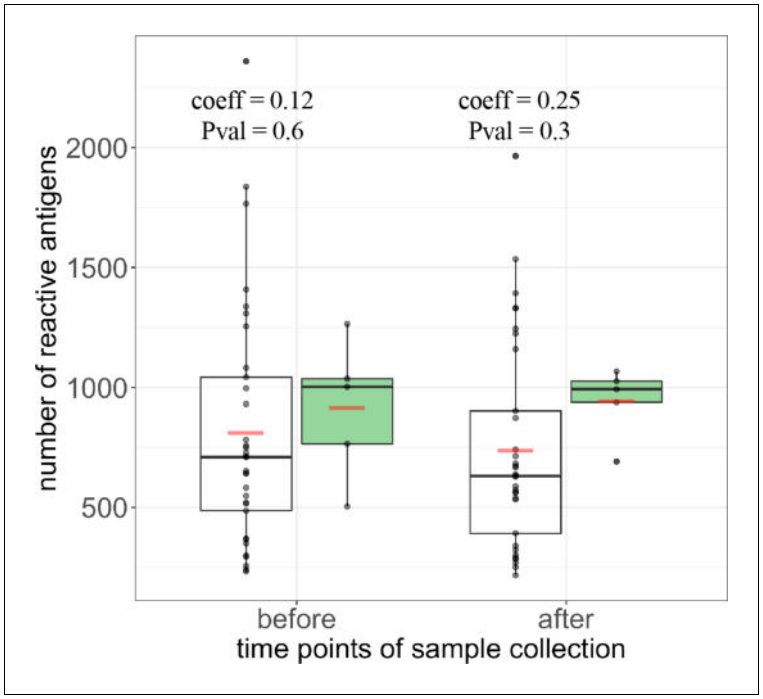

Figure 6. Breadth and magnitude of Pf-specific humoral immunity in protected and unprotected individuals. (a) Breadth counts of all PfSPZ vaccinees grouped by protection status following CHMI, with protected group in green and non-protected volunteers depicted in white, before and after immunization. The coefficient estimate with corresponding p-values from the negative binonial test is indicated for each time point. Boxplots show median, interquartile range (IQR) and 1.5xIQR limits and red bars represent the mean.

proteins in *Trypanosoma brucei*, and an N-terminal PRP1 splicing factor family involved in mRNA splicing (***Figure 7—source data 1***).

Discussion

We provide first time data on the proteome-wide antibody profiling study for malaria endemic populations enrolled in a PfSPZ Vaccine phase Ib trial with homologous CHMI. Our analysis provides not only insights to PfSPZ vaccine induced humoral immune response and potential association with protection, but also a comprehensive view of underlying naturally acquired immunity in healthy Tanzanian adult men. The protein microarray used in this study considered a near full-proteome coverage (***Aurrecoechea et al., 2009***), contained full length or fragmented proteins representing 4805 (91%) of the approximately 5400 protein-coding genes in the *P. falciparum* 3D7 genome, the remaining 3D7 genes being tiny (<150 bp), challenging to clone or express or non-protein-coding genes. We found that a large proportion of the proteome was immunogenic in this study population, with personalized profiles detected by the protein microarray only moderately altered in response to PfSPZ Vaccine immunization. The moderate number of protected individuals (compared to volunteer infection studies in naïve populations), the heterogeneous immune fingerprint in the study population, and the potentially higher breadth of humoral immune response in the protected individuals highlight a complex picture to understand if humoral immune mechanisms lead to protection. Importantly, this study suggests that whole sporozoite vaccines predominantly boost pre-existing immunity of pre-exposed adults as a result of the natural imprinting of individual immune responses. Additionally, the moderate number of protected individuals seem to indicate that boosting pre-existing humoral response might not be sufficient to induce sterile protection against infection. If confirmed, these findings have implications for the role of such vaccines in endemic populations and thus prompts the need for studies to define appropriate target ages for immunization in settings of variable levels of pre-exposure.

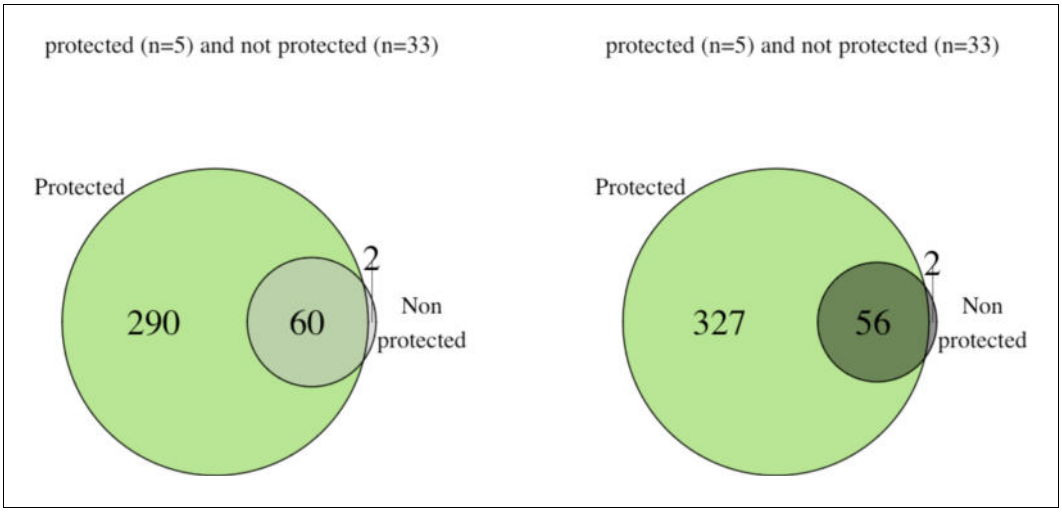

Figure 7. Protected individuals showed higher numbers of reactive antigens compared to the non protected group. The number of antigens that were reactive in at least 80% of the individual in each group are represented for (**a**) baseline for the protected group (green, n = 5) and the unprotected group (grey, n = 33) and for (**b**) after immunization for the protected group (green) and unprotected group (grey).
The online version of this article includes the following source data and figure supplement(s) for figure 7:

Source data 1. Table of commonly recognized antigens .
Figure supplement 1. The number of commonly recognized antigens, per threshold.

The humoral immune response in the malaria pre-exposed study population was broad, with 2239 unique proteins considered reactive among the serum samples tested. This large number of immunoreactive targets is surprising, especially considering that the majority of targets are predicted to be localized intracellularly. This is in stark contrast to a recent panproteome-wide analysis of antibody-binding targets for *Streptococcus pneumoniae* proteins, which identified a more restricted set of a few hundred antigens and a clear association of immunoreactivity with cell surface localized, albeit functionally diverse, pneumococcal proteins (***Croucher et al., 2017***). The tendency of malaria antigens to span a more comprehensive set of cellular compartments is likely due to the increased complexity of the life cycle and chronicity of infection. In hepatocytes and erythrocytes, the biological processes of schizogony occur in parasitophorous vacuoles culminating in the destruction of the host cell and likely release of numerous abundant parasite proteins into the immune system (***Cowman et al., 2016***). We speculate this results in substantial immune responses to intracellular parasite proteins and a significant dedication of host immune resources that are potentially functionally irrelevant for control of infection. However, the potential for these proteins to initiate cascades of cellular responses that aid in parasite clearance should not be discarded and could be the focus of future high throughput cellular antigen discovery.

Volunteers had a personalized antibody profile at baseline, and this profile remained at the same level of individual complexity after repeated PfSPZ Vaccine inoculation. Most individuals had an extensive breadth of immune response, but the number of reactive antigens that the samples have in common was low (around 10%). This implies that the majority of reactive antigens are distinctly recognized at the individual level and confirming uniqueness of reactivity profiles across individuals, further suggesting clonal imprinting is occurring in malaria immunity. Such imprinting is consistent with antigen recognition being less variable in adult populations experiencing seasonal exposure compared to children (***Taylor et al., 1996***), with adults consistently either seropositive or seronegative for a specific antigen throughout the transmission season. Of note, antibody 'fingerprints' were recently reported in volunteers of a *S. pneumoniae* whole cell vaccine trial, whereby the overall antibody profile remained consistent even following multiple exposures to killed pneumococci (***Campo et al., 2018***). A recent human cytomegalovirus (HCMV) vaccine study also found immune fingerprints where the vaccine boosted pre-existing immune responses implying a substantial effect of prior natural infection on vaccine induced immune responses for HCMV (***Baraniak et al., 2019***). These examples along with a review on this possible mechanism of fingerprinting in nine pathogens,

including Pf (**Vatti et al., 2017**), indicate that the phenomenon of 'original antigenic sin' which is known in the context of seasonal influenza vaccine goes beyond virus diseases. Our findings add to the increasing knowledge that – across the taxa - the personalized immune and metabolic status and history of pathogen exposure may affect vaccine take and the potential to elicit high levels of protection in vaccinated populations (**Vatti et al., 2017**; **Tsang et al., 2020**; **Hill et al., 2020**; **Baraniak et al., 2019**).

A consistent picture is emerging that pre-exposure limits the magnitude of vaccine induced responses. The same immunization regimen as group 3 in our Tanzanian study was administered to Malian adults, with antibody responses to PfCSP 6 to 7-fold lower in Malians than in the Tanzanians (**Jongo et al., 2018**; **Sissoko et al., 2017**). In contrast, higher anti-PfCSP levels were found following immunization in naïve U.S. vaccinees measured by same ELISA (**Epstein et al., 2017**; **Seder et al., 2013**) as well as in unpublished proteome-wide analysis (**Campo et al., 2018**), and in proteome-wide responses in European volunteers given chemo-attenuated sporozoites (PfSPZ-CVac) (**Mordmüller et al., 2017**). Studies of immunity induced after RTS,S vaccination have shown that anti-PfCSP titres are lower in Kenyan adults compared to naïve U.S. subjects, and a recent review on the immune response in RTS,S trials led to the hypothesis that pre-exposure might generate natural imprinting (**Vekemans, 2017**). It is possible that, as with PfCSP antibodies, the magnitude of vaccine-induced responses to other parasite antigens were lower in Tanzanian vaccinees than their U.S. and E.U. counterparts (**Kester et al., 2009**; **Polhemus et al., 2009**).

Although we cannot conclude on correlates of protection from this study, we did find an extensive breadth of humoral immune response in protected individuals indicating an underlying immune profile of wider antigen recognition compared to non-protected. Breadth is known to be associated with reduced risk of malaria (**Osier et al., 2008**; **Daou et al., 2015**), and anti-malaria protection likely to be the sum of protective immunity across different antigens, a concept known as the threshold of immune response (**Doolan and Hoffman, 1997**). Several recent protein microarrays studies found a range of antigens that were associated with protection (**Crompton et al., 2010**; **Trieu et al., 2011**; **Dent et al., 2015**). However, given the similar breadth profiles in the placebo group before and after placebo inoculation and the lack of any protected individuals in that group, it is more likely that the tendency for protected individuals to have higher antibody breadth represents a predisposition toward more effective PfSPZ Vaccine 'take', indicating that greater breadth prior to immunization may positively impact vaccination outcome. IgG specific to PfAMA1 and 3 variants of PfEMP1 were higher before CHMI in protected versus non-protected volunteers, but given the heterogeneous immune responses across volunteers, and the limited number of protected individuals, these antigens are not proposed as the mechanism for PfSPZ Vaccine induced immunity from this study. The three identified PfEMP1 antigens had higher levels in the protected individuals at baseline, indicating that higher levels of naturally primed, pre-existing PfEMP1 antibodies might be able to cross-react with other PfEMP1 proteins during CHMI.

We further found recognition of many more protein families in the protected group, including cell membrane proteins and numerous intracellular proteins. Protein families and functional categories uniquely identified in the protected group included the sporozoite protein SSP2/TRAP, a protein required for invasion of hepatocytes (**Mota and Rodriguez, 2004**). Interestingly, SSP2/TRAP antibodies were also detected in 26% of U.S. volunteers immunized with the same regimen of PfSPZ Vaccine as group 3 (**Epstein et al., 2017**). Additionally, the Rifin family, which are variant surface antigens exported to the infected erythrocyte surface and implicated in cytoadherence to the microvasculature and severe malaria (**Goel et al., 2015**) and the Mauer's cleft, which is involved in trafficking variant surface antigens, including Rifins and PfEMP1s, to the infected erythrocyte surface (**Mundwiler-Pachlatko and Beck, 2013**). While in many cases the magnitude of individual antibody responses was not significantly associated with protection, the analysis of protein families and functional categories can shed light on the types of antigens that may be targeted by a broad repertoire of antibodies conferring protection.

Given the small number of protected individual (n = 5) and the highly heterogeneous immune responses among the volunteers, results on potential association with protection of both individual antigens and breadth of humoral response in this study require further evaluation. Furthermore, four volunteers were protected in the highest immunization dose group (total of 1.35 million radiation attenuated sporozoites). Of particular note, the overall declining trend in antibody levels post-vaccination in both the lower dose and placebo groups was not evident in the higher dose group. Taken

together, this indicates that vaccination with a higher dose induced a maintained immune status over time, and thus increasing doses in African pre-exposed volunteers may rescue vaccine immunogenicity and an active memory B cell pool. Larger trials will be required to confirm or reject that higher immunization doses lead to increased protection level in pre-exposed adults, with recent studies indicating a four-fold increase in immunization dose did not increase efficacy compared to a two-fold increase (**Jongo et al., 2019**). Our results pertain to adults only and, consistent with the theory that high malaria pre-exposure reduces vaccine induced immune response, a PfSPZ Vaccine may well induce higher immune responses in children of endemic areas compared to adults (**Jongo et al., 2019**). Further studies are needed to understand the level of protection a PfSPZ Vaccine in all age groups and consequently, the likely vaccine efficacy achieved in a mass vaccination strategy.

Both PfSPZ Vaccine induced humoral and cellular immune response have been observed and associated with protection in studies in naïve volunteers, but an association of either or both of these responses with protection in pre-exposed populations is unclear. Previously, the primary immune response to PfSPZ immunization was thought to be cellular based (**Epstein et al., 2011**) including that increased cellular immune response following PfSPZ immunization observed in malaria naïve individuals (**Ishizuka et al., 2016**). However, these responses measured in peripheral blood are not correlated with protection in pre-exposed immunized adults assessed by CHMI (**Jongo et al., 2018**; **Jongo et al., 2019**), likely due to protective cellular immune effector cells residing in the liver (**Ishizuka et al., 2016**). Furthermore, there is accumulating evidence that PfSPZ Vaccine induces or boosts humoral immunity to a surprisingly limited number of antigens in pre-exposed adults, with no correlation between anti-CSP antibody titres and CHMI protection (**Jongo et al., 2018**; **Jongo et al., 2019**; **Sissoko et al., 2017**). This is despite previous reports that antibody responses are induced in US naïves (**Ishizuka et al., 2016**) and functional antibodies have been isolated from PfSPZ studies in Tanzania (**Tan et al., 2018**; **Zenklusen et al., 2018**).

Although we undertook an unbiased analysis approach, the use of microarrays comes with limitations. Firstly, false-positive discovery adjustments for protein microarray analysis potentially underestimate the association of antigens, especially where differences are subtle and heterogeneity between the samples is high. Secondly, as for most protein microarray studies, sample sizes replicates were not preformed. Nevertheless, the immune fingerprint was similar for both samples before and after immunization for each volunteer. As the array experiment was designed to balance samples across experimental nuisance factors, such as study day and sample order, it is unlikely that variability observed between volunteers is attributable to non-reproducibility of the experiment. Thirdly, the proteins and protein fragments of the microarrays are produced in a cell-free environment resulting in several epitopes lacking post-translational modifications and potentially folded into unnatural conformations that therefore might not be bound by specific serum antibodies, resulting in false-negative results (**Doolan et al., 2008**). For this reason, this technology has been described as a 'rule-in' and not a 'rule-out' method (**Stone et al., 2018**). Lastly, the paucity of information on the many functionally uncharacterized proteins present in the 5400 gene Pf proteome led to reliance on primarily *in silico* sequence prediction software to classify protein functional categories. Nevertheless, despite malaria-specific inaccuracies (details in Materials and methods), the DeepLoc analysis used in this study alongside gene ontology and protein family analysis provides valuable insight into overall distributions of the thousands of reactive antigens and those most recognized in protected individuals.

As information on the volunteers was limited, and as per design the study selected an apparently homogeneous population, it was impossible in the current analysis to examine associations of immune responses to different parasite or host factors. Thus, we cannot exclude associations between breadth of humoral immune responses, geographic location, immunogenetic background, transmission intensity, or other factors. Given the complexity and personalized immune response, we expect that much larger sample size, or a population meta-analysis, would be needed to identify any pattern of humoral response associated with host and parasite factors.

Further reduction and eventual elimination of malaria requires significant investment and research and development of new tools, including vaccines or other immune therapies (**Greenwood, 2008**). Our proteome-wide analysis indicates the breadth of antibody repertoire to Pf malaria is extensive and highly variable between individuals who are pre-exposed. Our findings and those from other PfSPZ Vaccine trials in Africa are subject to confirmation with future research studies before any

guidance can be made on the impact of pre-exposure on PfSPZ Vaccine efficacy and the implications for vaccination strategies. Nevertheless, we suggest, if these findings are confirmed, that the underlying, but difficult to assess, level of pre-exposure and resulting immune imprinting at the individual level may result in a more heterogeneous response to PfSPZ Vaccine. If personalized responses occur in pre-exposed individuals, then populations from different endemic regions cannot be considered homogeneous, and this will impact likely vaccination strategies. Similar to recent evidence for other pathogens (**Baraniak et al., 2019**; **Campo et al., 2018**; **Vatti et al., 2017**; **Tsang et al., 2020**), the potential impact of natural imprinting of humoral immune response in malaria deserves further investigation. Timing and duration of imprinting (infant age, childhood or throughout adulthood), as well as the role of co-infections and other yet to be identified host or environmental factors, are unknown. Without further fundamental studies, additional hurdles for future vaccine trials remain in regards to the validity of extrapolating vaccine outcomes from trials in naïve cohorts to pre-exposed populations and different age groups.

Materials and methods

Ethic statement

The study was approved by institutional review boards (IRBs) of the IHI (Ref. No. IHI/IRB/No:02–2014), the National Institute for Medical Research Tanzania (NIMR/HQ/R.8a/Vol.IX/1691), the Ethikkommission Nordwest-und Zentralschweiz, Basel, Switzerland (reference number 261/13), and by the Tanzania Food and Drug Authority (Ref. No.TFDA 13/CTR/0003); registered at Clinical Trials.gov (NCT02132299); and conducted under U.S. FDA IND 14826.

Study design of the original trial

The design and outcome of the clinical study is described in detail in **Jongo et al., 2018**. Briefly, volunteers were immunized five times with a lower (1.35×10^5) or a higher dose (2.7×10^5) of PfSPZ Vaccine by direct venous inoculation (DVI) at 4 week intervals for the first four vaccinations followed by a last booster with PfSPZ Vaccine after 8 weeks. After immunization, volunteers underwent CHMI either 3 weeks after last immunization, 24 weeks after last immunization, or both using 3200 non-attenuated aseptic, purified, cryopreserved, infectious PfSPZ of PfSPZ Challenge administered by DVI. Serum samples for microarray analysis were collected at baseline (before immunization) and 2 weeks after last immunization in the individuals who underwent CHMI at 3 weeks after last immunization (**Figure 1**).

The 36 volunteers were healthy, adult males between 18–35 years old, with no parasitemia at the start of the study (measured by TBS and antibodies to PfEXP1 by ELISA), no history of malaria episodes over the last 5 years, and no parasitemia before CHMI (measured by TBS and qPCR) (**Jongo et al., 2018**). They were all students in Dar Es Salaam at the time of the study, however home town or travel history was not specified; thus, history of geographic exposure is not known.

Protein array chip design

The protein microarray used in this study was produced by Antigen Discovery, Inc (ADI) and encompasses 7455 full-length or fragmented Pf proteins representing 4805 protein-coding genes and covering 91% of the proteome (**Mordmüller et al., 2017**). As previously described (**Felgner et al., 2013**) proteins were expressed from a library of Pf partial or complete open reading frames (ORFs) cloned into a T7 expression vector pXI using an in vitro transcription and translation (IVTT) system, the *Escherichia coli* cell-free Rapid Translation System (RTS) kit (5 Prime). This library was created via an *in-vivo* recombination cloning process with PCR-amplified Pf ORFs, and a complementary linearized expressed vector transformed into chemically competent *E. coli* was amplified by PCR and cloned into pXI vector using a high-throughput PCR recombination cloning method (**Davies et al., 2005**). Each expressed protein includes a 5′ polyhistidine (HIS) epitope and 3′ haemagglutinin (HA) epitope. Proteins were expressed according to manufacturer's instructions and then translated proteins were printed onto nitrocellulose-coated glass AVID slides (Grace Bio-Labs) using an Omni Grid Accent robotic microarray printer (Digilabs, Inc). Quality checks of the microarray chip printing and protein expression were performed by probing random slides with anti-HIS and anti-HA monoclonal antibodies with fluorescent labelling. In addition to the 7,455 Pf peptide fragments, each microarray

chip contained 302 IgG positive control spots as an assay control and 192 in vitro Transcription and Translation (IVTT) control spots (IVTT reactions with no Pf ORFs) as a normalization factor. All the spotted proteins were printed in three replicated pads per slide to accommodate one sample per pad. The experiment included two chips that made up the full proteome microarray, and samples were probed on each chip. Due to cost constraints, we did not replicate the experiment. Prior to probing samples, a balanced array experimental design was generated to mitigate nuisance factors, including pad position and day that sample was assayed, against sample grouping factors such as time point, dosing group and protection status. Sample balancing factors were provided as blinded, coded variables by Sanaria, Inc to ADI and unblinded following data acquisition.

Sample probing

Sample probing has been previously described elsewhere (***Campo et al., 2015***; ***Mordmüller et al., 2017***). Briefly, serum samples were diluted 1:100 in a 3 mg ml−1 *E. coli* lysate solution in protein arraying buffer (Maine Manufacturing) and incubated at room temperature for 30 min. Chips were rehydrated in blocking buffer for 30 min. Blocking buffer was removed, and chips were probed with serum samples by incubating in sealed, fitted slide chambers to ensure no cross-contamination of sample between pads. Chips were incubated overnight at 4˚C with agitation. Chips were washed five times with TBS-0.05% Tween 20, followed by incubation with biotin-conjugated goat anti-human IgG (Jackson ImmunoResearch) diluted 1:200 in blocking buffer at room temperature. Chips were washed three times with TBS-0.05% Tween 20, followed by incubation with streptavidin-conjugated SureLight P-3 (Columbia Biosciences) at room temperature protected from light. Chips were washed three times with TBS-0.05% Tween 20, three times with TBS, and once with water. Chips were air dried by centrifugation at 1000 g for 4 min and scanned on a GenePix 4300A High-Resolution microarray scanner (Molecular Devices), and spot and background intensities were measured using an annotated grid file (.GAL). Data adjusted for local background by subtraction were exported to Microsoft Excel as CSV files and subsequently imported into R (***R Development Core Team, 2015***) where all subsequent data processing occured.

Protein array data processing

Signal intensities were transformed by base two logarithm, and the median of IVTT control spots for each sample was subtracted from the sample-specific IVTT Pf antigen signals, a method that has been used previously in protein microarray analysis (***Mordmüller et al., 2017***; ***Felgner et al., 2013***). A seropositive threshold was defined as two times IVTT control signals, or 1.0 on the $\log_2$ scale. A value of 0.0 +/- 1 represents signal intensities that are equivalent to the background. Values below −2, representing less than 0.25 times the median IVTT control signals were adjusted to −2. This affected 1639 of the 685,860 signals included in the complete dataset and 42 of the 257,968 signals included in the set of reactive antigens. Reactive antigens were defined as proteins that were seropositive in at least 10% of the study population at one or more time points. High level group reactivity was defined as 80% seropositivity to one probe in vaccinees who received either the lower (group 2) or higher (group 3) doses of PfSPZ Vaccine.

Analysis

To visualize the high dimensional dataset of the microarray spots and understand potential patterns or clustering of the samples, t-SNE analysis (***Maaten and Hinton, 2008***) was used with a perplexity value of 30 and with 10,000 iterations. The t-SNE algorithm was applied the 92 samples of the entire dataset of the 2804 $\log_2$-transformed signal intensities or for the subset of 441 reactive proteins fragments predicted to be expressed at the sporozoite stage (***Florens et al., 2002***). The breadth of immune response for each individual was defined as the total number of positive reactive antigens for each serum draw. Breadth data were identified as over-dispersed after observing that the variance was greater than the means. Therefore, breadth between different groups was compared using negative binomial regression. The frequencies of reactive antigens summed into subcellular localization categories for each group were tested using 2-propotions Z-test and p-values adjusted using the BH method. Gene Ontology (GO) annotation for each protein was retrieved from PlasmoDB.org. Protein families were queried using amino acid sequences for each protein using the Pfam database

(*El-Gebali et al., 2019*). Fisher's exact tests were used to assess reactivity of each GO category of Pfam functional group, followed by p-value adjustment using the BH method.

The mean reactivity of each antigen per study group (control, group 2 and group 3) 2 weeks after immunization was compared with baseline levels (pre-immunization), using the paired t-test and by adjusting for false discovery rates (FDR) with the Benjamini-Hochberg method (BH) (*Benjamini and Hochberg, 1995*). One individual in group 2 who received 4 instead of 5 doses of immunization was excluded from this analysis. 'Delta' was defined as the fold change in antibody reactivity for each antigen before and after immunization. The average delta was compared between the vaccinated and the control groups using the unpaired t-test, and the resulting p-values were adjusted with the BH method. Because of the heteroscedastic nature of the normalized $\log_2$ signal intensities (*Figure 4—figure supplement 3*) we preferred the ordinary t-test over the empirical Bayes test (eBayes) (*Smyth, 2004*), which is sometimes used to compare signal intensities in microarray experiments. Due to the large number of positive probe signals and changes between time points, the eBayes could estimate a prior distribution that is over-dispersed relative to the paired t-test resulting in reduced power to detect changes in outlier responses, whereas eBayes may be more suitable for full proteome microarray studies with more restricted immunoreactivity profiles (*Campo et al., 2018*).

Common antigens to a group were defined as antigen which are reactive in at least 60%, 80% or 100% of the samples of the group and differences in frequencies of commonly recognized antigens between groups were assessed with a 2-sample test for equality of proportions with continuity correction. To account for the large difference in sample sizes between the protected (n = 5) and unprotected (n = 33) groups, bootstrap samples with n = 5 samples in each group were repeatedly drawn (repeated 1000 times), with replacement.

Amino acid sequences for each protein were queried using the DeepLoc online program to predict subcellular localization of each protein (*Almagro Armenteros et al., 2017*). Algorithms for prediction of subcellular localization of eukaryotic parasite proteins have not been trained like they have for Gram negative and positive bacteria, mammalian, plant and fungal cells, and DeepLoc program was trained to the latest UniProt dataset that reported higher accuracy using primary sequence input and deep neural networks over methods reliant on homology (*Almagro Armenteros et al., 2017*). No subcellular localization categories exist in these algorithms for the specialized organelles of malaria, such as the rhoptries and micronemes, which exocytose internal parasite proteins to the surface of the parasite membrane, parasitophorous vacuoles or host cell cytoplasm and membrane. Thus, many proteins such as the majority of PfEMP1 variants were misclassified as localized to the endoplasmic reticulum, golgi apparatus, cytoplasm or nucleus. The parasite apicoplast is also absent from these algorithms. Training to these organelles can only be done with a substantial database of sequences with which to train models, which is currently in limited supply for eukaryotic parasites.

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Additional information

Competing interests

Joseph J Campo, Timothy Q Le, Amit Oberai, Christopher Hung, Jozelyn V Pablo, Andy A Teng, Xiaowu Liang: is an employee of Antigen Discovery, Inc. B Kim Lee Sim, Stephen L Hoffman: is employed by Sanaria. Sanaria Inc manufactured PfSPZ Vaccine and PfSPZ Challenge. Thus, all authors associated with Sanaria have potential conflicts of interest. The other authors declare that no competing interests exist.

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Author contributions

Flavia Camponovo, Software, Formal analysis, Visualization, Methodology, Writing - original draft, Writing - review and editing; Joseph J Campo, Conceptualization, Data curation, Formal analysis, Supervision, Investigation, Writing - original draft, Writing - review and editing, Performed the protein localization; Timothy Q Le, Data curation, Investigation, Perform microarray experiment; Amit Oberai, Investigation, Performed the protein localization predictions; Christopher Hung, Jozelyn V Pablo, Andy A Teng, Data curation, Investigation, Performed the protein microarray experiments; Xiaowu Liang, Data curation, Investigation; B Kim Lee Sim, Funding acquisition, Conducted and supervised pharmaceutical operations, vaccine shipments of investigational products, Sanaria Inc sponsored the BSPZV1 study; Said Jongo, Resources, Conducted and supervised the BSPZV1 clinical trial; Salim Abdulla, Resources, Conducted and supervised the BSPZV1; Marcel Tanner, Supervision, Writing - review and editing; Stephen L Hoffman, Funding acquisition, Writing - review and editing, Sponsored the BSPZV1 study; Claudia Daubenberger, Conceptualization, Resources, Supervision, Writing - original draft, Writing - review and editing, Conducted and supervised the BSPZV1 study; Melissa A Penny, Conceptualization, Formal analysis, Supervision, Funding acquisition, Validation, Visualization, Methodology, Writing - original draft, Project administration, Writing - review and editing

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Decision letter and Author response

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Author response https://doi.org/10.7554/eLife.53080.sa2

Additional files

Supplementary files

- Source data 1. Gene Ontology prediction for the molecular function of the Pf genes.
- Source data 2. Gene Ontology prediction for the cellular component of the Pf genes.
- Source data 3. Gene Ontology prediction for the biological process of the Pf genes.
- Source data 4. Pfam database for the prediction of protein families.
- Supplementary file 1. Gene and protein families present in the protected versus non protected groups. This table lists Pfam protein family prediction (***El-Gebali et al., 2019***), and gene ontology prediction available on Plasmodb.org (***Huntley et al., 2015***) and identified protein characteristics and distinct functional categories which were identified as being reactive in at least 80% of the protected or non protected group before and after immunization. Reactive proteins were associated to each group using the Fisher's exact test, and p value correct using the Benjamini-Hochberg method (BH) (***Benjamini and Hochberg, 1995***). Pfam and GO description were found in https://www.ebi.ac.uk/QuickGO/ and https://biocyc.org/ and https://www.ebi.ac.uk/QuickGO/ and https://biocyc.org/, respectively. See also ***Source datas 1–4***.
- Transparent reporting form

Data availability

All data analyzed during this study are included in the manuscript and supporting files, or cited accordingly when published elsewhere.

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A public antibody lineage that potently inhibits malaria infection by dual binding to the circumsporozoite protein

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Abstract

Immunization with attenuated *Plasmodium falciparum* sporozoites (PfSPZ) has been shown to be protective, but the features of the antibody response induced by this treatment remain unclear. To

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Supplementary information is available on the online version of the paper.

Author Contributions J.T. characterized monoclonal antibodies, analyzed the data and wrote the manuscript; B.K.S. performed *in vivo* assays, analyzed the data and wrote the manuscript; D.O. performed structural analysis, analyzed the data and wrote the manuscript; I.Z. collected samples, conducted *in vitro* assays, analyzed the data and wrote the manuscript; L.Pi. characterized monoclonal antibodies, analyzed the data and wrote the manuscript; S.B. sequenced and expressed antibodies; M.F. performed bioinformatics analysis; C.S.F. immortalized memory B cells; J.M and L.Pe. immunized mice; S.J. supervised cohorts; S.A. oversaw the clinical trial and provided PBMCs to the laboratory team; G.C. provided PfCSP peptides; L.V. designed peptide and antibody mutants; F.S. and S.H.I.K. provided supervision; S.L.H. and B.K.L.S. produced PfSPZ Vaccine and PfCSP, prepared the syringes used to immunize, and provided PfSPZ for antibody assays, including screening of mAbs; C.D. handled cohorts and provided supervision; I.A.W. supervised structural analysis and wrote the manuscript; A.L. provided overall supervision, analyzed the data and wrote the manuscript.

Competing Financial Interests A.L. is the scientific founder and shareholder of Humabs BioMed. F.S. is a shareholder of Humabs BioMed. Sanaria Inc. manufactured PfSPZ Vaccine and PfSPZ Challenge.

investigate this response at high resolution, we isolated IgM and IgG monoclonal antibodies from Tanzanian volunteers who were immunized by repeated injection of irradiated PfSPZ and who were found to be protected from controlled human malaria infection (CHMI) with infectious homologous PfSPZ. All IgG monoclonals isolated bound to *P. falciparum* circumsporozoite protein (PfCSP) and recognized distinct epitopes in the N-terminus, NANP repeat region, and C-terminus. Strikingly, the most effective antibodies, as assessed in a humanized mouse model, bound not only to the repeat region, but also to a minimal peptide at the PfCSP N-terminal junction that is not in the RTS,S vaccine. These dual-specific antibodies were isolated from different donors and used VH3-30 or VH3-33 alleles carrying tryptophan or arginine at position 52. Using structural and mutational data, we describe the elements required for germline recognition and affinity maturation. Our study provides potent neutralizing antibodies and relevant information for lineage-targeted vaccine design and immunization strategies.

Malaria is a serious global health threat, causing 445,000 deaths and 216 million clinical cases in 2016[1]. Much of the effort to develop a vaccine against the disease has focused on *Plasmodium falciparum* sporozoites (PfSPZ), the asymptomatic parasite stage that is injected by mosquitoes into the host skin to initiate a malaria infection. After entering the skin, PfSPZ migrate to the liver, multiply in hepatocytes and then emerge in the blood, where the parasites cause malaria symptoms and differentiate into sexual stages for transmission. While natural infection by PfSPZ elicits little or no protective immunity to this stage of the life cycle[2,3], subunit or whole organism vaccines based on PfSPZ can induce robust immune responses[4–6]. The most advanced malaria vaccine candidate, RTS,S, incorporates part of the *P. falciparum* circumsporozoite protein (PfCSP), which coats the PfSPZ surface and plays a key role in parasite migration out of the skin, entry into the liver parenchyma and invasion of hepatocytes[7–12]. Multi-site clinical trials in sub-Saharan Africa have shown that RTS,S confers significant but modest and short-lived protection against clinical illness[10,13,14]. An alternative approach that has shown promise is the use of whole attenuated PfSPZ as immunogens. This line of research is based on key early discoveries that immunization with irradiated *P. berghei* sporozoites protected mice against subsequent challenge[15] and that immunization of humans with >1000 irradiated mosquitoes carrying PfSPZ conferred sterilizing protection against controlled human malaria infection (CHMI)[16–18]. These studies have led to efforts to develop whole attenuated PfSPZ as a vaccine[19], and recent trials have shown that immunization with attenuated PfSPZ was highly protective in malaria-naïve volunteers and gave significant protection in Malian adults[6,20–22]. While these results are promising, the specific mediators of this protective immune response have yet to be fully elucidated. Studies of the antibody response have mainly investigated polyclonal serum responses to PfSPZ and PfCSP[6,20–22], and high-resolution analysis of the monoclonal antibodies generated by vaccination and their target antigens on the PfSPZ surface remains to be performed. These experiments could provide useful information for the improvement of whole sporozoite-based vaccines and for the identification of new antigens as subunit vaccine candidates, as well as to generate tools for prophylaxis of *P. falciparum* infection.

Malaria-exposed individuals produce robust antibody responses to immunizations with PfSPZ Vaccine

We characterized the antibody response of Tanzanian volunteers living in malaria-endemic regions who were immunized by repeated intravenous injection of irradiated PfSPZ (PfSPZ Vaccine) and then underwent CHMI with live homologous parasites (Fig. 1a). Serum IgM and IgG antibodies, as measured by flow cytometry on live PfSPZ, increased following immunization, but did not show a clear association with protection from CHMI (Fig. 1b,c and Supplementary Fig. 1a,b). Memory B cells from five protected individuals were immortalized and screened by staining of intact PfSPZ to isolate human monoclonal antibodies against any surface antigen on PfSPZ (Supplementary Fig. 1c). Most of the IgG monoclonal antibodies bound to PfSPZ with high affinity (Fig. 1d,e). Interestingly, in the two donors from whom both PfSPZ-specific IgM and IgG monoclonal antibodies were isolated, the IgM antibodies were recovered at much higher numbers (Fig. 1f). The IgM antibodies had fewer, but still substantial, mutations compared to the IgG antibodies, consistent with an origin from IgM memory B cells (Fig. 1g). In particular, the finding of an antibody lineage containing both IgM and IgG members suggests an incomplete switch in this response despite repeated immunization (Supplementary Fig. 1d). These results demonstrate that immunizations with PfSPZ Vaccine induce a robust antibody response that retains a significant IgM component.

PfSPZ-specific monoclonal antibodies exhibit potent neutralizing capacity *in vitro* and *in vivo*

To identify the features of the most effective neutralizing monoclonal antibodies produced by the protected individuals, we tested a panel of IgG antibodies *in vitro* for their capacity to inhibit PfSPZ traversal and invasion of a human hepatocyte cell line (Fig. 2a). The invasion-inhibitory activity varied among antibodies and was significantly correlated with binding affinity to PfSPZ (Fig. 2b). A subset of antibodies was further tested in an *in vivo* mouse humanized liver model for their capacity to protect against natural, mosquito bite-transmitted infection by PfSPZ. Some antibodies, such as MGG3, MGG4, MGH2, MGU10 and MGU12, were very potent in reducing liver burden by up to 98.3%, while others, such as MGG8, MGH1, MGH3 and MGU1, were less effective (Fig. 2c). These findings suggest that *in vivo* neutralizing activity may be related to the fine specificity of the antibodies.

Highly neutralizing antibodies exhibit dual specificity for NANP and the N-terminus junction of PfCSP and show common usage of VH3-30^{f} genes

Next, we set out to identify the target antigens of the monoclonal antibodies. Strikingly, although we had used an antigen-agnostic approach to identify antibodies that bound to the PfSPZ surface regardless of specificity, we found that all of the antibodies bound to recombinant PfCSP (Fig. 2d,e), confirming that this is the most immunogenic protein on the PfSPZ surface[23–25]. To understand the basis for effective neutralization, we mapped the specificity of the monoclonal antibodies using synthetic peptides that cover the N-terminus, the NANP repeat region, the N-terminal junction (connecting region between the N-terminal

domain and NANP repeats), and the C-terminus of PfCSP. Binding to the classical NANP repeats (NANP18 peptide), the PfCSP C-terminus, recombinant PfCSP or PfSPZ did not correlate with efficacy (Supplementary Fig. 2a–d). Interestingly, however, binding to a minimal 15-mer peptide (NPDP15) that covers the junction between the N-terminal domain and the NANP repeats was a shared characteristic of the most potent *in vivo* neutralizing antibodies (Fig. 2f). These potent antibodies also recognized the NANP18 peptide, suggesting that the capacity to bind both to the NANP repeats and to the N-terminal junction of PfCSP is the main feature of efficient neutralization. Another distinctive characteristic of the most potent antibodies was the common usage of VH3-30 family alleles carrying tryptophan or arginine at position 52 (VH3-30^{f}, here defined as including VH3-30, VH3-30-3, VH3-30-5 and VH3-33 alleles sharing >96% identity) (Fig. 2e, Supplementary Fig. 3,4, Supplementary Table 1). Strikingly, VH3-30^{f} was the most common VH gene used by IgM antibodies isolated from donors G and U, with almost 90% of such antibodies carrying W52 or R52 (Supplementary Fig. 2e). Collectively, these data indicate that the most potent antibodies have a dual specificity and share common VH gene usage. Importantly, such antibodies were isolated from four out of five donors, suggesting that these antibodies belong to a public lineage and therefore have the potential to be readily induced by vaccination.

VH3-30^{f} antibodies acquire dual specificity and high affinity for PfCSP through somatic mutations

To investigate the influence of somatic mutations on binding of VH3-30^{f} antibodies to PfCSP, we focused on two clonally related and highly mutated antibodies, MGU1 and MGU10 (Fig. 3a). The unmutated common ancestor (UCA) of these antibodies, which carries tryptophan at position 52, was able to bind to PfCSP and PfSPZ with low affinity, while substitution to serine 52, which is commonly found in VH3-30 alleles, resulted in loss of binding (Fig. 3b,c). These findings, in conjunction with the high frequency of W52 in the IgM sequences and the identification of putative VH3-30^{f} alleles carrying W52 in the germline sequences obtained from non-B cells of donors G, U and W (Supplementary Fig. 3,4), identify VH3-30^{f} alleles carrying W52 as a preferred feature for the initiation of this lineage-specific antibody response to PfCSP. The branch point of this clone achieved, through several mutations, high affinity binding to PfSPZ, PfCSP and NANP18, while further mutations in MGU10, but not MGU1, increased breadth by conferring the unique ability to bind to NPDP15 (Fig. 3b–e, Supplementary Fig. 5). Despite their similarities in binding to PfSPZ, PfCSP and NANP18, MGU10 was substantially more potent than MGU1 in the *in vivo* assay (Fig. 2c), suggesting that acquisition of binding to NPDP15 is the key factor for potent neutralization. Interestingly, mutagenesis studies of MGU10 suggest that W52 remains a critical residue for binding to NPDP15, but becomes dispensable for high affinity binding to NANP and full-length PfCSP in the fully mutated antibody (Fig. 3f). In contrast, in a second clonal family consisting of MGU5 and MGU8, full binding to PfCSP, NANP18, NPDP15 and PfSPZ was already achieved by the branch point while the remaining mutations appeared redundant (Supplementary Fig. 6a–e).

Unmutated VH3-30^{f} antibodies recognize PfCSP NANP repeat units and subsequently acquire binding to specific motifs in the N-terminal junction

To investigate the original specificities of germline VH3-30^{f} antibodies, we analysed binding of the UCAs of various VH3-30^{f} antibodies to PfCSP peptides using a more sensitive bead-based assay. Most UCAs bound to NANP18 but not to NPDP15, suggesting that the antibodies generally started as NANP-specific and gained affinity for NPDP15 through somatic mutations (Supplementary Fig. 6f–m). These data delineate a pathway of antibody development that is dependent on specific VH alleles and leads to antibodies with dual specificity. To identify the minimal residues recognized by antibodies at the PfCSP N-terminal junction, we performed a mutational analysis on NPDP19, a 19-mer version of NPDP15 that was used to provide a longer scaffold for binding (Fig. 3g). The loss of binding to certain peptides identified a specific motif (DPNANP) that was recognized by most antibodies regardless of VH gene usage. These findings, combined with data from peptide array experiments (Supplementary Fig. 7), identify the N-terminal junction binding site of the most potent neutralizing antibodies as including the first unit of the NANP repeat region and flanking non-repeat sequences, providing a molecular basis for the dual specificity of these antibodies.

Structural analysis of the MGG4 antibody reveals the basis for recognition of the PfCSP N-terminal junctional peptide

To gain structural insights into the recognition of the N-terminal junction, we attempted to crystallize several VH3-30^{f} antibodies and successfully crystallized MGG4 in complex with the Ac-^{1}KQPADGNPDPNANP^{14}-NH_2 peptide (Fig. 4a **and** Supplementary Table 2). Only the C-terminal half (NPDPNAN, residues 7–13) of the peptide was visible, with most of the contacts being made by heavy-chain residues as shown by the Fab buried surface area (Fig. 4b). Specifically, the heavy-chain CDR loops form a groove in which the peptide resides (Fig. 4a **and** Supplementary Fig. 8a). In addition, three interfacial waters are involved in an extensive hydrogen-bonding network, connecting the side chain of N11 to the base of CDR H1 and CDR H3 (Supplementary Fig. 8b). The CH/**π** interaction between P10 and W52 (Fig. 4a **and** Supplementary Fig. 8b) confirms that the latter residue is critical for binding as indicated by the mutagenesis experiments. While most peptide residues, except for N13, display a relatively large buried surface area (Fig. 4c), weak electron density for the residues visible at the peptide's termini (N7, P8, A12 and N13) (Fig. 4e **and** Supplementary Fig. 8c) indicates structural flexibility and highlights the sequence DPN as the principal binding motif. Interestingly, the DPN sequence displays a pseudo 3_{10} turn, which is stabilized by hydrogen bonding of the aspartate side chain to the asparagine backbone amide. Such a conformation is very similar to turns observed for unbound NANP peptides in solution and in crystal structures of free and antibody-bound peptides (Fig. 4d)[25–29]. An isolated DPN motif is also present in the 282–383 C-terminal peptide, which may explain its binding to MGG4 (Fig. 2e), notwithstanding the significant differences between the DPN flanking residues in the 282–383 C-terminal and the N-terminal junctional peptides (**N**DPN**R** versus **P**DPN**A**, respectively). Binding to both NPDP and NANP repeats suggests that the aspartate residue in the DPN motif is interchangeable with an asparagine. Binding studies of MGG4

to NPDP19 peptide mutants validate the specificity of MGG4 toward DPN and NPN motifs (Fig. 3g). Only when the central DPN and NPN motifs within the NPDP19 peptide are mutated to DPA and AAN is MGG4 binding completely abrogated. In contrast, a third DPN motif present at the C-terminus of the NPDP19 peptide does not contribute to binding, possibly indicating the importance of flanking residues for optimal binding. Overall, these data imply that potential peptide-binding promiscuity allows MGG4 to bind to diverse epitopes on PfCSP. Since other VH3-30^{f} antibodies cannot bind to the 282–383 peptide, we expect that they will be less promiscuous and bind slightly larger or more specific sequences.

Immunization of mice with the NPDP19 peptide generates a robust but non-inhibitory anti-PfSPZ response

The finding that the most potent monoclonal antibodies recognize a defined N-terminal junctional peptide suggests that this region could be a component of an effective subunit vaccine. In an initial attempt to investigate whether the NPDP19 peptide might be sufficient to induce a protective response, we immunized BALB/c mice with NPDP19 conjugated to KLH. All mice produced IgG antibodies that were specific for the NPDP19 peptide, but were at best weakly reactive for the NANP18 peptide (Supplementary Fig. 9a,b). Strikingly, in spite of their ability to bind to PfSPZ, the mouse sera were unable to inhibit PfSPZ invasion of a hepatocyte cell line *in vitro*, suggesting that dual specificity for NANP and the N-terminal junction may be required for potent neutralizing function (Supplementary Fig. 9c,d). The reliance of the dual-specific antibodies that we isolated on specific human VH3-30^{f} alleles suggests that mice, which do not have the counterparts of human VH3-30^{f} genes, may not be the most suitable model organism to test a novel CSP-based vaccine. Rather, an organism such as the *Aotus* monkey, which has a more similar VH gene repertoire to humans and contains VH3-30^{f}-like genes carrying the equivalent of W52[30], may be a more suitable choice.

Discussion

This study shows that the antibodies produced by vaccinated and protected African individuals contain a highly mutated IgG component, as well as an important IgM component with fewer but substantial mutations, as also seen in the response to blood-stage *Plasmodium* antigens[31]. The large IgM component would be consistent with stimulation of marginal zone B cells in the spleen following intravenous immunization with a particulate antigen[32]. Strikingly, all of the IgG antibodies that we identified recognized PfCSP, consistent with previous studies describing the immunodominance of this protein and its abundance on the PfSPZ surface[23–25,33,34]. Our findings are in agreement with previous work that separately describes the importance of the PfCSP NANP repeat region and of the N-terminus[23,35–37], but, importantly, highlight the fact that antibodies that target epitopes in both regions simultaneously are more potent than antibodies that exclusively recognize each individual site. Interestingly, the structural analysis and peptide mutational data indicate that these antibodies, which are originally specific for NANP motifs, do not acquire a completely unrelated specificity, but rather gain promiscuity for sequences centred on a DPN motif. This

dual specificity is to a large extent encoded by VH3-30[f] alleles, but also requires extensive somatic mutations. The importance of dual specificity is highlighted by the fact that immunization with a single NPDP19 peptide is not sufficient to confer protection despite eliciting PfSPZ-binding antibodies.

The increased potency of the dual-specific antibodies could be due to the proximity of the N-terminal junction to region I (KLKQP) of PfCSP, which is involved in the cleavage of the N-terminus to allow PfSPZ invasion of hepatocytes[9,12]. This N-terminal junction region is not included in the most advanced malaria vaccine candidate RTS,S, which may explain its limited efficacy in malaria-endemic regions[10]. These findings support continued work to develop whole PfSPZ vaccines, which contain the entire PfCSP, and provide a rationale for further attempts to develop refined vaccination approaches to elicit dual-specific antibodies using prime-boost strategies with improved carriers[38,39].

The finding that the most potent antibodies share common VH gene usage in multiple donors is consistent with a public antibody response that can be readily induced by vaccination. These results are reminiscent of previous work on the use of particular VH genes and their allelic forms in the response to the stem of influenza haemagglutinin[40,41] and justify further efforts to investigate the role of VH gene polymorphisms in protective antibody responses. Nevertheless, whether these antibodies are sufficient to protect humans and why some individuals were not protected remain to be established. Possible reasons for the latter include the lack of a VH3-30[f] allele, the incomplete maturation of the VH3-30[f] antibodies, or insufficient production of the potent antibodies. The antibodies described could be used to obtain proof of concept that antibodies alone can be protective *in vivo* in humans, as previously shown in mice and non-human primates[42–44], and pave the way for the use of antibodies in the prophylaxis of *P. falciparum* infection and for the development of improved antibody-based subunit malaria vaccines.

Methods

Sporozoites

Aseptic, purified cryopreserved PfSPZ of the NF54 strain provided by Sanaria® were used in serum and monoclonal antibody binding experiments. PfSPZ produced by the Center for Infectious Disease Research, Seattle was used in all *in vitro* and *in vivo* functional assays.

Clinical trial and donors

Following informed consent, blood samples used in this study were collected from malaria pre-exposed volunteers during a clinical phase 1 clinical trial of the safety, immunogenicity and protective efficacy of the Sanaria® PfSPZ Vaccine in Bagamoyo, Tanzania between 2014 and 2015. The trial was performed in accordance with Good Clinical Practices and the protocol was approved by Institutional Review Board of the Ifakara Health Institute (IHI-IRB) (Ref. No. IHI/IRB/No:02-2014), the National Institute for Medical Research Tanzania (NIMR/HR/R.8a/Vol.IX/1691) and the Ethikkommission Basel (EKNZ), Basel, Switzerland (reference number 261/13). The protocol was approved by the Tanzania Food and Drug Authority (TFDA) (Ref. No. TFDA13/CTR/0003), registered at Clinical Trials.gov

(NCT02132299) and conducted under U.S. FDA IND 14826. Detailed information on the study procedures of the trial are given in Jongo *et al.* 2017 (manuscript submitted). Briefly, healthy male volunteers aged 20–30 years were randomized to direct venous inoculation (DVI) of 5 doses of normal saline or 1.35×10^5 or 2.7×10^5 PfSPZ of the Sanaria® PfSPZ Vaccine. Vaccine efficacy was assessed by homologous controlled human malaria infection (CHMI) by DVI inoculation of 3,200 infectious PfSPZ Challenge, at 3 and 24 weeks after the last PfSPZ vaccination.

Sample collection and preparation

For serum preparation, whole blood was collected in Vacutainer tubes (BD) containing clot activators and kept at room temperature until a clot was formed. The tube was centrifuged at 2,000 × *g* for 10 min at 22°C and the serum fraction was stored at −80°C. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll density gradient centrifugation and resuspended in freezing medium for long-term storage in liquid nitrogen.

Flow cytometry assay to detect antibody binding to PfSPZ

Cryopreserved PfSPZ (Sanaria®) were thawed and stained with different concentrations of Tanzanian sera or monoclonal antibodies in 3.3× SYBR Green I (ThermoFisher Scientific) for 30 min at 4°C. The PfSPZ were washed twice by centrifugation at 3220 × g for 5 min. Human serum antibody binding was detected using 2.5 μg ml^{-1} Alexa Fluor 647-conjugated goat anti-human IgG (Jackson ImmunoResearch, 109-606-170) or Alexa Fluor 647-conjugated goat anti-human IgM (Jackson ImmunoResearch, 109-606-129). Mouse serum antibody binding was detected using 1 μg ml^{-1} PE-Cy7-conjugated goat anti-mouse IgG (Biolegend, 405315) or PE-Cy7-conjugated rat anti-mouse IgM (BD Biosciences, 552867). FACS Diva (version 6.2) was used for acquisition of samples and Flow-Jo (version 10.1) was used for FACS analysis. The PfSPZ were gated based on high fluorescence in the FITC channel. Median fluorescence intensity (MFI) of the PfSPZ in the Alexa Fluor 647 or PE-Cy7 channel was calculated to quantify IgG or IgM binding. The concentration of antibody needed to achieve MFI 10,000 ($Conc_{10000}$) was calculated by interpolation of binding curves fitted to a sigmoidal curve model (Graphpad Prism 7) as a measure of affinity. The gating strategy can be found in Supplementary Figure 1.

B-cell immortalization and isolation of monoclonal antibodies

IgM or IgG memory B cells were isolated from frozen peripheral blood mononuclear cells (PBMCs) by magnetic cell sorting with 0.5 μg ml^{-1} anti-CD19-PECy7 antibodies (BD, 341113) and mouse anti-PE microbeads (Miltenyi Biotec, 130-048-081), followed by FACS sorting using 3.75 μg ml^{-1} Alexa Fluor 647-conjugated goat anti-human IgG (Jackson ImmunoResearch, 109-606-170), 5 μg ml^{-1} Alexa Fluor 647-conjugated goat anti-human IgM (Invitrogen, A21215) and 1/40 PE-labeled anti-human IgD (BD, 555779). As previously described[47], sorted B cells were immortalized with Epstein-Barr virus (EBV) and plated in single cell cultures in the presence of CpG-DNA (2.5 μg ml^{-1}) and irradiated PBMC-feeder cells. Two weeks post-immortalization, the culture supernatants were tested (at a 2/5 dilution) for binding to PfSPZ by flow cytometry using a no-wash protocol. Briefly, cryopreserved PfSPZ were thawed, stained with the supernatants in 6.25× SYBR Green I for 30 min at room temperature, and incubated with 2.5 μg ml^{-1} Alexa Fluor 647-conjugated

goat anti-human IgG or anti-human IgM for 1 hour at 4°C. Only supernatants that did not bind to control beads were selected to exclude polyreactive antibodies.

In vitro inhibition of PfSPZ invasion and traversal

The ability of monoclonal antibodies and serum to prevent PfSPZ invasion and traversal *in vitro* was tested as previously described[44,48]. Briefly, monoclonal antibodies at 10 μg ml^{-1} were mixed with freshly dissected PfGFP_luc sporozoites in DMEM media containing FITC-dextran, 10% FBS, pen/strep, fungizone and L-glutamine and incubated at 37°C for 15 min. These PfSPZ were then added to HC04 hepatoma cells plated one day prior at 80,000 cells/well in a 96-well plate for a final MOI of 0.3 (26,000 PfSPZ: 80,000 HC04). Plates were then spun at 500 × g for 3 min and the PfSPZ were left to infect for 90 min at 37°C. Cells were then fixed, stained with 10 μg ml^{-1} of the monoclonal antibody 2A10 conjugated to AlexaFluor-647 and analyzed by flow cytometry for invaded cells (2A10/AlexaFluor-647 positive) or traversed cells (FITC-dextran positive).

In vivo P. falciparum mosquito bite challenge in humanized liver mice

FRG huHep mice were purchased from Yecuris, Inc. and infected by bite of 50 PfGFP_luc mosquitos 16–24 hours following intraperitoneal injection of 150 μg/mouse of each monoclonal antibody or human IgG control as described previously[44]. Parasite liver burden was determined by bioluminescent imaging using an IVIS imager at day 6 at the peak of liver burden. Reductions in liver burden were calculated by normalization to the mean of control mice injected with an equivalent dose of human IgG within each bite experiment. All animal procedures were conducted in accordance with and approved by the Center for Infectious Disease Research Institutional Animal Care and Use Committee (IACUC) under protocol SK-16. The Seattle Biomed IACUC adheres to the NIH Office of Laboratory Animal Welfare standards (OLAW welfare assurance # A3640-01).

Sequence analysis of antibody cDNA

cDNA was synthesized from selected B-cell cultures and both heavy chain and light chain variable regions (VH and VL) were sequenced as previously described[49]. The usage of VH and VL genes and the number of somatic mutations were determined by analyzing the homology of VH and VL sequences of monoclonal antibodies to known human V, D and J genes in the IMGT database (version 3.4.8)[50]. Antibody-encoding sequences were amplified and sequenced with primers specific for the V and J regions of the given antibody. Sequences were aligned with Clustal Omega (version 1.2.4)[51]. Unmutated common ancestor (UCA) sequences of the VH and VL were inferred with Antigen Receptor Probabilistic Parser (ARPP) UA Inference software, as previously described[52], or constructed using IMGT/V-QUEST[50]. Phylogenetic trees were generated with the DNA Maximum Likelihood program (Dnaml) of the PHYLIP package, version 3.69[40,53].

Production of recombinant antibodies and antibody variants

Antibody heavy and light chains were cloned into human IgG1, Igκ and Igλ expression vectors and expressed by transient transfection of Expi293F Cells (ThermoFisher Scientific)

using polyethylenimine. Cell lines were routinely tested for mycoplasma contamination. The antibodies were affinity purified by protein A chromatography (GE Healthcare).

X-ray crystallography

The KQPADGNPDPNANP peptide was ordered from Innopep Inc. with a purity of >98% and containing chlorine counter ions. The peptides have N-terminal acetylation and C-terminal amidation to eliminate charges at the peptide termini. The MGG4-peptide complex was crystallized from a solution containing MGG4 at 11.4 mg ml^{-1} in TBS buffer (50 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, pH 8.0) with a 5:1 molar ratio of Ac-KQPADGNPDPNANP-NH_2 peptide to Fab. Crystals were grown using sitting drop vapor diffusion with a well solution containing 0.04 M KH2PO4, 20% glycerol, 16% PEG8000 at 277 K and typically appeared within 3 days. Crystals were cryo-cooled without additional cryoprotection. X-ray diffraction data were collected at the Advanced Light Source (ALS) 5.0.3. Data collection and processing statistics are outlined in Supplementary Table 2. Data sets were indexed, integrated, and scaled using the HKL-2000 package (version 712)[54]. The structures were solved by molecular replacement using PHASER (version 2.7.17)[55] with a homology model (SWISS-MODEL[56–58] and PIGSPro[59]) for MGG4 as a search model. After refinement of the Fab using phenix.refine (version 1.12-2829)[60] combined with additional manual building cycles in Coot (version 0.8.8)[61], positive Fo-Fc density was observed in the Fab combining site for the peptide. The peptide was manually built into the difference density Fo-Fc map, followed by additional rounds of refinement of the complex in phenix.refine[60] and manual building cycles in Coot[61]. Buried surface areas (BSA) were calculated with the program MS (version 1.0)[62], using a 1.7-Å probe radius and standard van der Waals radii[63].

ELISA

Total IgGs were quantified using half-area, high-binding 96-well plates (Corning) with 10 μg ml^{-1} goat anti-human IgG (SouthernBiotech, 2040-01) using Certified Reference Material 470 (ERMs-DA470, Sigma-Aldrich) as a standard. To test specific antibody binding, ELISA plates were either directly coated with 1 μg ml^{-1} of recombinant PfCSP (Sanaria®, sequence previously shown[21]), 2 μg ml^{-1} of peptide 22–110 or 1 μg ml^{-1} of peptide 282–383, or first with 10 μg ml^{-1} of avidin (Sigma-Aldrich), followed by 10 μg ml^{-1} of NANP18 (NANPNANPNANPNANPNA), NPDP19 (KQPADGNPDPNANPNVDPN), NPDP15 (KQPADGNPDPNANPN) or various NPDP19 peptide mutants. Non-specific binding to plates coated with an irrelevant control peptide was tested to exclude polyreactivity of the antibodies. All peptides and mutants were synthesized with biotin attached to the C-terminus (A&A Labs). Plates were blocked with 1% bovine serum albumin (BSA) and incubated with titrated antibodies, followed by 1/500 AP-conjugated goat anti-human IgG (Southern Biotech, 2040-04). Plates were then washed, substrate (p-NPP, Sigma) was added and plates were read at 405 nm.

Bead-based assay to detect binding to PfCSP peptides

Streptavidin beads with different levels of FITC labelling (SVFB-2552-6K, Spherotech) were coated with 10 μg ml^{-1} of biotinylated NANP18, NPDP15, NPDP19 or a negative control peptide for 30 min at room temperature. The beads were washed and incubated with

titrations of monoclonal antibodies for 20 min at room temperature. Antibody binding was detected with 2.5 μg ml^{-1} Alexa Fluor 647-conjugated goat anti-human IgG or anti-human IgM. The UCAs were compared for binding to NANP18 and NPDP15 at a concentration of 22 μg ml^{-1} (Supplementary Fig. 6f).

Surface plasmon resonance (SPR) assays

Biotinylated NPDP15 and NANP18 peptides were diluted (20 nM) in HEPES buffered saline (HBS) (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant Tween-20). HBS was also used as running buffer. An irrelevant biotinylated 19-mer peptide was used as a control for non-specific interactions. A NeutrAvidin-immobilized NLC ProteOn sensor chip (Biorad) was pre-conditioned with an NaCl solution (1 M) and the biotinylated peptides were injected onto the chip. The monoclonal antibodies were diluted and titrated in HBS (50-16.7-5.6-1.9-0.6 nM) and injected onto chip; one channel of the chip was injected with HBS and used as reference for the analysis. All injections were made at a flow rate of 100 μl/min. Injection time and dissociation time were 240 s and 900 s, respectively. Each binding interaction of the monoclonal antibodies with the biotinylated peptides was assessed using a ProteON XPR36 instrument (Biorad) and data were processed with ProteOn Manager Software (version 3.1.0.6). K_a, K_d and K_D were calculated by applying the Langmuir fit model.

Peptide array analysis

Peptides of 15-amino acid lengths spanning the entire PfCSP (with a shift of a single amino acid between peptides) were synthesized and coated onto a microarray chip (PEPperMAP® Linear Epitope Mapping, PEPperPRINT GmBH). The peptides were incubated with 1 μg ml^{-1} of monoclonal antibodies for 16 h at 4°C, followed by incubation with DyLight680-conjugated goat anti-human IgG to detect antibody binding.

Immunization of mice

Female BALB/c mice (6–9 weeks of age) were obtained from ENVIGO Laboratories. All procedures were performed in accordance with guidelines by the Swiss Federal Veterinary Office and after obtaining ethical approval from the Ufficio Veterinario Cantonale, Bellinzona, Switzerland (approval number: 332016). Keyhole limpet haemocyanin (KLH)-conjugated NPDP19 (Genscript) was reconstituted in water and formulated with 50% MF59 (Addavax, Invivogen) according to the manufacturer's instructions. Mice were immunized subcutaneously with 50 μg of peptide on day 0 and 10. Mice were bled on day 21. Recovered sera were used for staining of PfSPZ by flow cytometry and for binding to PfCSP and PfCSP peptides by ELISA.

Statistical analysis

The number of mutations in the heavy chains of IgG (n=19 antibodies) and IgM (n=65 antibodies) isolated from the Tanzanian volunteers were compared by a two-sided t-test. Results are shown as mean ± s.d.. In this test, n refers to the number of antibodies, $P = 0.002$, $t = 3.196$, $df = 82$. A two-tailed Spearman's correlation was performed to correlate invasion with binding affinity to PfSPZ (from n=1 representative experiment out of 2), $P =$

0.0037. In the *in vivo* test of the monoclonal antibodies, error bars show s.d. and were calculated from n = 4 or 5 mice for each antibody. A one-sided ANOVA with Kruskal-Wallis post test was used to compare the percentages of liver burden with that of control mice injected with irrelevant human IgG; for the ANOVA, $P = 0.0001$, F = 4.652, df1 = 9, df2 = 55. For the Kruskal-Wallis post test, the results for each individual antibody are presented as * P<0.05, ** P<0.01, **** P<0.0001. A two-tailed Spearman's correlation was performed to correlate affinity for NPDP15, PfCSP, NANP18, 282–383 or PfSPZ with *in vivo* antibody efficacy (from n=1 representative experiment out of 2). $P = 0.0183$, 0.7435, 0.3125, 0.6944 and 0.7081, respectively. The confidence intervals were not determined by Prism as n<10 for each correlation. In all other cases, n refers to the number of independent experiments.

Data availability

Sequence data of the monoclonal antibodies isolated in this study will be deposited in GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The X-ray structure factors and coordinates have been deposited in the Protein Data Bank (PDB ID 6BQB).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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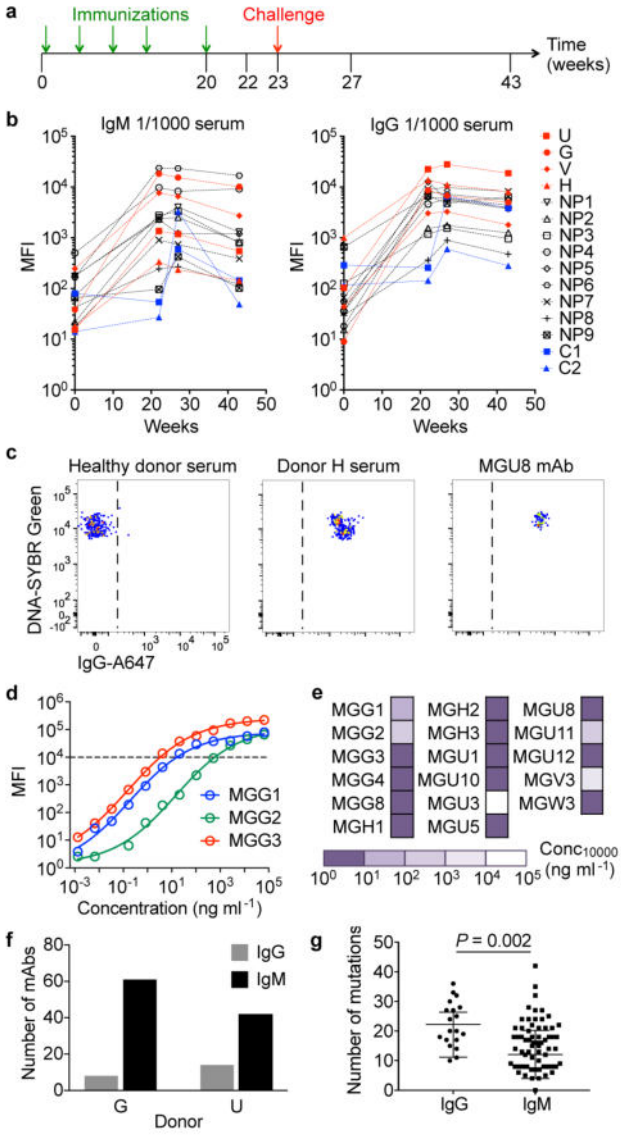

Figure 1. Immunization with PfSPZ Vaccine induces robust antibody responses in malaria-exposed individuals

a, Protocol of PfSPZ immunization of Tanzanian adults. **b,** Binding of serum IgM and IgG antibodies to PfSPZ. Median fluorescence intensity (MFI) values are for binding at a 1/1000 serum dilution (representative of n=2 independent experiments). Samples in red, black and blue are from protected (U, G, V, H), non-protected (NP) and placebo (C) volunteers, respectively. Results for donor W are not shown as this donor was immunized with a lower dose of PfSPZ. **c,** Staining of PfSPZ by serum from a European blood donor, serum from a protected individual (donor H) and a monoclonal antibody (MGU8) (representative of n=3 independent experiments). **d,** Dose-dependent binding of three representative antibodies to PfSPZ measured by flow cytometry (representative of n=2 independent experiments). **e,** Binding values of the panel of IgG monoclonal antibodies to PfSPZ (representative of n=2 independent experiments). The values indicate the concentration of antibody required to

reach a 10,000 MFI. **f,** Number of PfSPZ-binding IgG and IgM monoclonal antibodies isolated from protected donors G and U. **g,** Number of mutations in the heavy chains of IgG (n=19 antibodies) and IgM (n=65 antibodies) isolated from the Tanzanian volunteers. These values were calculated by adding the number of VH and JH mutations. Results are shown as mean ± s.d.. A two-sided t-test was used to compare the number of mutations.

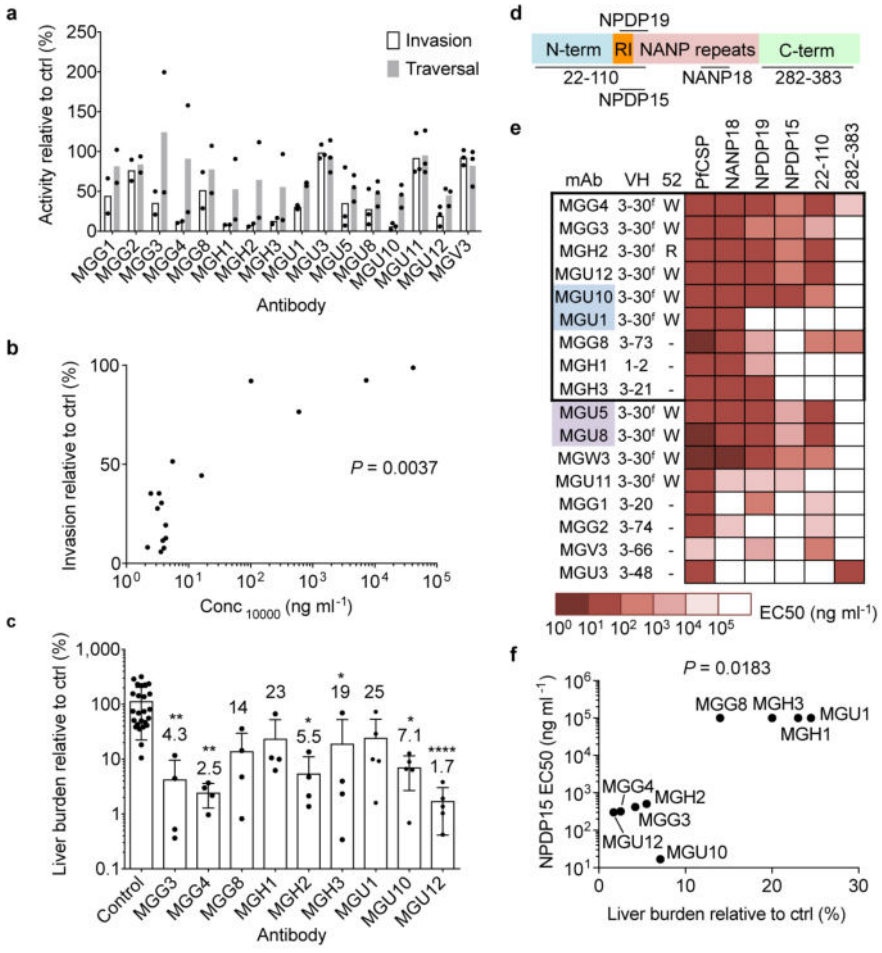

Figure 2. Highly neutralizing antibodies use VH3-30^{f} genes and exhibit dual specificity for NANP and the N-terminus junction

a, *In vitro* inhibition of PfSPZ traversal and invasion by monoclonal antibodies (n=2 or 3 independent experiments). Bars show mean percentages relative to control experiments with irrelevant human IgG. **b,** Correlation of invasion with binding affinity to PfSPZ (from n=1 representative experiment out of 2). A two-tailed Spearman's correlation was performed, 95% confidence interval 0.2878–0.8887. **c,** *In vivo* activity of monoclonal antibodies in humanized liver mice infected by *P. falciparum* via mosquito bites (n = 4 or 5 mice per antibody tested). Bars show the mean percentage of liver parasite burden relative to that of control mice injected with irrelevant human IgG. Error bars show s.d.. A one-sided ANOVA with Kruskal-Wallis test was used; *P9.05, ** P9.01, **** P<0.0001. **d,** Scheme of PfCSP (not to scale). **e,** Binding of monoclonal antibodies to full-length PfCSP and PfCSP peptides (representative of n=2 independent experiments). The antibodies are classified according to the VH gene used and the residue at position 52 for VH3-30^{f} antibodies. The antibodies in the box are those tested in the *in vivo* assay. Antibodies belonging to the same clone are highlighted in the same colour. NANP18, NANPNANPNANPNANPNA; NPDP15, KQPADGNPDPNANPN; NPDP19, KQPADGNPDPNANPNVDPN. 22-110 and 282-383 are long N-terminal and C-terminal peptides from Pf3D7 CSP, respectively[36,45]. **f,**

Correlation of affinity for NPDP15 with *in vivo* antibody efficacy. A two-tailed Spearman's correlation was performed (from n=1 representative experiment out of 2). Confidence interval not determined by Prism for n<10.

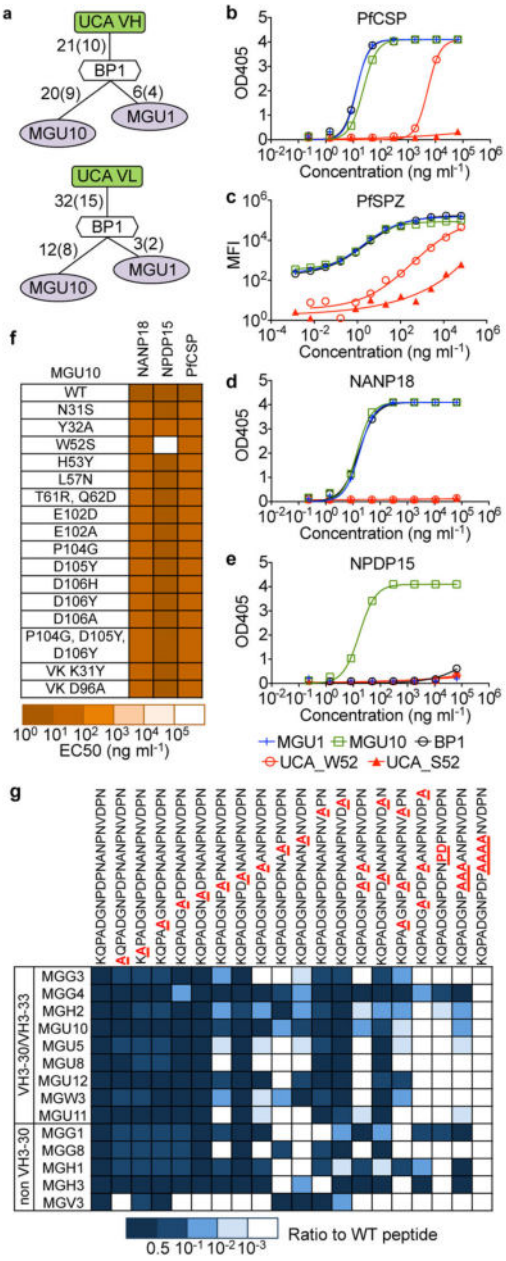

Figure 3. Somatic mutations increase affinity for PfCSP and lead to the acquisition of dual specificity

a, VH and VL genealogy trees of MGU1 and MGU10. Shown are the nucleotide and amino acid substitutions, with the latter in parentheses. UCA, unmutated common ancestor; BP, branch point. **b–e,** Binding of members of the MGU1 and MGU10 clonal family to PfCSP, PfSPZ, NANP18, and NPDP15, respectively (representative of n=2 independent experiments). **f,** Binding of MGU10 mutants to NANP18, NPDP15 and full-length PfCSP (representative of n=2 independent experiments). **g,** Binding of monoclonal antibodies to

NPDP19 peptide mutants (n=1 experiment). The letters highlighted in red show the mutated residues in the peptide.

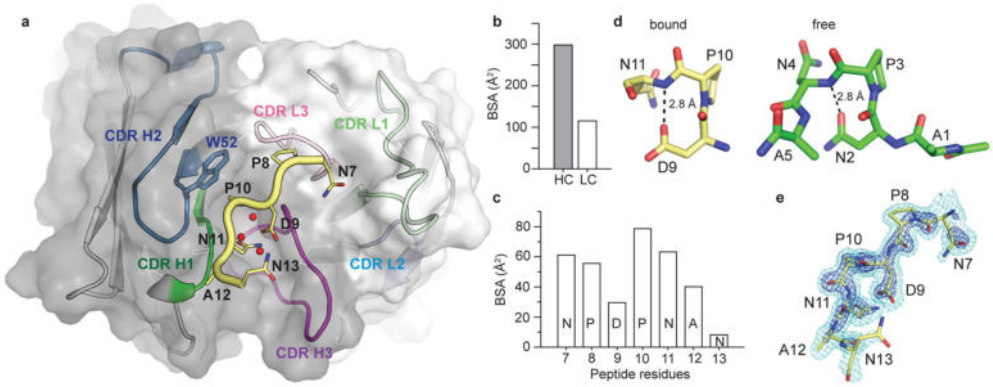

Figure 4. Structural basis for recognition of an N-terminal junctional peptide by MGG4

a, Binding interface of MGG4 in complex with the ^{1}KQPADG**NPDPNAN**P^{14} peptide; only residues 7 to 13 of the peptide have interpretable electron density (indicated in bold). The peptide is shown in the cartoon representation with sidechains as sticks, while the heavy and light chains of MGG4 are shown as dark and light grey surfaces respectively. The CDR loops are in the cartoon representation: CDRH1 (green), CDRH2 (blue), CDRH3 (magenta), CDRL1 (light green), CDRL2 (light blue) and CDRL3 (pink). The W52 sidechain is shown as blue sticks and the interfacial waters are highlighted as red spheres. **b,** Buried surface area (BSA) for the heavy chain (HC) and light chain (LC) with the peptide. **c,** BSA for individual peptide residues with the Fab. **d,** Pseudo 3_{10} turn for the DPN motif of the bound peptide (yellow carbons) and type I β-turn for the previously published crystal structure of the unbound ANPNA peptide (green carbons)[27]. Stabilizing hydrogen bonds between the sidechain of D9/N2 and the amide backbone of N11/N4 in the two structures are highlighted by the dashed line. **e,** 2Fo-Fc electron density map for the N-terminal peptide contoured at 2.0σ (dark blue) and 0.8σ (light blue).

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Role of human Pegivirus infections in whole *Plasmodium falciparum* sporozoite vaccination and controlled human malaria infection in African volunteers

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Abstract

Background: Diverse vaccination outcomes and protection levels among different populations pose a serious challenge to the development of an effective malaria vaccine. Co-infections are among many factors associated with immune dysfunction and sub-optimal vaccination outcomes. Chronic, asymptomatic viral infections can contribute to the modulation of vaccine efficacy through various mechanisms. Human Pegivirus-1 (HPgV-1) persists in immune cells thereby potentially modulating immune responses. We investigated whether Pegivirus infection influences vaccine-induced responses and protection in African volunteers undergoing whole *P. falciparum* sporozoites-based malaria vaccination and controlled human malaria infections (CHMI).

Methods: HPgV-1 prevalence was quantified by RT-qPCR in plasma samples of 96 individuals before, post vaccination with PfSPZ Vaccine and after CHMI in cohorts from Tanzania and Equatorial Guinea. The impact of HPgV-1 infection was evaluated on (1) systemic cytokine and chemokine levels measured by Luminex, (2) PfCSP-specific antibody titers quantified by ELISA, (3) asexual blood-stage parasitemia pre-patent periods and parasite multiplication rates, (4) HPgV-1 RNA levels upon asexual blood-stage parasitemia induced by CHMI.

Results: The prevalence of HPgV-1 was 29.2% (28/96) and sequence analysis of the 5′ UTR and E2 regions revealed the predominance of genotypes 1, 2 and 5. HPgV-1 infection was associated with elevated systemic levels of IL-2 and IL-17A. Comparable vaccine-induced anti-PfCSP antibody titers, asexual blood-stage multiplication rates and pre-patent periods were observed in HPgV-1 positive and negative individuals. However, a tendency for higher protection levels was detected in the HPgV-1 positive group (62.5%) compared to the negative one (51.6%) following CHMI. HPgV-1 viremia levels were not significantly altered after CHMI.

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Conclusions: HPgV-1 infection did not alter PfSPZ Vaccine elicited levels of PfCSP-specific antibody responses and parasite multiplication rates. Ongoing HPgV-1 infection appears to improve to some degree protection against CHMI in PfSPZ-vaccinated individuals. This is likely through modulation of immune system activation and systemic cytokines as higher levels of IL-2 and IL17A were observed in HPgV-1 infected individuals. CHMI is safe and well tolerated in HPgV-1 infected individuals. Identification of cell types and mechanisms of both silent and productive infection in individuals will help to unravel the biology of this widely present but largely under-researched virus.

Keywords: Malaria, Human pegivirus, Controlled human malaria infection, Immune activation, Antibody response, PfSPZ vaccine

Background

Vaccination is an invaluable tool in public health that has contributed to control of many, and in some cases, to the elimination of infectious disease like smallpox [1]. Malaria, a disease caused by *Plasmodium* species remains a major public health burden particularly in the tropics and sub-tropical regions where it accounted for approximately 405,000 deaths in 2018 [2]. A major goal in malaria research is to develop an efficacious vaccine that complements currently used control tools based on vector control and treatment of clinical malaria infections [3]. However, these vaccine development efforts are challenged by an incomplete understanding of the immune mediators leading to highly protective, long-lasting vaccine induced immunity in the field [4]. A number of studies testing cryopreserved, purified, metabolically active and radiation-attenuated whole sporozoites of *P. falciparum* as vaccine approach (PfSPZ Vaccine) have been published recently [5–9]. Strikingly, the comparison of PfSPZ vaccine-induced antibody titers specific for the *P. falciparum* circumsporozoite protein (PfCSP) showed significantly lower titers in malaria pre-exposed than malaria-naive individuals immunized with the PfSPZ Vaccine using comparable regimen [6–9]. These differences in PfSPZ vaccine-induced immunity was also observed between vaccinees residing in malaria endemic countries including Tanzania, Mali and Equatorial Guinea [10–12].

Recently our group demonstrated that age, location and iron status influence the immune system development of children as well as vaccine-induced responses to the most advanced malaria vaccine candidate, the RTS,S [13]. Additionally, communicable and non-communicable diseases have been implicated in suboptimal vaccine-induced responses [14] Chronic, asymptomatic viral infections at time of immunization might contribute to reduced magnitude and longevity of vaccine-induced immune responses [15–17]. To date, the number of human viruses investigated in this context is limited and their mechanisms in modulation of vaccine-induced responses remain unclear.

Human Pegivirus-1 (HPgV-1), a predominantly asymptomatic virus causing a chronic infection, is common in Africa where an estimated 18–28% of its roughly 750 million global infections occur [18]. The virus establishes its persistence potentially by replicating in immune cells including T cells, B cells, monocytes, and natural killer (NK) cells [19, 20]. Interestingly, seminal field studies have linked HPgV-1 co-infection status to significant survival advantages in HIV-1 and Ebola infected humans [21–24]. These favourable outcomes are thought to be based on immune-modulatory properties of HPgV-1 such as reduced activation of T cells, B cells and NK cells [20, 25] and the altered regulation of cytokine and chemokine expression [26–28]. Different HPgV-1 genotypes might influence the extent of immune modulation resulting in varied disease outcomes [21–23].

Given the high prevalence of HPgV-1 infection in *P. falciparum* endemic countries, we expected an overlapping geographical distribution and aimed to investigate within-host interactions between the two infections. We were therefore interested to study whether HPgV-1 infection status might influence PfSPZ vaccine-induced immune responses. We characterized prevalence and genotype distribution of HPgV-1 in three cohorts of adult volunteers participating in PfSPZ Vaccine studies [9, 29, 30]. We explored the influence of HPgV-1 infection status on cytokine and chemokine levels in serum and correlated HPgV-1 infection on vaccine-induced anti-PfCSP-antibody titers and protection against homologous CHMI. We also aimed to characterize for the first time the potential impact of a CHMI study on HPgV-1 viremia in these volunteers.

Methods

Study population

We used samples from volunteers enrolled in four studies conducted in Bagamoyo, Tanzania (acronyms BSPZV1, BSPZV2, and BSPZV3a) and on Bioko Island in Equatorial Guinea (acronym EGSPZV2) registered at Clinicaltrials.gov with registration numbers NCT03420053, NCT02132299, NCT02613520, and NCT02859350, respectively. Detailed trial designs and study procedures

such as pre-defined inclusion and exclusion criteria have been described previously [9, 10, 29, 30]. Briefly, these trials evaluated the safety, immunogenicity and efficacy of live, cryopreserved, purified, whole *P. falciparum* sporozoites in malaria pre-exposed volunteers. The analyses in these studies were performed on samples collected from adult volunteers in which vaccine efficacy was evaluated by homologous CHMI based on direct intravenous inoculation of 3200 fully infectious, aseptic purified cryopreserved *P. falciparum* sporozoites. The current study was performed in two parts (i) a pilot virome study that included samples from a subset of volunteers from BSPZV1 (NCT03420053) (Additional file 1: Fig. 1); (ii) the main study which utilized samples from volunteers participating in the BSPZV2 (NCT02132299), BSPZV3a (NCT02613520) and EGSPZV2 (NCT02859350) trials. Samples were selected based on availability and scientific aims. Tested sample types and sizes are described in further detail in each section and in Additional file 1: Fig. 1A.

Identification of human Pegivirus RNA in RNA-seq data from whole blood

Whole venous blood samples were used from a subset of participants (n = 28) (Additional file 1 Fig. 1A) participating in the BSPZV1 vaccine trial (NCT03420053) based on their availability. All volunteers were healthy males, aged 18 to 35 years and confirmed as negative for HIV-1, Hepatitis B and C at enrolment. Venous blood was collected at different time points including: before vaccination (baseline), 2 and 7 days after first vaccination, 7 days after the second vaccination, before CHMI, 2 and 9 days after CHMI. Each of the placebo (n = 6) and the vaccine (n = 22) participants had a total of 3 and 7 blood sampling time points screened respectively, resulting in 172 samples in total. All blood samples (n = 172) were stored in Paxgene RNA tubes and subjected to RNA-seq analysis performed as published [31]. Briefly, RNA-seq data was generated from whole blood RNA (depleted for globin/rRNA) that was fragmented; the first strand cDNA synthesis was done by random priming and dTTP was used, whereas 2nd strand cDNA synthesis used dUTP which eliminates 2nd strands in the downstream PCR amplification that enabled strand specific RNA-seq sequencing [31]. From the RNA-seq sample set (n = 172), 800 million non-human reads were identified. Given the naturally occurring fluctuation of viremia of many viruses, we performed a longitudinal assessment of viral infection status, and obtained reads from all available time points for each of the volunteers. The analyses was performed in an in-house developed viral metagenomics analysis pipeline outlined in (Additional file 1: Fig. 1B–C). The pipeline is a combination of several published algorithms adapted from commonly used viral metagenomic analytical tools [32–36]. Briefly, our analyses were carried out in three consecutive steps: viral identification, in silico validation and RT-PCR confirmation. In the viral identification step, we analysed approximately 3 million non-human, unmapped paired end reads from each volunteer. The initial reads were first searched for "suspected" viral hits by running bowtie2 against the NCBI database containing more than 7424 viral genomes. Thereafter, low quality and complexity reads as well as reads mapping to human genome, transcriptome and repeat regions were removed from the resulting "suspected" viral reads using bowtie2, knead data and tandem repeat finder algorithms, respectively. The "clean" viral reads were then comprehensively searched for viral hits using virome scan [32] and Taxonomer [33] and for viral proteins using adapted Diamond tool containing a custom made database with more than 100,000 viral proteins [35]. The initial unmapped reads were also analysed by Fast virome explorer without filtering for host reads to allow the identification of endogenous retroviral elements and other viruses that may have been missed previously [34]. Only viral hits known to be associated with a human host were selected, and viral contaminants such as lymphotropic murine virus and synthetic constructs coding for either HIV-1 or hepatitis B were removed based on documented literature [37]. In a following *in-silico* confirmatory step, the suspected viral hits were blasted and mapped against specific viral whole genomes using a Geneious bioinformatics tool [38]. As a last step, we performed reverse transcription PCR (RT-PCR) analysis. Due to limited sample availability we were unable to screen the entire BSPZV1 cohort for HPgV-1. Hence, the presence of the most prevalent virus (human pegivirus-1, HPgV-1) was confirmed by RT-PCR in plasma samples of volunteers found positive by RNA-seq transcriptome analysis only.

RT-qPCR for detection and quantification of HPgV-1 and HPgV-2

Plasma samples collected from male and female individuals (n = 96), aged 18–45 years, and participating in the BSPZV2, BSPZV3a and the EGSPZV2 studies were included. Plasma was prepared by density gradient centrifugation of whole blood and cryopreserved. At analysis cryopreserved plasma was thawed and used for detection of HPgV-1 and HPgV-2 RNA in all study participants. Plasma samples collected at 3 time points for each volunteer were included, namely before vaccination (baseline), before CHMI and 28 days after CHMI. Presence or absence of HPgV-1 and HPgV-2 was determined simultaneously using RT-qPCR based on published methods [25]. Briefly, total nucleic acids were extracted from 300 ul plasma using Zymo quick DNA/RNA viral kit (Zymo

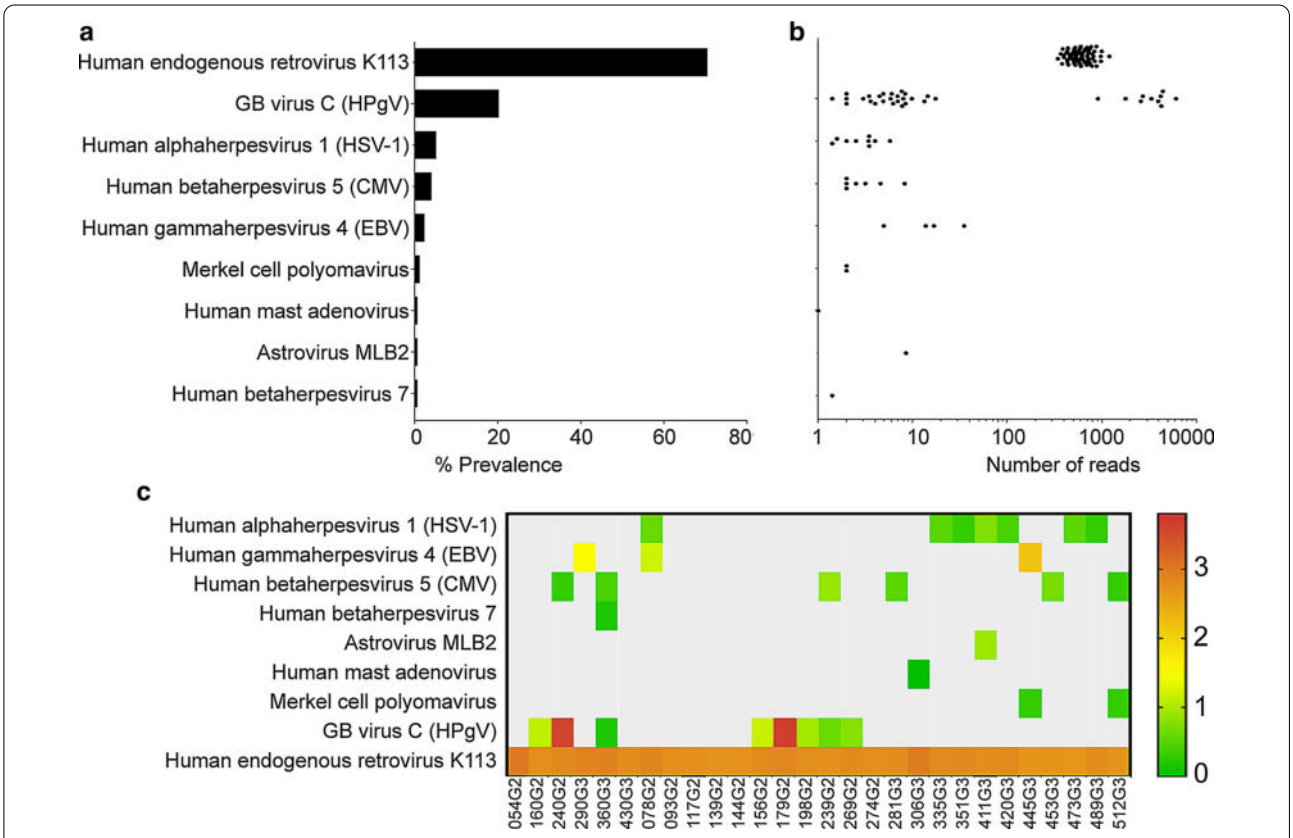

Fig. 1 Unbiased search for RNA molecules encoding human viruses in RNA-seq transcriptomics data. **a** Overall prevalence of 9 human viruses detected in 172 whole blood samples **b** Number of viral RNA-seq reads detected for each of the identified viruses. Virus names are plotted on the y-axis and proportion (**a**), number of reads (**B**) on the x-axis. **c** Distribution of the 9 different viruses across the 28 individuals included. Virus names are plotted on the y-axis and volunteer IDs on the x-axis. Each bar indicates viral reads for an individual. The log viral RNA-seq reads are plotted, in increasing order ranging from 0 to 3; green indicating low number and red high number of reads

Research, Irvine, USA) and eluted in 50 ul of DNase/RNase free water. 5 ul of the recovered DNA/RNA solution was used as amplification template together with 2X.Lunar universal one step qPCR master mix (10 ul, 1X), Luna warm start reverse transcriptase enzyme mix (1 ul, 1X) (New England Biolabs, MA, USA) and primers binding to the 5′ untranslated regions of HPgV-1 and HPgV-2 (each at 2 ul, 0.4 uM) [25, 39]. In addition, human RNaseP primers were added as internal control. Each sample was run in triplicate in a one-step multiplex RT-qPCR using the CFX96 real time PCR system (Bio-Rad, Hercules, CA, USA). The RT-qPCR cycling conditions were: 55 °C for 10 min, 95 °C for 1 min, 45 cycles at 95 °C for 15 s and 55 °C for 1 min. The generated data were uploaded to an in-house available analysis platform where quantification cycle values (Cq) were calculated automatically [40]. HPgV viral quantification was done as described by Stapleton et al. using in vitro transcribed (IVT) viral RNA [25]. In each RT-qPCR experiment, we included a positive (HPgV-1 IVT-RNA), a negative (from HPgV-1 negative volunteer) and a non-template control.

Genotyping of HPgV-1

The Fire Script cDNA kit was used to synthesize cDNA in accordance to manufacture instructions (Solis Biodyne, Tartu, Estonia). Briefly, 5 ul of extracted RNA as described above was added into a master mix containing forward and reverse primers specific to 5′ UTR of HPgV-1 (each at 1,1 uM), deoxribonucleotide triphosphate mix (dNTP) (0.5 ul, 500 uM), reverse transcription buffer with DTT (2 ul, X1), RiboGrip Rnase inhibitor (0.5 ul, 1 U/ul), Fire script reverse transcriptase (Solis Biodyne, Tartu, Estonia) (2 ul, 10 U/ul) and RNase free water (9 ul to 20 ul). Amplification conditions included 50 min at 50 °C and 10 min at 94 °C. 3 ul of cDNA generated by reverse transcription were used for the first round of PCR amplification with forward primer 5′-AAAGGTGGT GGATGGGTGATG-3′ [41] and reverse primer 5′-ATG CCACCCGCCCTCACCAGAA-3′ combination [41]. 1.2 ul of this amplification product was then used for the nested PCR amplification using the forward primer 5′-AATCCC GGTCAYAYTGGTAGCCACT-3′ and reverse primer 5′-CCCCACTGGCZTTGYCAACT-3′

combination [41]. Both PCR reactions included primers specific for HPgV-1 (1 ul, 1 uM), firepol master mix (4 ul, X5) (Solis Biodyne, Tartu, Estonia) and RNase free water to a final volume of 20 ul. Cycling conditions were 5 min at 95 °C, followed by 28 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 oC for 30 s with a final extension step at 72 °C for 10 min. The E2 region was amplified as described by Souza et al. [42]. The final PCR products from 5′ UTR amplification (256 base pairs) and E2 amplification (347 base pairs) were sequenced by the Sanger sequencing method (Microsynth, Switzerland).

HPgV-1 phylogenetic analysis

Nucleotide sequence analysis and phylogenetic analysis was performed with the Geneious software version 8.1.9. Chromatograms were examined for quality, and only sequences with quality threshold above 86% were included in analysis. CLUSTALW algorithm was used to align 5` UTR nucleotide sequences from volunteers to selected reference sequences corresponding to 5` UTR of HPgV-1 (genotype 1 to 7) available through the NCBI database. Thereafter, phylogenetic trees were constructed by the neighbour joining method and the Kimura two parameter models. The references sequences for 5` UTR of HPgV-1 included AF488786, AF488789, KC618399, KP710602, U36388, JX494177, Y16436, and MF398547 (Genotype 1); AB003289, AF104403, D90600, JX494179, MG229668, JX494180, U4402, U59518 (Genotype 2; 2a), MH000566, U59529, U63715, MH053130 (Genotype 2; 2b); AB008335, KR108695, JX494176, D87714 (Genotype 3); AB0188667, AB021287, HQ3311721 (Genotype 4); DQ117844, AY949771, AF488796, AF488797 (Genotype 5); AB003292, AF177619 (Genotype 6), HQ331235, HQ 3312233 (Genotype 7). The hepatitis C nucleotide sequence deposited under AJ132997 was used as an outgroup. For the E2 region the sequences were KP701602.1, KM670109, U36380, KP710600, KC618399, AB003291 (Genotype 1); AF121950, MK686596, D90600 (Genotype 2a) and U63715 (Genotype 2b); D87714 (Genotype 3); AB0188667 (Genotype 4); AY949771, KC618401, AY951979 (Genotype 5); AB003292 (Genotype 6). A Chimpanzee HPgV-1 strain deposited under AF70476 was used as an out-group and U4402 (Genotype 2) was used for mapping of our sequences to identify regions of similarity.

Ex vivo *cytokine and chemokine measurements*

Serum samples available from 44 volunteers collected from EGSPZV2 and BSPZV3a (only HIV-1 negative volunteers) at baseline were used for the assessment of the systemic immune activation status. Cytokine and chemokine concentrations were measured using the Cytokine/Chemokine/Growth Factor 45-Plex Human ProcartaPlex™ Panel 1 (Affymetrix Biosciences, USA) and acquired on a validated Luminex XMAP technology platform as described [43]. The investigated cytokines and chemokines included BDNF, Eotaxin/CCL11, EGF, FGF-2, GM-CSF, GRO alpha/CXCL1, HGF, NGF beta, LIF, IFN alpha, IFN gamma, IL-1 beta, IL-1 alpha, IL-1RA, IL-2, IL-4, IL-5,IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12 p70, IL-13, IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-27, IL-31, IP-10/CXCL10, MCP-1/CCL2, MIP-1 alpha/CCL3, MIP-1 beta/CCL4, RANTES/CCL5, SDF-1 alpha/CXCL12, TNF alpha, TNF beta/LTA, PDGF-BB, PLGF, SCF, VEGF-A and VEGF-D. Only cytokines and chemokines with levels above the pre-defined lower detection limit of the specific standard curves were included in the group comparisons. Absolute concentrations were normalized to account for the inter-plate variations before analysis in R software version 3.5.1.

Serological analysis

Serum samples for anti-PfCSP antibody evaluation were collected before vaccination (baseline) and 14 days post last vaccination. Anti-PfCSP total IgG levels were measured by enzyme linked immunosorbent assay (ELISA) as described previously [9, 10, 29, 30].

Quantitative detection of Plasmodium falciparum

Asexual blood-stage malaria parasitemia during CHMI was assessed using thick blood smear (TBS) microscopy and retrospectively analysed using stored whole blood samples and qPCR as described [9, 10, 29, 30]. Whole blood samples for the assessment of parasitemia were taken before CHMI and during the observation period beginning at day 9 after parasite challenge inoculation until volunteers either became asexual blood-stage malaria positive or until day 21. TBS were performed twice a day from day 9 to 14 and then once a day for day 15 to 21. TBS were also performed on day 28 before malaria drug treatment. Pre-patent periods were calculated from the time of PfSPZ challenge to first positivity detected by qPCR and TBS [9, 10, 29, 30]. Parasite multiplication rate (PMR) was assessed using a linear model fitted to log10-transformed qPCR data as published [44]. PMR was calculated for all volunteers that developed asexual blood-stage parasitemia which lasted for at least two 48-h cycles [44].

Statistical analysis

Figures and statistical analyses were generated in R version 3.5.1 and GraphPad Software (Prism V5). Wilcoxon rank sum test or Mann–Whitney test were used to compare continuous variables. Chi-square test was used to compare categorical variables. Absolute values for antibody titers and concentrations of cytokines and

chemokines were plotted. Data were log transformed only when investigating the anti-PfCSP antibody titres and viremia levels. Spearman correlation was used to investigate the potential effect of HPgV-1 infection status and viremia with antibody titres and cytokine levels. Data for cytokines, chemokines and growth factors were not analysed for multiple correction as we considered this question as exploratory. P value ≤ 0.05 was considered significant. Differences in viral diversity, abundance and prevalence were assessed using Linear discriminant analysis effect size [45] and GraphPad Software (Prism V5), respectively.

Results

Unbiased search for RNA molecules encoding human viruses

We aimed to identify viruses present in peripheral blood of our volunteers participating in PfSPZ Vaccine studies. These analyses included samples from 28 participants of the BSPZV1 study collected at multiple time points including baseline, 2 days after first vaccination, 7 days after the first and second vaccination and before CHMI, 2 and 9 days after CHMI. Sequences were identified from a pool of RNA-seq data reads that did not map to the human reference transcriptome. A total of 800 million non-human RNA-seq reads derived from 172 whole blood samples were analysed with our virome discovery platform based on previously established metagenomics pipelines and tools (Additional file 1: Fig. 1B, C) [32–35].

In total, RNA molecules encoding 9 human viruses were detectable including the Human simplex virus (HSV-1), Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Merkel cell polyomavirus (MCV), Human mast adenovirus (HAdV), Astrovirus MBL2, Human betaherpesvirus 7 (HHV-7), Human endogenous retrovirus K113 (HERV-K113) and HPgV-1 (Fig. 1a). The number of reads for each of the identified viruses was quantified and is given in Fig. 1b. After identifying 9 different viruses present in 172 whole blood samples, we further assessed the distribution of viruses within our cohort. HERV-K113 was detected with high number of reads in all 28 individuals, while HSV-1 and CMV were present in seven and six individuals, respectively (Fig. 1c). MBL2, HHV-7 and HAdV were present in low read counts in one individual each, and MCV was found in two individuals. Eight individuals carried HPgV RNA with read counts ranging from low to high (Fig. 1c). Three out of 8 HPgV-positive individuals were co-infected with CMV (Fig. 1c). Our analysis showed that a high proportion of Tanzanian adults (28.6%, 8/28) harboured HPgV -1 infection. To reconfirm our findings, we extracted RNA from plasma samples collected from these 8 volunteers and amplified HPgV-1 by RT-PCR. We reconfirmed in 2 out of 8 volunteers the in silico identified presence of HPgV-1 RNA. Interestingly, these 2 volunteers had the highest RNA read counts for HPgV-1 in our bioinformatics analysis (Fig. 1c). These results were important for selecting HPgV-1 as our further research focus and the optimization of RT-PCR assay used for assessment of HPgV-1 infection status in the main study described below.

Prevalence of HPgV-1 in East and West African volunteers

After having established that HPgV-1 is highly present in our Tanzanian cohort (BSPZV1), we aimed to explore the prevalence of HPgV-1 and HPgV-2 in two larger cohorts from Tanzania (BSPZV2 and BSPZV3a) and one from Equatorial Guinea (EGSPZV2). Plasma samples collected from 96 participants, including 12 HIV-1 positive individuals, were analysed for presence of HPgV-1 and HPgV-2 using optimized RT-qPCR. The overall prevalence of HPgV-1 was 29.2% (28/96) (Fig. 2a), while HPgV-2 was not detected. The proportion of HPgV-1 positive individuals by gender and geographic location were comparable, with slightly more HPgV-1-positive individuals in Equatorial Guinea (31.4%) than Tanzania (26.7%) (Fig. 2b, c). Of the 12 HIV-1 positive individuals from the BSPZV3a study, two (16.7%, 2/12) were positive for HPgV-1 (Fig. 2d).

HPgV-1 viral loads and distribution

Next, we quantified the HPgV-1 viral load in plasma samples using RT-qPCR. HPgV-1 viral loads were comparable between individuals from the two countries (Fig. 3a). However, based on viral loads with a predefined threshold of 10^6 viral RNA copies/ml of plasma, both cohorts could be divided into HPgV-1 low and high viremic individuals (Fig. 3b). High and low HPgV-1 viremia were found in 17 (60%) and 11 (40%) of the 28 HPgV-1 positive volunteers, respectively (Fig. 3b). Of the 17 high viremic individuals, 8 were from Tanzania and 9 from Equatorial Guinea. Of the 11 low viremic individuals, 4 were Tanzanians and 7 Equatorial Guineans.

Genotyping of HPgV-1 isolates

Seven different genotypes of HPgV-1 have been described globally with genotype 1 and 5 being highly prevalent in sub-Saharan Africa [26]. Therefore, we determined the phylogenetic relatedness of the isolates by amplifying and sequencing the 5′ UTRs. From the 28 positive individuals, 2 were excluded due to poor quality of the sequences obtained. Genotype 1 was found in 2 volunteers (7.7%) and surprisingly, genotype 2, described as dominant in Europe and America, was found in 24 of 26 volunteers (92.3%) (Fig. 4). Most genotype 2 strains clustered closely with the related genotype 2a sequences described from Venezuela (Fig. 4). To further increase the resolution of

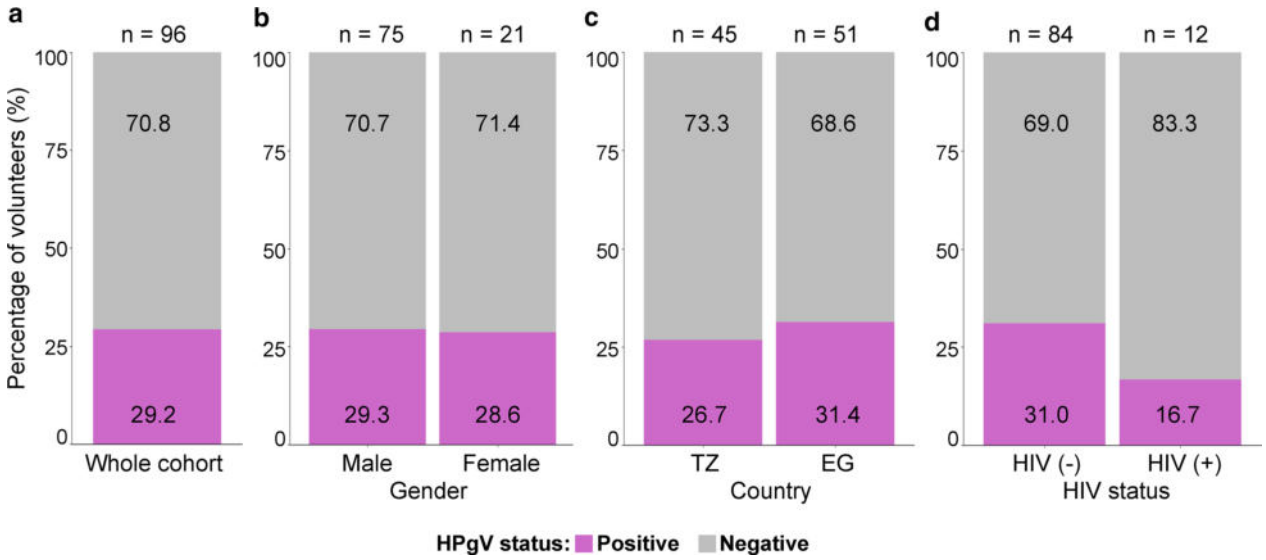

Fig. 2 Proportion of individuals with (purple) and without (grey) HPgV- 1 infection. **a** Total cohort of 96 vacinees, **b** separated by gender, **c** Country of origin, **d** HIV-1 infection status. All individuals are between 18 and 35 years of age. Chi square with Yates correction for group comparisons (*$P < 0.05$)

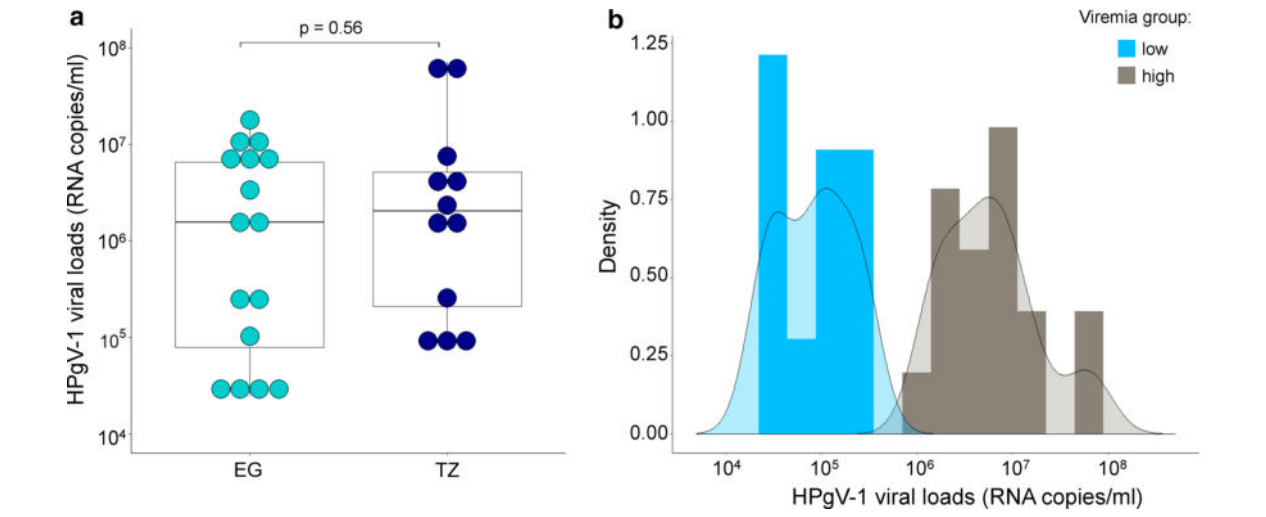

Fig. 3 Comparisons of HPgV-1 viral loads. No differences in HPgV-1 viral loads between Equatorial Guinea (green, n = 16) and Tanzania (blue, n = 12) volunteers **(a)**. Two distinct groups with low (blue) and high (grey) viremia levels in plasma are found in HPgV-1 infected individuals **(b).** The two groups were divided based on a cut off value of 600,000 RNA copies/ml plasma

the genetic relatedness of our isolates, we amplified in addition the polymorphic E2 region of HPgV-1. E2 RNA was successfully amplified and sequenced in 9 of the 28 positive volunteers (32%). According to the E2-sequences of our HPgV-1 isolates, our strains clustered within genotypes 1, 2, and 5 (Fig. 5). Notably, genotypic clustering of isolates 6EG and 14EG differed based on E2 and 5′ UTR derived PCR products (Figs. 4, 5). In summary, these results show that a range of HPgV-1 genotypes are circulating in Tanzania and Equatorial Guinea, clustering to published genotypes 1, 2 and 5.

Effect of HPgV-1 positivity on systemic cytokine and chemokine levels

To dissect whether ongoing HPgV-1 infection affects cytokine and chemokine levels in serum, we measured 45 cytokines, chemokines and growth factors in a subset of 44 volunteers from BSPZV3a (HIV-1 negative only) and

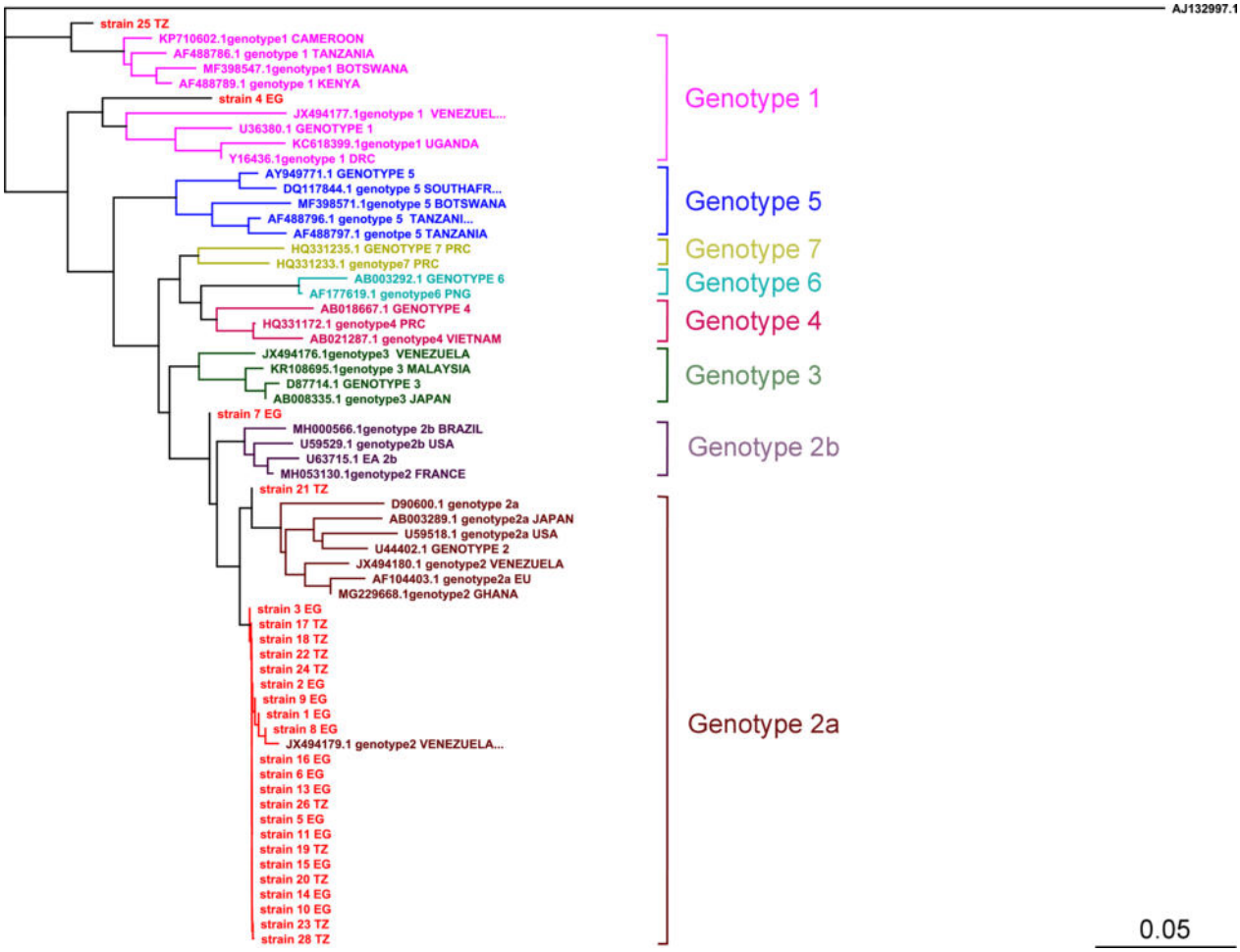

Fig. 4 Phylogenetic inferences of the HPgV-1 isolates based on 5′ UTR. Phylogenetic tree was constructed using Neighbour joining method and Kimura two-parameter model of the 5′ UTR. The 5′ UTR sequences from Tanzania and Equatorial Guinea (n = 26) were compared to selected references spanning genotype 1 to 7 from different countries available in the NCBI database. The accession numbers for the reference sequences were: AF488786, AF488789, KC618399, KP710602, U36388, JX494177, Y16436, and MF398547 (Genotype 1, Pink); AB003289, AF104403, D90600, JX494179, MG229668, JX494180, U4402, U59518 (Genotype 2; 2a light brown), MH000566, U59529, U63715, MH053130 (Genotype 2; 2b Brown); AB008335, KR108695, JX494176, D87714 (Genotype 3, Green); AB0188667, AB021287, HQ3311721 (Genotype 4, Maroon); DQ117844, AY949771, AF488796, AF488797 (Genotype 5, Light blue), AB003292, AF177619 (Genotype 6, Bright green); HQ331235, HQ 3312233 (Genotype 7, Golden) and Hepatitis C (AJ132997, Black) was used as outgroup

EGSPZV2. 23 cytokines, chemokines and growth factors were detected above their pre-defined lower limits of detection (Additional file 5: Table 1). Although there was a trend of overall higher cytokine levels in HPgV-1 infected individuals (Additional file 2: Fig. 2), only IL-2 and IL-17A reached significance levels (Fig. 6). There was no statistically significant difference in cytokine and chemokine levels when HPgV-1 high and low viremic individuals were compared (data not shown). Also, we could not find differences in chemokine and cytokine levels when comparing the different HPgV-1 genotypes (data not shown). Taken together, these data suggest that the presence of HPgV-1 infection increases IL-2 and IL-17A levels in peripheral blood.

Effect of HPgV-1 infection status on PfSPZ vaccine-induced humoral immune response

IL-2 and IL-17A might contribute to differentiation of naïve B cells into plasma cells and support the survival of activated B cells [46, 47]. We examined the potential of HPgV-1 infection to impact on PfSPZ vaccine-induced humoral immunity. PfCSP is the most immuno-dominant protein recognized after PfSPZ vaccination. Anti-PfCSP titres were measured at baseline in all volunteers (n = 70) and 14 days past last vaccination in all vaccinated

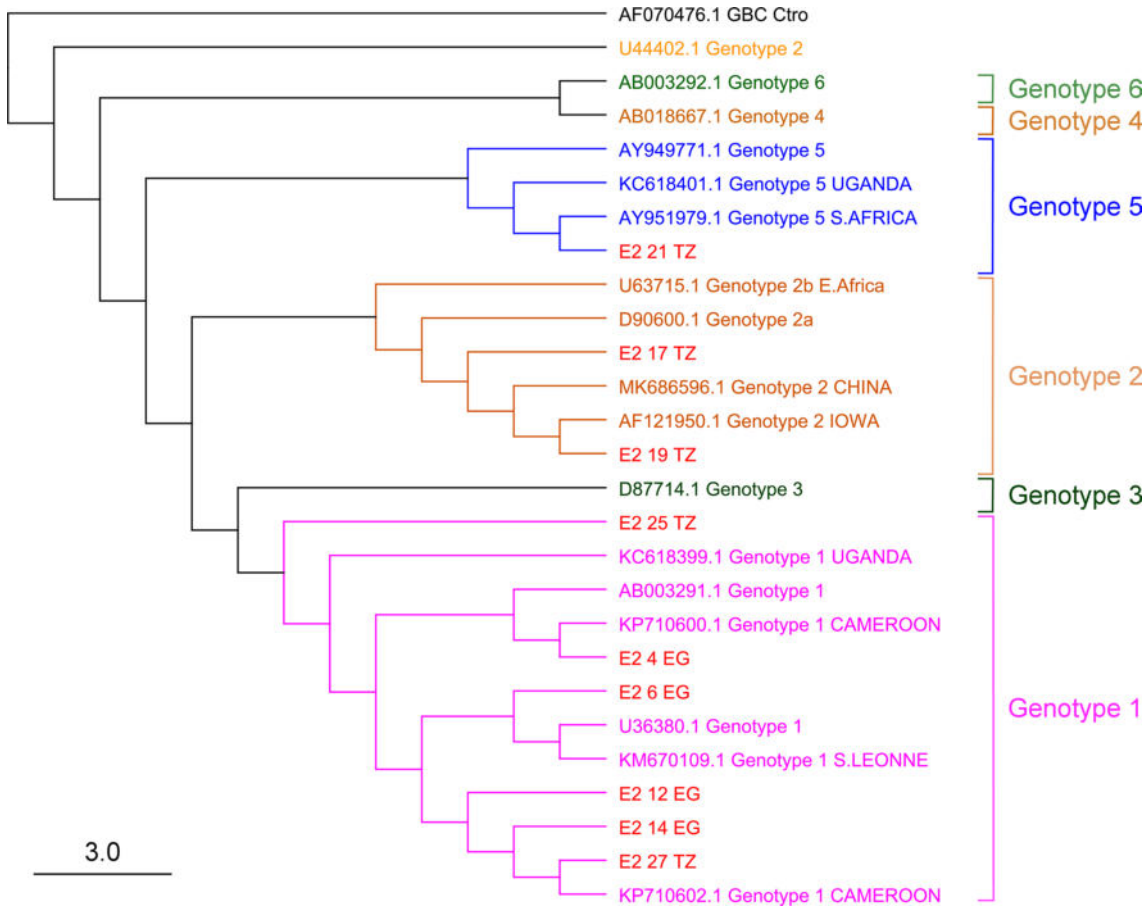

Fig. 5 Phylogenetic inferences of the HPgV-1 isolates based on E2 region. Phylogenetic tree was constructed using Neighbour joining method and Kimura two-parameter model of the E2 region of HPgV-1. The E2 sequences from Tanzania and Equatorial Guinea (n = 9) were compared to selected references spanning genotype 1 to 6 from different countries available in the NCBI database including; KP701602.1, KM670109, U36380, KP710600, KC618399, AB003291 (Genotype 1, Pink); AF121950, MK686596, D90600 (Genotype 2; 2a Brown), U63715 (Genotype 2; 2b Brown) D87714 (Genotype 3, Green); AB0188667 (Genotype 4, Brown); AY94977, KC618401, AY951979 (Genotype 5, Light blue) and AB003292 (Genotype 6, Green). Equatorial Guinean and Tanzanian strains identified in this study are denoted by strain number followed with letters EG or TZ, respectively (Red). Chimpanzee HPgV-1 strain (AF70476, Black) was used as outgroup and U4402 (Genotype 2, Golden) was used for mapping of our sequences to identify regions of similarity. The scale bar under the tree indicates nucleotide substitution per site

volunteers (n = 54) participating in BSPZV2, BSPZV3a and EGSPZV2 (Fig. 7 A-B). Similar results were observed when PfSPZ vaccine-induced antibody responses were analysed as net increase (titres at 14 days post last vaccination minus baseline titres) (Fig. 7 C) and as fold change (titres 14 days past last vaccination divided by baseline titres) (Fig. 7 D). There was no significant correlation between HPgV-1 infection status and anti-PfCSP antibody titre at baseline and after vaccination.

Effect of HPgV-1 infection on PfSPZ vaccine efficacy

The high prevalence of HPgV-1 positive volunteers in our cohort allowed us to investigate a potential impact of ongoing viral infection during PfSPZ vaccination on vaccine-induced protection. Protective efficacy of the vaccine was evaluated by presence or absence of asexual blood-stage parasitemia following homologous PfSPZ challenge (CHMI) (Additional file 3: Fig. 3). While none of the placebo-receiving participants was protected (0/20), the overall protection in the vaccinated group was 55% (26/47). The HPgV-1 prevalence was comparable in these two groups (placebos and vaccines) with 35% (7/20) versus 34% (16/47), respectively, suggesting that HPgV-1 infection does not facilitate protection against CHMI. We further compared the CHMI protection levels in HPgV-1 positive and negative participants in the vaccinated group only (Fig. 8a). HPgV-1 positive vaccinees showed slightly higher protection levels after CHMI (62.5%; 10/16) compared to HPgV-1 negative individuals (51.6%; 16/31). We also assessed anti-PfCSP antibodies

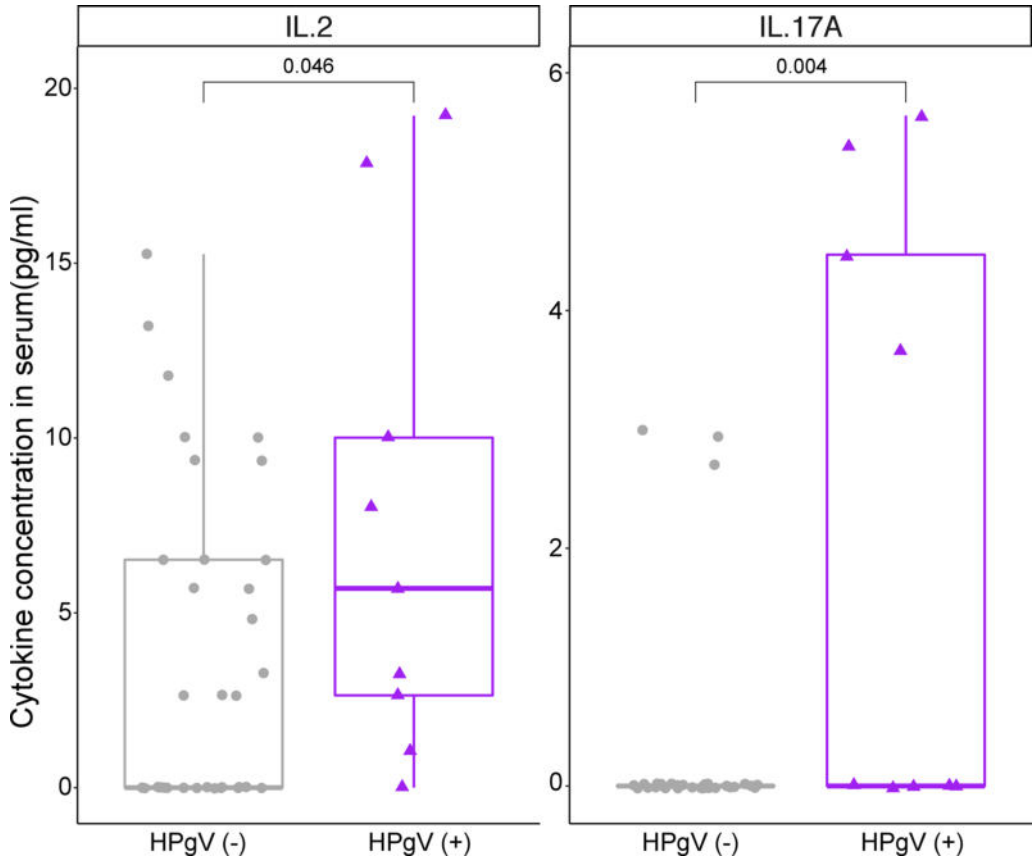

Fig. 6 HPgV-1 infection is associated with increased systemic levels of IL-2 and IL-17A. Cytokine, chemokine and growth factors levels were analysed by Luminex and levels compared between HPgV-1 negative (5′ UTR-, grey, n = 35) and HPgV-1 positive (5′ UTR+, purple, n = 9) volunteers. Absolute serum concentrations levels (pg/ml) of Interluekin-2 (IL-2) and Interluekin-17A (IL-17A) at samples taken before vaccination are shown. Significantly higher IL-2 and IL-17A are seen in the HPgV-1^{+} compared to the HPgV-1^{-}. Wilcoxon rank sum test was used to determine significance (*P* value * < 0.05) which are indicated on top of top for each group comparison

titres at the peak response which is 14 days past last vaccination. Slightly higher anti-PfCSP levels were seen in protected compared to non-protected individuals (Fig. 8b), without reaching statistical significance. PfCSP antibody levels tended to be lower in the HPgV-1 positive individuals (Fig. 8b).

Interaction of HPgV-1 and controlled human malaria infection induced asexual blood-stage parasites

HPgV-1 co-infection has been associated with favourable clinical outcomes in HIV-1 and Ebola co-infected individuals [21–23]. So far, the HPgV-1 impact on *P. falciparum* infection and immunity is unknown. We evaluated parasite multiplication rates and pre-patent periods in the placebo volunteers only, that have not been vaccinated (n = 20) undergoing CHMI. Comparable asexual blood-stage multiplication rates and pre-patent periods were observed between HPgV-1 positive and negative individuals (Fig. 9a, b).

Effect of CHMI on HPgV-1 viremia levels

P. falciparum infection is known to impact viremia levels of some common viruses like HIV-1 and EBV [48, 49]. We therefore evaluated the effect of an acute *P. falciparum* infection on HPgV-1 viremia by comparing the viral load before and 28 days post CHMI in 21 eligible trail participants. Detectable levels of HPgV-1 at both time points were quantified in 9 individuals; 5 of those showed an increased HPgV-1 viral load and the other 4 had decreased viremia post CHMI (Fig. 9c). In addition, 12 trial participants were HPgV-1 positive only for one of the two tested time points; 6 were positive before CHMI and 6 were positive at 28 days post CHMI (Fig. 9c) (Additional file 4: Fig. 4 showing four representative volunteers individually).

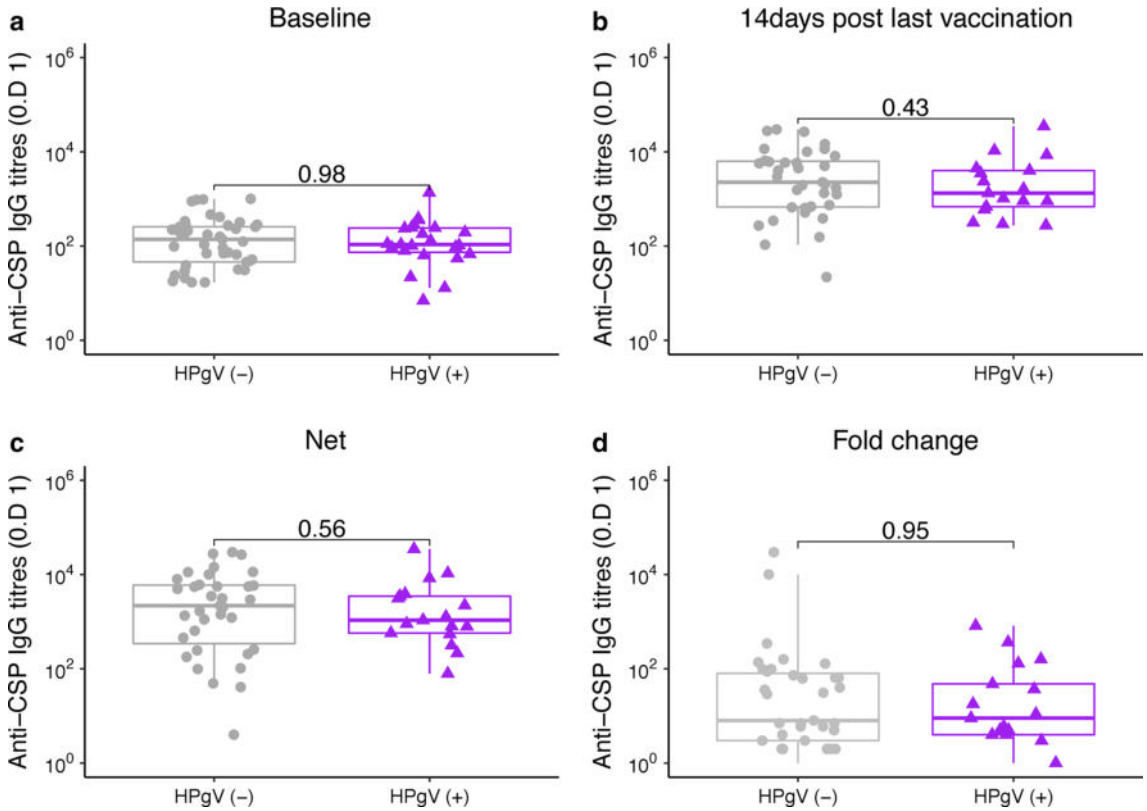

Fig. 7 HPgV-1 infection status does not impact on anti-PfCSP antibody titres. Total IgG antibodies recognizing full length PfCSP were measured by ELISA in HPgV-1 negative (5′ UTR-, grey), HPgV-1 positive (5′ UTR+, purple) volunteers. **a** Shows baseline (pre-vaccination) anti-PfCSP IgG levels of HPgV-1 negative (n = 47) compared to HPgV-1 positive (n = 23) volunteers. **b** Anti-PfCSP IgG levels at 14 days past last vaccination in HPgV-1 positive individuals (n = 17) versus the HPgV-1 negative (n = 37) group. **c**, **d** Comparison of vaccine-induced changes in anti-PfCSP IgG titres as net responses (14 days post last immunization—baseline) as well as fold (14 days post last vaccination/baseline). Only vaccinated individuals were included for 14 days post last immunization, net and fold change responses. One HPgV-1^{+} individual was not included in these subsequent analyses due to missing antibody data. Log anti-PfCSP titres expressed in arbitrary units are shown. Each point represent an individual, box plot with horizontal bar show median values for each group. Statistical significance was calculated by using Wilcoxon rank sum test (*P* value * < 0.05). *P* values are indicated on top for each group comparison

Discussion

The role of chronic asymptomatic viral infections in modulating immune responses in health and disease is increasingly appreciated [50]. The present study sought to better understand the prevalence and genotype distribution of HPgV-1 in East and West-central Africa. We aimed to investigate the potential influence of HPgV-1 infection on experimental malaria vaccine-induced humoral immunity and vaccine-induced protection. By studying a cohort of volunteers undergoing CHMI, we were in a unique position to investigate if an acute malaria episode has an impact on HPgV-1 viremia in chronically infected volunteers.

In a first step, we used an unbiased approach by generating RNA-seq data to identify prevalent viruses circulating in peripheral blood of our BSPZV1 study volunteers that lead to the identification of HPgV-1 as highly present in this group. Confirmation by RT-PCR was possible only in 2/8 HPgV-1 positive volunteers. Several factors might have contributed to these discrepancies in results, such as the volume and type of samples used. 300 ul of plasma was used as starting material for RNA extraction and RT-PCR, while roughly 10 times more whole blood was applied as starting material for the RNA-seq data generation. Also, the variation in HPgV-1 viremia levels between serum and cellular components have been demonstrated previously [19, 25]. The drawbacks of RT-PCR based methods over deep RNA sequencing methods in virus identification have been previously reported. To overcome these limitations, the combination of qPCR working with predefined primers and unbiased deep sequencing approaches is recommended [51, 52]. The unbiased virome analyses were important for focusing our analyses on HPgV-1 in larger cohorts in East and West Africa.

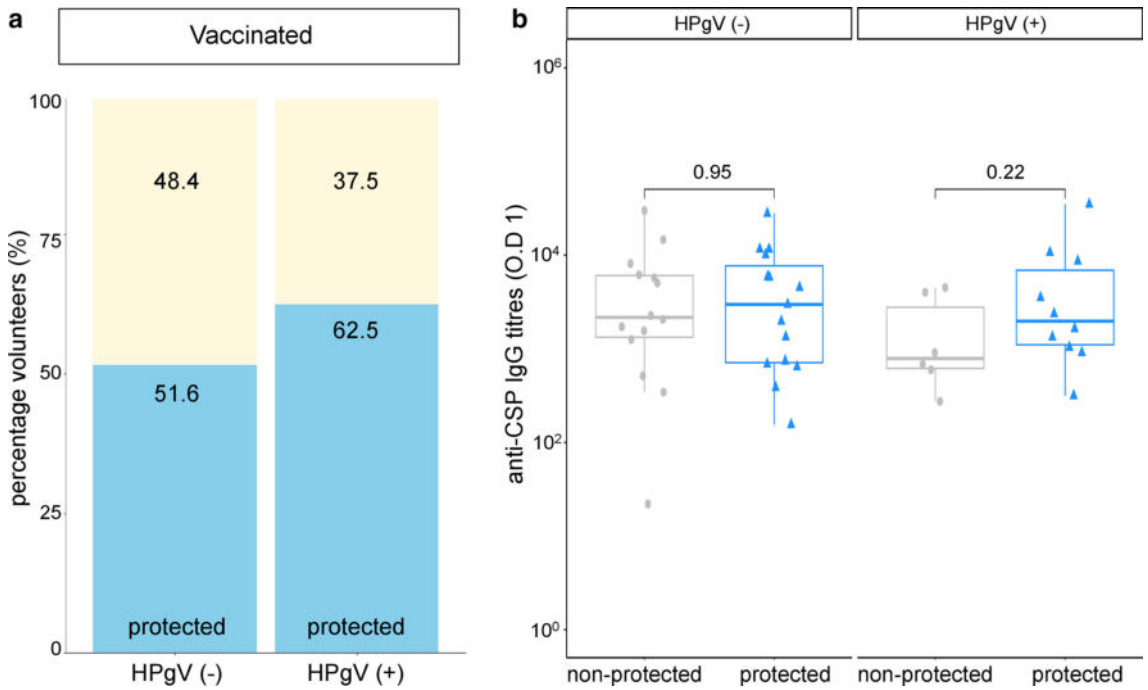

Fig. 8 HPgV-1 infection does not influence *P. falciparum* pre-patent periods and parasite multiplication rates during CHMI. Parasitemia was determined in whole blood by qPCR and thick blood smear microscopy (TBS). The analysis included only placebo participants, positive and negative for HPgV-1. **a** Shows log-fold change of parasitemia in 48 h between HPgV-1 negative (5′ UTR-, grey, n = 13) and HPgV-1 positive (5′ UTR +, purple, n = 7) volunteers. **b** Comparison of days post CHMI to malaria positivity by microscopy in HPgV-1 negative (5′ UTR-, grey, n = 11) and HPgV-1 positive (5′ UTR +, purple, n = 7). **c** HPgV-1 viral loads before (red) and 28 days post CHMI (green) in HPgV-1 infected individuals. Each point represents an individual, box plots show data distribution with horizontal bar denoting viral load at each visit. Lines connect viremia levels in individuals found positive for HPgV-1 on both time points. Geometric means were compared between groups and unpaired t-test was used to calculate significance. Horizontal bars represent mean with standard deviation Wilcoxon rank sum test was used to compare viremia levels before and after CHMI. P-values are indicated on top of each comparison

The overall prevalence of HPgV-1 in our main study cohort was 29.2%, roughly the same for Tanzania and Equatorial Guinea. The prevalence reported here is likely underestimated as we observed fluctuations of HPgV-1 viral loads longitudinally, with some volunteers showing HPgV-1 positivity in one, two or all three time points assessed**.** These detection variations might indicate either viral clearance or continuously ongoing viral replication with viremia fluctuations sometimes below the lower detection limit of our RT-qPCR assay [19, 25]. We did not detect HPgV-2 RNA in any of our volunteers but we cannot completely exclude the possibility of the presence of circulating HPgV-2 as antibody titers against the HPgV-1 and HPgV-2 E2 envelope proteins were not measured. Our study focussed on the potential impact of HPgV-1 on PfSPZ vaccine induced humoral responses and protection, thus HIV-1 positive individuals of the BSPZV3a study were excluded from the HPgV-1 association analyses. It is well known that HIV-1 infection negatively impacts immunity in widely used routine vaccines [53].

Similar to a study in Mexico, we observed two broad groups, low and high, of HPgV-1 viremic individuals, defined by a cut off value of 600,000 RNA copies/ml. This observation likely reflects the different viral replication states within infected volunteers [22]. We observed similar numbers of high and low viremic individuals, who are infected with HPgV-1 genotype 2. The potential role of a distinct viral genotype on this pattern remains unclear, given the small number of volunteers in this study and limited heterogeneity of the detected HPgV-1 genotypes.

Currently, 7 HPgV-1 genotypes are described globally [54, 55] and some of these genotypes have been implicated in varied clinical outcomes [23, 26, 56]. HIV-1/HPgV-1 co-infection studies revealed lower CD4 T cell counts in individuals infected with HPgV-1 genotype 2a than genotype 2b [56, 57] and higher HPgV-1 viral loads in individuals with genotype 1 compared to genotypes 2a and 2b [58]. Higher serum levels of IFN-ɣ were described in HIV-1 positive women co-infected with genotype 2 compared to genotype 1 [23].

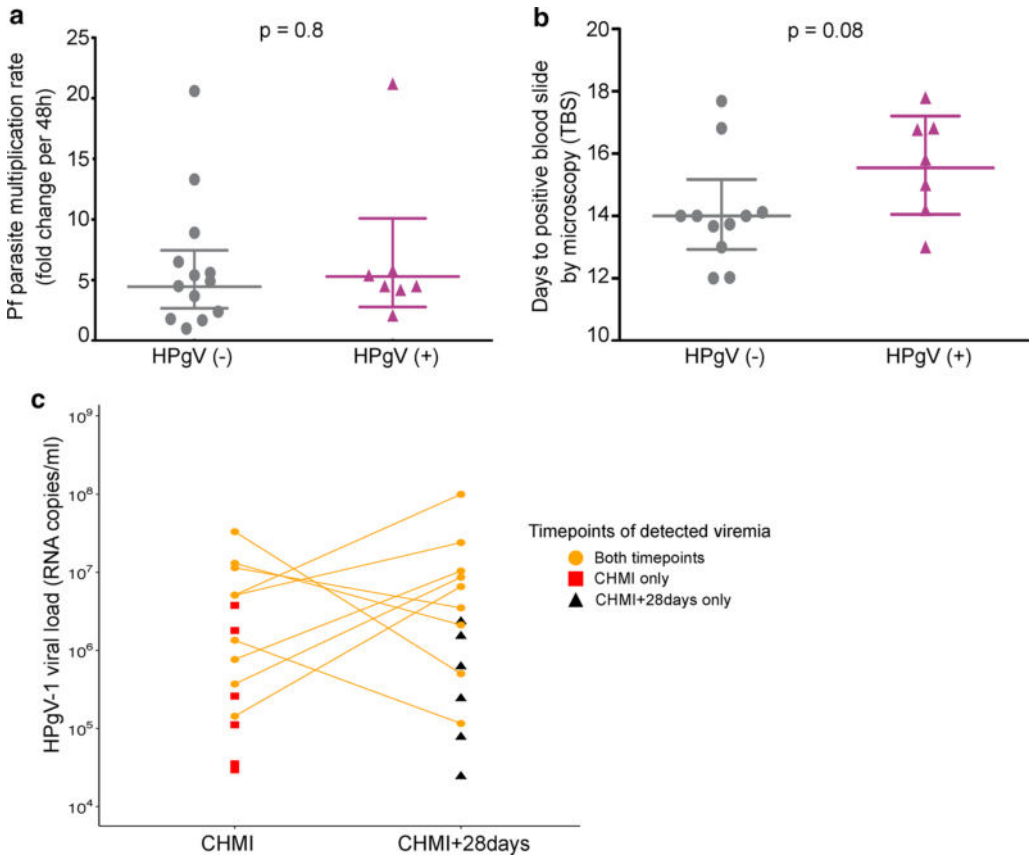

Fig. 9 Association of HPgV-1 infection status with PfSPZ CHMI outcome and anti-CSP titers in immunized volunteers. Individuals were treated with either normal saline (placebo) or PfSPZ Vaccine (vaccinees). Presence or absence of malaria parasites was determined in whole blood by thick blood smear microscopy (TBS) and confirmed by qPCR. Total IgG antibodies recognizing full length PfCSP were measured by ELISA. **a** Proportion of non-protected (cream) and protected (blue) in vaccinated volunteers with and without HPgV-1 infection. Proportions are indicated inside the bar and volunteer numbers on top. **c** Total anti-CSP IgG levels at 14 days past last vaccination in the protected (malaria negative) and non-protected (malaria positive) groups, with and without HPgV-1 infection. Log anti-PfCSP titres expressed in O.D units are shown**.** Each point represent an individual, and box plot with horizontal bar show median values for each group. Chi square with Yates correction was used for group comparisons of categorical values (*$P < 0.05$). Wilcoxon rank sum test was used to compare anti-CSP titres in the two groups. *P* values are indicated on top of each comparison

Phylogenetic analyses in our cohort demonstrated the presence of genotype 1 (n = 2, 7.1%) and 2 (n = 24, 92.3%). Most of our genotype 2 strains clustered within group 2a, originally described from Venezuela. Genotype 1 and 2 have been previously reported in Tanzania but there are no published data available for Equatorial Guinea [59, 60]. The predominance of genotype 2 in our study is somewhat surprising. Given the diverse geographic origin of our volunteers recruited from East and West-central Africa, we had expected to find higher HPgV-1 genetic diversity. Studies in neighbouring countries including Cameroon, the Democratic Republic of Congo and Gabon revealed a high prevalence of genotype 1 [61–65]. Genotypes 2 and 5 were also seen when phylogenetic studies included molecular markers other than 5′ UTR region like envelope protein 1 (E1), non-structural protein 3 (NS3) and non-structural protein 5A [62, 66]. The limitations of amplification of the 5′ UTR, a highly conserved region, to discriminate closely related isolates is known [67]. Due to its high variability, E2 provides better genotyping resolution compared to 5′ UTR. We amplified and sequenced the E2 region from subjects with high viremia levels in serum (n = 9). Based on the E2 sequences, these 9 isolates clustered with strains described elsewhere in Africa. It is possible that the failure to amplify E2 from all volunteers positive by 5′ UTR detection is either due to the low sensitivity of the assay used or the high genetic diversity of the E2 region [42]. While it is known that the detection of HPgV-1 based on amplification of the E2 region

is highly specific, it requires higher amounts of RNA input [42] and individuals with low HPgV-1 viremia are likely missed. Alternatively, it is possible that E2 genetic variants could not be amplified with the primers used due to nucleotide sequence mismatch. The E2 region is highly variable and this diversity contributes to structural, functional and immunogenic properties of the virus [68]. The inconsistent genotyping results of isolates 6EG and 14EG based on 5′ UTR and E2 amplification might be resolved by whole genome sequencing of the virus. Vitrenko et al., reported similar findings in samples from Ukrainian females donating fetal tissues [67].

Cytokines, chemokines and growth factors are important for inter-cellular communication and regulation of immune processes [69]. Any changes in levels of these immune mediators can act as markers of inflammation, immunity or vaccine uptake [26, 70, 71]. We therefore investigated if altered levels of cytokines and chemokines unique to ongoing HPgV-1 infection could be identified. We analysed serum samples taken at baseline for 45 cytokines in a Luminex platform. Volunteers with chemokine and cytokine levels above the lower limit of detection were stratified according to the HPgV-1 infection status. Of all 23 differentially detected cytokines and chemokines, IL-2 and IL-17A were significantly higher in HPgV-1 positive compared to HPgV-1 negative individuals.

IL- 2 is an essential survival factor for T and B lymphocytes [47, 72] and induces the development and survival of regulatory CD4 T cells critical for the maintenance of immune tolerance [73]. Fama et al., showed increased levels of circulating soluble IL-2 receptor (sIL-2R) in HPgV-1 positive volunteers but the authors did not quantify IL-2 levels [74]. The increased concentrations of IL-2 seen amongst the HPgV-1 positive individuals could be linked to either on-going antiviral immunity [75] or serves as a survival mechanism used by the virus to establish persistence in immune cells. A similar mechanism has been described in the apicomplexan pathogen *Theileria parva* that infects T and B lymphocytes in cattle [76]. Contrary to our observations are results from HPgV-1/HIV-1 coinfection studies which have shown reduced T-cell activation and IL-2 release in coinfected individuals [77, 78]. The HPgV-1 envelope protein 2 (HPgV1-E2) has been implicated in these outcomes, due to its ability to inhibit T cell-receptor mediated signalling and IL-2 signalling pathways [77, 78].

IL-17A induction has been associated with bacterial, fungal, autoimmune and inflammatory diseases [79]. IL-17A stimulates production of chemokines such as monocyte chemoattractant protein-1 which mediates tissue infiltration of monocytes. The role of IL-17A in the context of HPgV-1 infection is unknown. However, in other viral infections like HIV-1 and Hepatitis C, IL-17A has been shown to promote T-cell mediated anti-viral responses through activation and recruitment of dendritic cells, monocytes and neutrophils [80, 81]. Other cytokines and chemokines which could be detected, albeit not significantly different in HPgV-1 positive individuals included SCF (lower) and IL-1beta, IL-12p70, MCP-1, LIF, VEGF.A, HGF and TNF-α (higher). BDNF, EGF, Eotaxin, GRO-alpha, IFN-ɣ, IL-7, IP-10, MIP1-a, Mip-1b, PDGF.BB, PIGF.1, RANTES, SDF-1a, and VEGF.D were comparable between the two groups. The levels of these measured cytokines and chemokines are within ranges previously reported [26, 27]. While most of the previous HPgV-1 studies had focused on at risk populations, particularly on HIV-1 positive persons, our investigations are in healthy individuals [26, 74], therefor some of the observed differences could be due to health status.

Here, we observed lower, albeit not statistically significant, median anti-PfCSP titres in the HPgV-1 positive versus the negative group at baseline and 14 days past last vaccination. These observations mirror findings by Avelino-Silva et al., who showed no association between HPgV-1 infection status/viremia with yellow fever specific neutralizing antibody titres in HIV-1 positive individuals immunized with yellow fever vaccine [82]. While studies have extensively tried to understand potential inhibition mechanisms induced by HPgV-1 (and other Flaviviruses) on T cell activation [77, 83], activation pathways that might be affected in B cells are less explored. It is also possible that the effect of HPgV-1 viruses on immune responses against vaccines is negligible when studied singly, but this impact is significantly synergized in the presence of other, co-infecting viruses like EBV, CMV and HSV [85, 86]. Hence, the potential role played by the combined human virome in shaping vaccine-induced responses in different populations needs to be further explored in larger cohorts.

Clinically silent, chronic viral infections are known to modulate host immunity [16] and in turn, acute co-infections are known to drive the re-activation of asymptomatic viral infections [49]. Several viruses, like HIV-1, Ebola and HCV have been implicated in the pathogenesis and clinical outcome of ongoing malaria infections through a range of different mechanisms [84–86]. It has been suggested that HIV-1 infections worsen *P. falciparum* presentations by depleting the CD4 T-cell compartment, essential for driving malaria-specific antibody responses and for clearance of malaria infected red blood cells [84]. In contrast, better survival outcomes have been reported in Ebola infected individuals with *P. falciparum* co-infections [85]. Reports have also suggested delayed emergence of *P. falciparum* asexual blood-stages

in Gabonese individuals chronically infected with HCV [86]. Thus, we studied the impact of HPgV-1 positivity on asexual *P. falciparum* parasitemia and multiplication rates during CHMI. Vice versa, we also looked for the first time at the impact of PfSPZ vaccination and PfSPZ challenge on HPgV-1 viremia. We could not find evidence of an association between HPgV-1 infection status and asexual blood-stage parasite multiplication rates after CHMI. A slight trend towards longer pre-patent period was seen in HPgV-1 positive individuals. HPgV-1 positivity appears to increase malaria vaccine-induced protection, since slightly higher proportion of CHMI protected individuals were HPgV-1 positive (62.5% vs 51.6%). However, our study is limited by the sample size and further investigations with larger cohorts are required to corroborate these findings. Importantly, PfSPZ vaccination and PfSPZ challenge did not impact HPgV-1 viremia levels in our cohort suggesting that the conduct of CHMI is safe in HPgV-1 infected volunteers.

Conclusions

Notable effects have been reported in HPgV-1 co-infections with other RNA viruses such as HIV-1 and Ebola. Although our study is constrained with limited sample size, we have highlighted the epidemiology and genetic distribution of HPgV-1 in areas endemic for malaria. We have reported for the first time HPgV-1 genotype distribution in Equatorial Guinea. We examined the potential influence of HPgV-1 infection status on PfSPZ vaccine-induced PfCSP-antibody titres and CHMI outcome without finding any striking correlation. Our study provides first time evidence that intravenous vaccination using large numbers of attenuated *P. falciparum* sporozoites and CHMI does not increase HPgV-1 viremia in already infected volunteers.

Supplementary information

The online version contains supplementary material available at https://doi.org/10.1186/s12985-021-01500-8.

Additional file 1. Fig. 1 Flow chart of volunteers included in virome pilot study and analyses pipeline. A) Flow chart of volunteers included in virome pilot study and analyses. Samples for transcriptomic studies were selected from a subset of volunteers of BSPZV-1 (n=28). RNA sequencing was performed and, differential gene expression and blood transcriptome modules were analysed. Non –human reads data was used for virome analyses. B) Virus identification: Pilot virome study analysis pipeline-"Bagamoyo viromescan" i) Non-human (un-mapped reads) were searched for "suspected" viral hits in NCBI database containing more than 7424 viral genomes using bowtie 2. ii) Removal of low quality and complexity reads as well as reads mapping to human genome, transcriptome and repeat regions by bowtie 2, knead data and tandem repeat finder algorithms respectively. iii) Search for viral hits in the "clean" viral reads using virome scan and Taxonomer and for viral proteins using Diamond tool. iv) The non-human unmapped reads were also analysed by Fast virome explorer, without filtering host reads to allow the identification of endogenous retroviral elements and other viruses that may have been missed by Taxonomer and viromescan. C) Viral confirmation: i) Pre-selection criteria for suspected viral hits by each tool ii) In-silico confirmation of suspected viral hits through blasting in NCBI and mapping against specific viral whole genomes in geneous tool; and removal of viral contaminants. iii) Laboratory confirmation of viruses by reverse transcription polymerase chain reaction.

Additional file 2. Fig. 2 Impact of HPgV-1 infection on systemic cytokines and chemokines. Absolute cytokines, chemokines and growth factor levels at baseline are shown based on HPgV status: HPgV-1 negative (-), grey (n=35) and HPgV-1 positive (+), purple (n=9). Comparable median levels of Brain derived neutrophil factor (BDNF), Epidermal growth factor (EGF), Eosinophil chemoattractant cytokine (Eotaxin/ CCL11), Growth regulated oncogene-alpha (GRO-alpha), Interferon gamma (IFN-ɣ), Interluekin-7 (IL-7), Interferon gamma induced protein- 10 (IP-10), Macrophage inflammatory protein 1-alpha(MIP1-a), MIP1-b (Macrophage Inflammatory protein 1-beta), Platelet derived growth factor BB (PDGF.BB), Placental growth factor (PlGF.1), Regulated on activation normal T cells and excreted (RANTES), Stromal derived factor 1 alpha (SDF-1a) , and Vascular endothelial growth factor D (VEGF.D); Lower median levels of Stem cell factor (SCF); and higher median levels of Monocyte chemoattractant protein 1 (MCP-1), Leukemia inhibitory factor (LIF), Vascular endothelial growth factor A (VEGF.A), Hepatocyte growth factor (HGF) and Tumor Necrosis Factor-alpha (TNF-α) in the HPgV-1 positive individuals. Cytokines, chemokines and growth factors with values above their predefined lower detection limit were considered substantial. Wilcoxon rank sum test was used to compare the two groups and P-values are indicated on top for each comparison

Additional file 3. Fig. 3 Vaccine trial design and procedures. Volunteers are enrolled and randomized into placebo (black icons) and vaccine groups (green icons). Immunized with specified dose of radiated-attenuated whole sporozoites or whole sporozoites with antimalarial drug (V1, V2; V3 etc.) and subsequently challenged with homologous PfSPZ parasites used for vaccination (CHMI). Volunteers are monitored in a controlled setting up to 21 days with venous blood drawn daily to monitor presence (malaria positive, not protected) or absence (malaria negative, protected) of asexual blood-stage parasitemia. All volunteers were treated with an anti-malarial drug either once turning TBS positive or at day 28 after start of CHMI. Further monitoring of volunteers occurred at 56 days post CHMI. HPgV-1 infection was evaluated in plasma samples from the time points highlighted in blue.

Additional file 4. Fig. 4 HPgV-1 RNA positivity and viremia across study visits (Baseline, CHMI and CHMI+28) in Tanzania and Equatorial Guinea. HPgV-1 viral plasma RNA was measured by RT-qPCR at baseline (pre-vaccination), before (CHMI) and 28 days post immunization (CHMI+28 days) in Tanzanian (n=45) and Equatorial Guinean (n=51) volunteers. Here four volunteers from the whole cohort are displayed as a representation. The figure depicts inter-individual variability in HPgV-1 RNA detection with some individuals negative or positive at one, two or all three measured time points. Log 10 viral loads are plotted on the y-axis and the time points on the x-axis. Each square plot represents an individual with volunteer identification numbers indicated on top. Each dot corresponds to a single time point connected to the next by a solid line. The horizontal dashed line indicates the threshold value of zero viremia.

Additional file 5. Table 1 Sensitivity and standard curve ranges for the 45 cytokines, chemokines and growth factors analysed in this study. The tables shows the 45 cytokines, chemokines and growth factors their sensitivities and standard curve ranges as provided by manufacturer.

Abbreviations

CHMI: Controlled human malaria infection; CSP: Circumsporozoite protein; E1/2: Envelope glycoproteins (1 and 2); HPgV: Human pegivirus; IVT: In vitro transcription; LEfSe: Linear discriminant analysis effect size; NK: Natural killer cells; NS5A: Non-structural protein 5A; PfCSP: *Plasmodium falciparum* Circumsporozoite protein; PfSPZ: Plasmodium falciparum sporozite; PMR: Parasite Multiplication Rate; RNaseP: Ribonuclease P; SSA: Sub-Saharan Africa; TBS: Thick blood smear; UTRs: Untranslated regions.

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Authors' contributions
Study concept and design: AT, TS, CD, Investigation: AT, TS, JP, MP, KS, Technical support and resources: SM, DM, Analyses and interpretation of data: AT, TS, NOF, CD, Drafting the manuscript and reviewing: AT, TS, NOF, CD, and all other authors reviewed the manuscript, Study supervision: CD; AO, SJ, Funding acquisition: CD, MT, KS. All authors read and approved the final manuscript.

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Availability of data and materials
Data are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate
The studies were registered at Clinicaltrial.gov. under the registration numbers NCT02132299 (BSPZV1), NCT02613520 (BSPZV2), NCT03420053 (BSPZV3a) and NCT02859350 (EGSPZV2). All clinical trials were approved by the Institutional Review Board for the Ifakara Health Institute (IHI-IRB), Tanzanian Food and Drug Administration (TFDA), Tanzanian National Institute for Medical Research (NIMR) and the Ethical Committee of Northern and Central Switzerland (EKNZ). Written informed consent was obtained from all participants prior enrolment. All trial procedures were conducted in accordance to good clinical practice (GCP) and under the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Malaria Journal

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Incidence of *Plasmodium falciparum* malaria infection in 6-month to 45-year-olds on selected areas of Bioko Island, Equatorial Guinea

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Abstract

Background: Extensive malaria control measures have been implemented on Bioko Island, Equatorial Guinea over the past 16 years, reducing parasite prevalence and malaria-related morbidity and mortality, but without achieving elimination. Malaria vaccines offer hope for reducing the burden to zero. Three phase 1/2 studies have been conducted successfully on Bioko Island to evaluate the safety and efficacy of whole *Plasmodium falciparum* (Pf) sporozoite (SPZ) malaria vaccines. A large, pivotal trial of the safety and efficacy of the radiation-attenuated Sanaria® PfSPZ Vaccine against *P. falciparum* is planned for 2022. This study assessed the incidence of malaria at the phase 3 study site and characterized the influence of socio-demographic factors on the burden of malaria to guide trial design.

Methods: A cohort of 240 randomly selected individuals aged 6 months to 45 years from selected areas of North Bioko Province, Bioko Island, was followed for 24 weeks after clearance of parasitaemia. Assessment of clinical presentation consistent with malaria and thick blood smears were performed every 2 weeks. Incidence of first and multiple malaria infections per person-time of follow-up was estimated, compared between age groups, and examined for associated socio-demographic risk factors.

Results: There were 58 malaria infection episodes observed during the follow up period, including 47 first and 11 repeat infections. The incidence of malaria was 0.25 [95% CI (0.19, 0.32)] and of first malaria was 0.23 [95% CI (0.17, 0.30)] per person per 24 weeks (0.22 in 6–59-month-olds, 0.26 in 5–17-year-olds, 0.20 in 18–45-year-olds). Incidence of first malaria with symptoms was 0.13 [95% CI (0.09, 0.19)] per person per 24 weeks (0.16 in 6–59-month-olds, 0.10 in

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5–17-year-olds, 0.11 in 18–45-year-olds). Multivariate assessment showed that study area, gender, malaria positivity at screening, and household socioeconomic status independently predicted the observed incidence of malaria.

Conclusion: Despite intensive malaria control efforts on Bioko Island, local transmission remains and is spread evenly throughout age groups. These incidence rates indicate moderate malaria transmission which may be sufficient to support future larger trials of PfSPZ Vaccine. The long-term goal is to conduct mass vaccination programmes to halt transmission and eliminate *P. falciparum* malaria.

Keywords: Malaria, *Plasmodium falciparum*, Incidence, PfSPZ Vaccine, Malabo, Bioko Island, Equatorial Guinea

Background

The burden of malaria is concentrated in sub-Saharan Africa, where 94% of the estimated 229 million malaria cases occurred in 2019, the large majority caused by *Plasmodium falciparum* (Pf). Children and pregnant women are the most vulnerable for dying of the disease, and progress with burden reduction has plateaued [1]. The World Health Organization (WHO) is currently promoting the "high burden to high impact" approach prioritizing eliminating malaria deaths as an immediate, focused response [2]. However, the WHO and multiple international stakeholders have renewed their commitment to long-term malaria control and elimination, and emphasized the need for new tools for tackling the disease. New vector control measures, drugs, and vaccines are under development to control and eliminate malaria, including subunit and whole PfSPZ Vaccines [3].

The Government of Equatorial Guinea (EG) is partnering with Medical Care Development International (MCDI), Ifakara Health Institute (IHI), Swiss Tropical and Public Health Institute (Swiss TPH), and Sanaria Inc. to evaluate the safety and efficacy of the whole *P. falciparum* sporozoite (PfSPZ) malaria vaccine approach, with radiation-attenuated Sanaria® PfSPZ Vaccine and the chemo-attenuated Sanaria® PfSPZ-CVac, the two leading products under development [4, 5]. PfSPZ Vaccines are intended to be offered to whole populations through mass vaccination programmes (MVPs) to eliminate malaria in defined geographic areas.

Three phase 1/2 studies of PfSPZ Vaccines have been conducted in EG. The first study was the first clinical trial in the history of EG and evaluated the safety and tolerability of PfSPZ Vaccine in adults [6]. The second study assessed the safety and tolerability of PfSPZ Vaccine in 6 month- to 65-year-olds and compared the safety, tolerability, and vaccine efficacy (VE) against controlled human malaria infection (CHMI) of PfSPZ Vaccine and PfSPZ-CVac [7]. The third study assessed the safety, tolerability, and VE against CHMI of four different dosage regimens of PfSPZ Vaccine (Jongo SA et al., pers. commun.). These studies and the experience from other studies in Africa, Europe, and the USA have encouraged further development of PfSPZ Vaccines. Planning is underway to conduct a larger randomized, double-blind, placebo-controlled trial of the lead product, PfSPZ Vaccine, to assess the safety and VE against natural exposure to malaria in 2- to 50-year-olds (1400 vaccinees and 700 controls), split into two age groups, 2–12 years and 13–50 years.

An efficacious pre-erythrocytic vaccine, like PfSPZ Vaccine, which interrupts malaria transmission since the development of asexual blood stages and associated sexual stages is prevented [8]. Therefore, the planned trial of PfSPZ Vaccine will focus on VE against the incidence of malaria parasitaemia as detected by thick blood smear (TBS) in 2- to 50-year-olds in selected areas of Bioko Island, EG as the primary efficacy objective. Combined active (biweekly) and passive surveillance will be used to identify incident infections, reading ~0.54 µL of blood from a TBS and scoring the TBS as positive or negative. VE against malaria with symptoms (clinical malaria) will also be an objective.

Information on the expected incidence of malaria and malaria with symptoms on Bioko Island is needed to properly design the upcoming trial, including estimating the appropriate sample size [9, 10]. Hence, this study, called EGMALEP (Equatorial Guinea Malaria Epidemiology Project), was conducted to assess the incidence of malaria (parasitaemia) and malaria with symptoms in a closely followed cohort of participants from areas with relatively high malaria prevalence, in and around Malabo. This study used the same outcome variable as will be used in the upcoming vaccine trial—new malaria infection identified by TBS through active and passive surveillance. In addition to measuring incidence, the study assessed the concomitant illnesses that occurred in the study population and piloted tools and procedures, including the active and passive surveillance methods.

Methods

Study population

The study was conducted in communities in an urban/peri-urban area of Malabo District in the Bioko North Province on Bioko Island, EG, where the capital city, Malabo, is located. The population of Malabo District is estimated to be 191,671 individuals living in 68,306 households with an average household size of

2.8 individuals (2018 BIMCP unpublished health census data). The population is composed of several ethnic groups, including the Bubi, Fang, Annobones, Ndowe, Bisio, and Fernandino. The study area (Fig. 1) includes a health facility in Sampaka and two primary health posts in Sacriba and Basupú communities. Serious illnesses in these areas are generally cared for at the Malabo Regional Hospital, a government-owned public hospital. There are also two private hospitals in close distance to these communities.

Malaria on Bioko Island

Malaria has been a major public health problem in EG, and malaria transmission occurs throughout the year, with more cases occurring during and after the rainy season (March–December) [11]. The main malaria species, *P. falciparum*, constitutes over 95% of cases, although *Plasmodium malariae, Plasmodium ovale,* and *Plasmodium vivax* circulate on Bioko Island with low prevalence [12–15].

The major vectors maintaining residual transmission on Bioko are *Anopheles melas* and *Anopheles coluzzii* [16]. Significant reduction of malaria has been achieved on Bioko Island in the last 16 years following intensive malaria control efforts [17]. Since 2004, the Bioko Island Malaria Control Project (BIMCP) and the National Malaria Control Programme (NMCP) have implemented repeated rounds of indoor residual spraying (IRS), mass distribution of long-lasting insecticidal nets (LLINs), larval source management, behaviour change communication activities, and improved case management resulting in 63% reduction in malaria prevalence in 2–14-year-olds in 2019 [11]. Despite these efforts, pockets of high malaria prevalence remain throughout the island, and visitors import additional cases from mainland EG where transmission is more intense [11, 18].

For this study, communities with high malaria prevalence (parasitaemia above 15% by rapid diagnostic test (RDT)) based on a 3-year average (2016–2018) of data from the annual cross-sectional malaria indicator survey were selected. The selected communities were

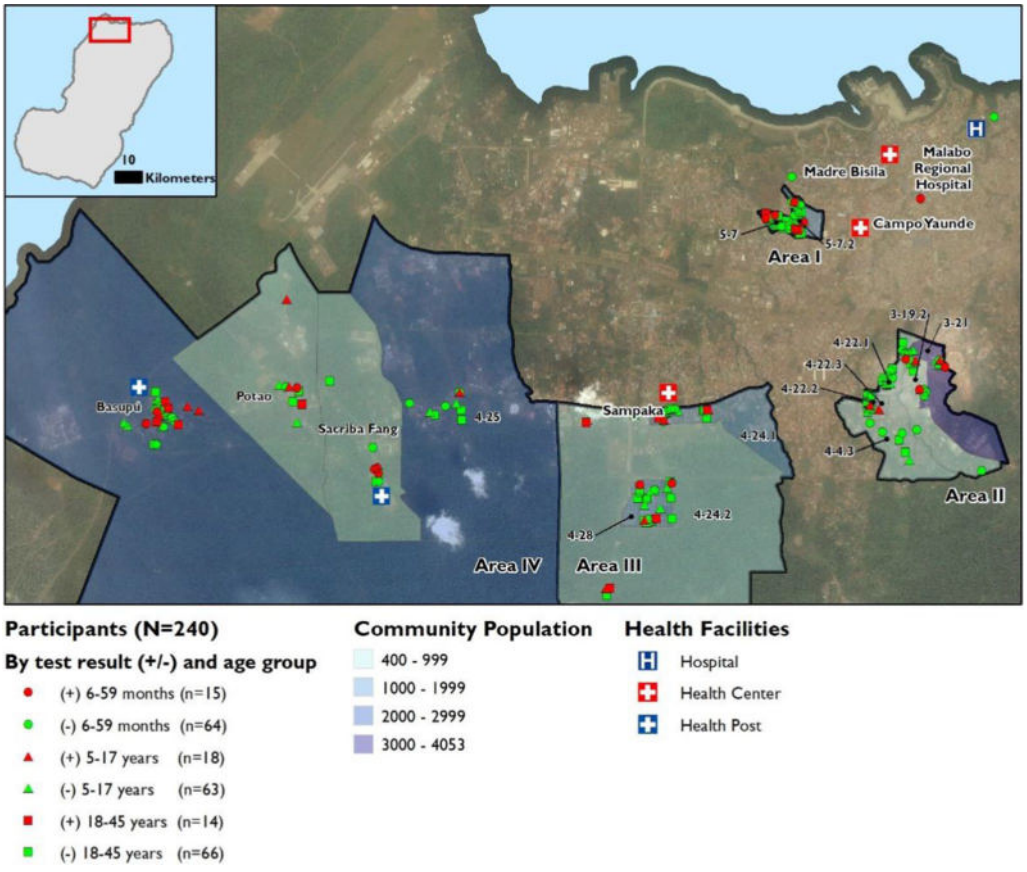

Fig. 1 Map of the study areas in the northwest sector of Bioko Island showing population estimates for each community and distribution of study participants showing the place of residence, age group, and whether parasitaemia positive or negative during surveillance

mainly from the western and southwestern regions of the Malabo District; they were categorized based on the administrative boundaries and geolocation into four study areas (area I–IV) (Fig. 1). The study was conducted out of the centrally located Sampaka Health Center (SHC) (Fig. 1).

Study design and procedures

The EGMALEP was a prospective cohort study conducted from January 28th to September 12nd, 2019. The primary objective was to estimate malaria incidence diagnosed using TBS in the areas planned for the future large trial of PfSPZ Vaccine following clearance of any existing parasitaemias. The secondary objective was to describe the characteristics and clinical presentation of the incident infections as well as assess the performance of RDT. Participants were followed for 24 weeks with biweekly TBSs (active surveillance) augmented by assessing any acute febrile illnesses (passive surveillance). Information on co-morbidities that occurred was collected from participants during study visits through updates on medical history, physical examination, and laboratory investigations. The study targeted the enrollment of 240 participants split evenly into three age groups: 6–59 months, 5–17 years, and 18–45 years.

Community sensitization and LLIN distribution

Permission was obtained from local government officials and community representatives to use the household database from the BIMCP/NMCP to identify household members to be invited to participate in the study [19]. Subsequently, community meetings were held in the study areas to sensitize the population about the study. LLINs were distributed in the study areas by the BIMCP/NMCP before the start of the study.

Participant selection

A multistage sampling was done, first communities were selected primarily due to relatively high parasite prevalence in non-travelling children aged 2–14 years and proximity to the Sampaka Health Centre (no more than 6 kms from the facility) based on the BIMCP/NMCP 2018 health census. Individual demographic information from the 15 selected communities, was aggregated to the household level to determine the number of eligible individuals per age group permanently residing in inhabited households within the study area. Households with at least one eligible individual were included in the sampling frame, representing 20,240 eligible individuals residing in 7901 eligible households.

Thereafter, Python 2.7, PANDAS package was used to randomly select and order individuals from unique households for each of the three study age groups. The number of individuals to sample within each of the communities was pre-determined to ensure a representative geographic distribution of sampled individuals proportionate to community sizes. Once an age group was filled, individuals were only invited to participate if contributing to the unfilled age groups.

Eligibility and enrolment

Potential adult participants, or the parents or guardians of potential pediatric participants, present during household visits were asked to come to SHC to provide written consent, 9–17-year-olds were asked to provide written assent, and 6–8-year-olds were asked to provide verbal assent. Screening evaluation included the history of previous illnesses, and physical examination included vital signs and anthropometric measurements. Blood samples were collected for analysis of malaria parasitaemia (TBS, RDT and quantitative PCR (qPCR), the latter performed retrospectively), complete blood count with differential, and biochemistry including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, creatinine, and glucose. A urine sample was collected for human chorionic gonadotropin pregnancy testing in women aged 9–45 years. Socio-demographic data were also collected.

Participants were considered eligible for the study if they were permanent residents of the study area, age between 6 months and 45 years, and provided consent/assent. Participants were excluded if they had a history or clinical manifestations of serious or chronic disease requiring frequent medical care including human immunodeficiency virus (HIV) infection, clinical tuberculosis, sickle cell disease, malignancies, diabetes mellitus, hypertension, mental illness, or seizures. Additional exclusion criteria were mid-upper arm circumference (MUAC) < 11.5 cm for children under 5 years of age (indicating malnourished state), grade 3 abnormal laboratory parameters (based on local normal range and toxicity grades for haematology and biochemistry), pregnancy, and history of allergy or serious adverse reaction to artemether/lumefantrine (AL). Participants with issues that increased the risk of non-adherence to study procedures, including the intention to move from the study area during the study period, were excluded.

Eligible participants were given a directly observed curative 3-day, six-dose course of artemether–lumefantrine (AL) (Lumet 80® Cipla Ltd, India) according to manufacturer's instructions [20]. All the AL doses were directly observed by clinical staff or community health care workers. To ensure compliance with taking all doses at home, the prescribed medications were maintained at the clinic pharmacy and single doses were then collected by the community health workers, were taken to

participants and were administered under supervision for each specific dosing time point. The participants were rechecked for parasitaemia 14 days later and those who were negative by blood slide were officially enrolled.

Active surveillance

The enrolled participants were actively followed up at home every 2 weeks for 24 weeks except the 12th and 24th week time points when they were invited for medical evaluation at the SHC. During the home visits, axillary temperature was measured and the participants were asked about their well-being, history of fever in the prior 24 h and travel history in the preceding 2 weeks, and the use of ITNs, IRS and mosquito repellent. If clinically well, a blood sample for TBS, RDT and qPCR was collected by venipuncture. If symptomatic with fever or history of fever, the participant was transported to study clinic for medical evaluation. At the clinic, a medical history, travel history and malaria control intervention history were taken, a clinical examination performed, and the same malaria diagnostic blood sample obtained. All blood samples for TBS, RDT and PCR were collected using ethylene diamine tetra acetic acid (EDTA) tubes and kept in a cooler box to maintain ambient indoor temperature until reaching the laboratory.

Participants were treated based on TBS results. For those TBS positive participants who were at the clinic, treatment with an anti-malarial was initiated before they were allowed to go home. For those who were seen at home they were invited back to the clinic by the community health workers and were then reviewed again at the clinic and an anti-malarial treatment initiated.

Compliance with study visits and procedures was encouraged by the provision of a study calendar to participants, by using multiple contact methods (phone calls to participants/parents/guardians or close contacts provided on enrollment to remind participants of upcoming home or clinic visits), by home visits by community mobilizers, and by provision of transportation to the study center. Participants were compensated for time spent during scheduled study related clinic visits (approximately 12 United States dollars), but not for home visits.

Passive surveillance

Study doctors were available to see study participants 24 h–7 days a week. Participants were encouraged to visit the SHC or call the study doctors whenever they were sick. Medical history, physical examination and laboratory investigations were done as needed for clinical evaluation. All participants with history of fever had their blood checked for malaria by TBS, RDT and later qPCR.

Laboratory assessments

Thick and thin blood smears and the RDTs for assessment of malaria parasites were prepared from samples collected during home visits or at the study clinic. These were prepared and independently analysed at the SHC laboratory to allow blinding and standardization of the preparation and reading of the results. All remaining study related laboratory analyses were done at the public health laboratory in Baney which is located 24 km from the SHC. TBSs were prepared and read as described previously [21]. In short, thick and thin smear were made, air dried and then stained with 4% Giemsa for 45 min for samples from asymptomatic participants and 10% Giemsa for 10 min for samples from symptomatic participants. The TBSs were read using a light microscope with a high-power field (immersion oil, 100× objective) of 0.18 mm diameter; 6 passes (0.54 µL of blood) for asymptomatic or 24 passes (2.14 µL of blood) for symptomatic participants, were read before the TBS was declared negative. The slides were read by two independent expert microscopists and any discrepancies were resolved by a third microscopist. Parasite densities were calculated as the number of asexual parasites counted per volume of blood examined. All positive TBSs were verified retrospectively at the public health laboratory in Baney using qPCR, as described previously [15]. In short, the qPCR multiplex assay targeted two independent *Plasmodium* genes namely the Pan-*Plasmodium* 18S RNA sequence (Pspp18S) and the *P. falciparum*-specific acidic terminal sequence of the *var* genes (PfvarATS). The human Ribonuclease P gene (HsRNaseP) was used as a DNA extraction and qPCR amplification control. All qPCR assays were run in duplicate and both non-template control (Molecular grade nuclease-free water) and *P. falciparum* 3D7 DNA were included as negative and positive controls, respectively. The sample was considered positive if the quantitation cycles (Cq) for each of the two replicates was Cq < 40 for *Plasmodium* spp. gene and Cq < 28 for qPCR amplification control. Haematology parameters were established using ABX Pentra 60 C+ (Horiba Medical, USA) and biochemistry parameters using Cobas Integra 400 Plus (Roche, Switzerland). Urine pregnancy tests were done using Hexagon hCG 1-step rapid tests (Human Diagnostics Worldwide, Germany).

Data management

Information was collected on paper-based study case reports forms and these were entered into a customized electronic database, Castor EDC^{R}. Data were entered and verified by independent teams.

Sample size

The design assumed an incidence of new malaria infections (parasitaemia) over 24 weeks of 10% for each age group. In order to detect an incidence rate of 10% per person per 24 weeks with a relative precision of 20% (from 8 to 12%) for each age group at 90% confidence level, and considering a 15% rate of loss to follow up, a sample of 80 participants was targeted for recruitment in each age category.

Statistical analysis

Data were analysed using STATA version 15 (StataCorp, Texas, USA). Descriptive statistics were used to summarize the data. Household socioeconomic status (SES) was calculated as a weighted sum of data on household possessions and utilities, using principal components analysis and the scores divided into quintiles. Elements included in the measure: ownership of radio, TV, bicycle, refrigerator, mobile phone, computer, bed net, and source of energy (i.e., binary variable scoring presence or absence of electricity/gas/solar). The incidence rate of first infections was computed for 24 weeks of follow up as the total number of first infections observed after enrolment divided by total person time at risk.

Person-time was censored for loss to follow up or identification of a first infection. Incidence rate for multiple infections was computed for the 24 weeks of follow-up as the total number of malaria infections developing after enrolment divided by the total person-time at risk. Twenty-eight days were censored off from time at risk after each treatment of malaria infection. Kaplan Meier survival curves were used to compare time to first infection between groups, and Cox regression analysis was used to explore factors associated with first incidence of malaria infection. Lastly, proportional incidence of infection was computed as the number of participants developing first *P. falciparum* infection divided by the total number of participants at the start of follow up for each age category.

Results

Following the ordered sampling frame of participants identified from the BIMCP/NMCP household database who lived in the selected study areas (see "Methods"), 545 were contacted, and 306 expressed an interest to participate. After providing informed consent and undergoing screening, 66 were excluded: 37 did not meet the inclusion and exclusion criteria, 8 withdrew consent, 8 were not enrolled because the target number for recruitment was reached, 6 were lost to follow up, 2 did not finish the screening process, 2 had plans to travel outside the country, 1 left the study area, 1 did not complete AL pre-treatment and 1 had malaria by TBS 14 days after presumptive treatment (Fig. 2). 240 individuals completed pre-treatment, had a negative TBS thereafter and were enrolled. Screening and pre-treatment extended from 3rd of December 2018 to 15th of March 2019. One participant from the youngest group became 5 years old by the start of follow up and hence was re-categorized into the middle age group, leaving 79, 81 and 80 participants in groups 1 (6–59 months), 2 (5–17 years) and 3 (18–45 years), respectively, at the start of surveillance 14 days after AL.

The median age in years of those enrolled was 9.80 with range (IQR) from 1.02 to 42.51 (Table 1). The median ages and range for those in groups 1–3 were 2.65 (1.02, 4.99), 9.67 (5.01, 17.66) and 29.58 (18.3, 42.5) years, respectively. There were no infants recruited in Group 1 (also see Table 1). Overall, similar numbers of males and females were enrolled (ratio 0.91:1). The numbers of participants from the selected administrative areas of the city were well-balanced: Area I: 53; Area II: 63; Area III: 59; Area IV: 65. The distribution of wealth categorizes also appeared balanced between each age category (Table 1).

Clinical parameters collected at screening for those enrolled in the three age categorizes attested to good health.

Surveillance began 14 days after completing AL administration, and extended for 24 weeks. The period of surveillance for the first participants began on 28th January 2019 and ended on 15th July 2019, and for the last participants began on 2nd April 2019 and ended on 12th September 2019. During the follow up 33 participants dropped out of the study (13.8% with 95% confidence intervals (CI) 10.0, 18.8), 6 participants in group 1, 11 participants in group 2, and 16 participants in group 3 (Fig. 2). The reasons for drop-out were: 20 migrated outside study area, 12 withdrew consent (3 in group 1, 4 in group 2 and 5 in group 3) and 1 was lost to follow-up.

There were 58 malaria infection events, 47 first events and 11 secondary events, of which 9 were second episodes and 2 were third episodes. All infection events positive by TBSs were confirmed as positive for Pf by retrospective qPCR. The results of the comparison between the different malaria diagnostics tests performed, including TBS, RDT and qPCR, will be presented elsewhere. Five of the second episodes and all third episodes were in participants from Area IV the most rural study area. Four of the second episodes were among participants aged 5–17 years and 3 were in those aged 18–45 years. All individuals with third episodes were aged 5–17 years.

The overall incidence rate throughout the study period was 0.25 (95% CI 0.19, 0.32) per person per 24 weeks follow-up. The overall incidence of first infection was 0.23 (95% CI 0.17, 0.30) per person per 24 weeks. The

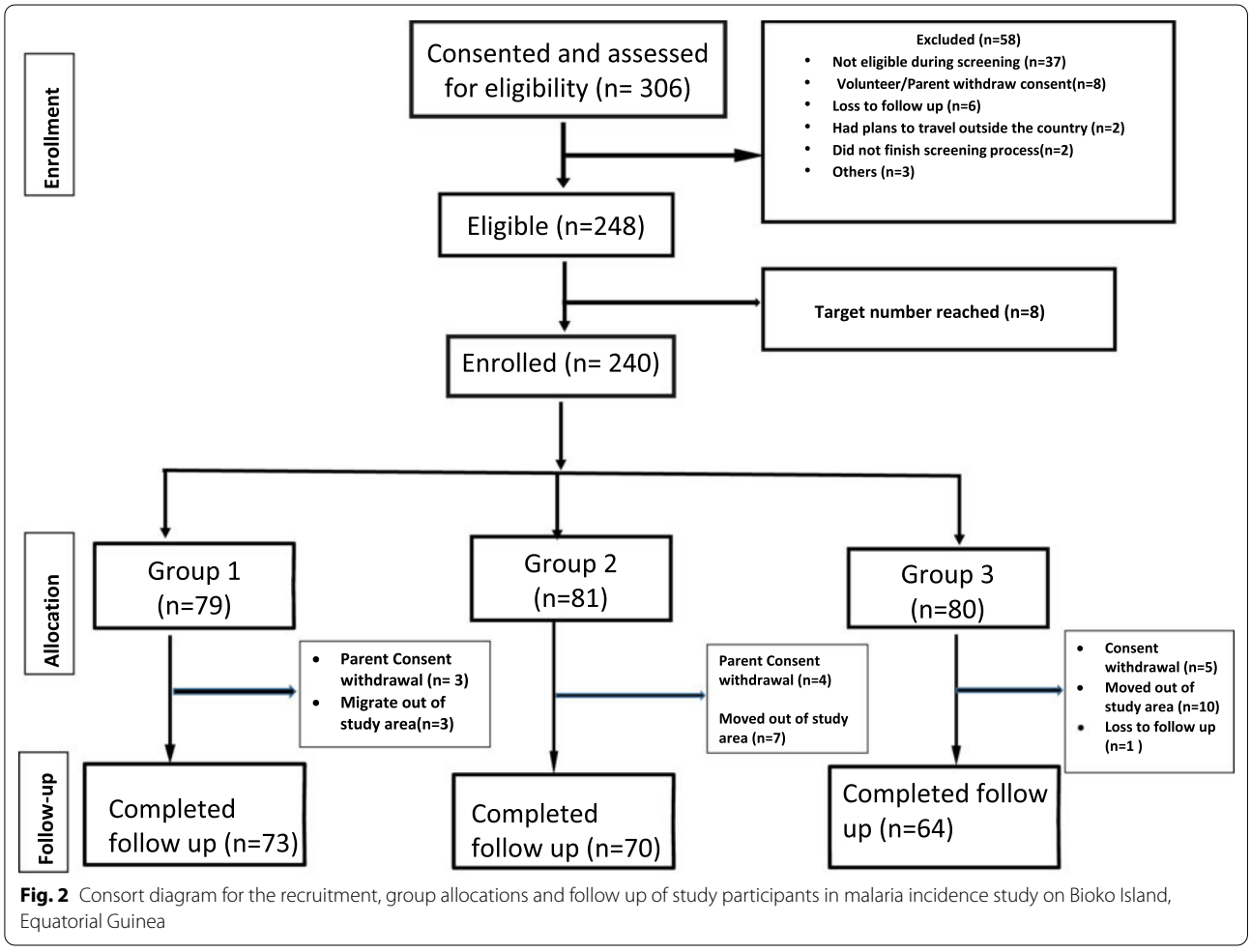

Fig. 2 Consort diagram for the recruitment, group allocations and follow up of study participants in malaria incidence study on Bioko Island, Equatorial Guinea

observed incidence rate for first infections was similar in the three age categories: 0.22 (95% CI 0.13, 0.36), 0.26 (95% CI 0.17, 0.42) and 0.20 (95% CI 0.12, 0.34) infections per 24 weeks in groups 1, 2 and 3, respectively (Table 2). First infection rates using a re-categorization of age ranges separating out 6–23-month-olds and, for those age 2 years and above, using the adjusted age ranges planned for the upcoming large trial, showed similar results: 0.15 (95% CI 0.05, 0.46) for 6–23-month-olds, 0.22 (95% CI 0.15, 0.34) for 2–12-year-olds, and 0.25 (95% CI 0.16, 0.38) for 13–45-year-olds.

Incidence of infection appeared to be greater in: (1) males, 0.31 (95% CI 0.22, 0.45) versus females, 0.16 (95%CI 0.10, 0.25), the trend observed in all areas, and (2) those living in Areas III, 0.26 (95% CI 0.15,0.45) and IV, 0.39 (95% CI 0.25, 0.61) compared to those in Areas I, 0.14 (95% CI 0.07, 0.30) and II, 0.12 (95%CI 0.06, 0.25). Incidence was lowest in the highest socioeconomic group (Table 2).

The Kaplan Meier estimator showed trends toward significant differences for the chosen parameters (Fig. 3). The use of ITNs was universal and there were no differences related to the use of malaria control interventions during the study. None of the malaria infections were preceded by history of travel in the previous 2 weeks, indicating mainly local transmission. However, there were 47 participants who traveled at least once in the 24 weeks of follow up, and these individuals appeared to have a higher incidence rate (32%) than those who did not travel (22%) (Table 2). Multivariate assessment of the important factors predicting infection showed that study area, gender, being malaria positive at screening, and socio-economic status independently predicted the observed incidence of first malaria infection (Table 3).

There were 31 cases of malaria with symptoms; these were 76% (n = 13), 42% (n = 10) and 47% (n = 8) of the total cases of malaria recorded in groups 1, 2 and 3 respectively. Of these, 27 were first cases of malaria with symptoms. The incidence rate for all cases of malaria with symptoms was 0.14 (95% CI 0.09, 0.19) and for first malaria with symptoms was 0.13 (95% CI 0.09, 0.19). The incidence rates of first cases of malaria

Table 1 Characteristics of 240 participants enrolled in malaria incidence study on Bioko Island, Equatorial Guinea (number (%))

Variable	Total	Age groups		
		6 to 59 months n = 79	5 to 17 years n = 81	18 to 45 years n = 80
Median age in years (range)	9.80 (1.02, 42.51)	2.65 (1.02, 4.9)	9.67 (5.01, 17.66)	29.58 (18.3, 42.5)
Sex				
Male	114 (47.5)	37 (46.8)	40 (49.4)	37 (46.3)
Female	126 (52.5)	42 (53.2)	41 (50.6)	43 (53.8)
Area				
Area I	53 (22.1)	18 (22.8)	18 (22.2)	17 (21.3)
Area II	63 (26.3)	27 (34.2)	21 (25.9)	15 (18.8)
Area III	59 (24.6)	10 (12.7)	19 (23.5)	30 (37.5)
Area IV	65 (27.1)	24 (30.4)	23 (28.4)	18 (22.5)
Household SES				
Lower	68 (28.3)	17 (21.5)	23 (28.4)	28 (35)
Upper lower	31 (12.9)	14 (17.7)	07 (8.6)	10 (12.5)
Middle	51 (21.3)	25 (31.6)	14 (17.3)	12 (15.0)
Upper middle	43 (17.9)	13 (16.5)	16 (19.8)	14 (17.5)
Upper	47 (19.6)	10 (12.7)	21 (25.9)	16 (20.0)
Use bed net as vector control	239 (99.6)	79 (100)	80 (98.8)	80 (100)
Clear vegetation around the house	185 (77.1)	61 (77.2)	59 (72.8)	65 (81.3)
Drying standing water	157 (65.4)	59 (74.7)	54 (66.7)	44 (55.0)
TBS positive before enrollment[b]	15 (6.3)	2 (2.5)	9 (11.1)	4 (5)
Haemoglobin level (mean ± SD)				
Male	11.9 ± (1.7)	10.7 ± (0.9)	11.4 ± (1.3)	13.6 ± (1.4)
Female	10.9 ± (1.3)	10.6 ± (1.2)	11.0 ± (1.3)	11.1 ± (1.3)
Creatinine level in mg/dL (median, range)[a]	0.47 (0.2, 2.04)	0.29 (0.20, 0.49)	0.49 (0.27, 0.80)	0.76 (0.23, 2.04)
Glucose level in mg/dL (median with range)	86 (61, 154)	88 (63, 154)	86 (62, 134)	84 (61, 147)

All values are number (%), unless specified otherwise

[a] N = 239, one subject in group 2 lacks creatinine results

[b] Malaria positive at screening or between screening and enrollment (within 2 months before enrolled into the study; however all participants were given presumptive treatment and retested before the day of initiation of the study and were negative by TBS before entering surveillance. Retrospective qPCR confirmed negative results in all included study subjects 14 days after AL treatment

with symptoms in groups 1, 2, 3 were 0.16 (95% CI 0.09, 0.30), 0.10 (95% CI 0.05, 0.22), 0.11 (95% CI 0.06, 0.22), respectively. Younger children and those living in Area IV tended to have more clinical episodes, but the numbers were small and the differences between areas were not statistically significant (Table 4). The incidence numbers for first malaria with symptoms using the adjusted age ranges consistent with the upcoming large trial were 0.10 (95% CI 0.03, 0.40) for 6–23-month-olds, 0.13 (95% CI 0.08, 0.23) for 2–12-year-olds, and 0.14 (95% CI 0.08, 0.24) for 13–45-year-olds.

The observed parasite density for symptomatic infections was higher than for asymptomatic infections with geometric mean parasites/μL of 1351.57 (95% CI 512.79, 3562.38) and 157.48 (95% CI 58.25, 425.72), respectively. The geometric mean parasite density for all cases of malaria with symptoms was 1898 (95% CI 431–8364), 357 (95% CI 88–1449) and 112 (95% CI 26–493) for age groups 6 to 59 months, 5 to 17 years and 18 to 45 years, respectively.

Common illnesses that occurred in the study participants included respiratory tract infections, gastroenteritis, diarrhoea, and skin infections. About 70% (95% CI 60, 79) of illnesses required medical attention during the 24 weeks of follow up, and the young age group had more events compared to the older age groups (Table 5).

Discussion

The areas of Malabo surveyed in this study are characterized by ongoing moderate malaria transmission despite over 16 years of intensive malaria control interventions. As expected, there was variation in the incidence of infection in different locations of the city; the semi-rural locations of the study (Area III and Area IV) had significantly higher burden compared to the more urban locations. This heterogeneity indicates that malaria control

Table 2 Incidence rate of malaria infection by TBS among participants enrolled in malaria incidence study on Bioko Island, Equatorial Guinea

	n	Events	Proportion positive (95% CI)	Incidence rate[a] (95% CI)
All infections				
Total	240	58	0.24 (0.19, 0.30)	0.25 (0.19, 0.32)
First infections				
Total	240	47	0.19 (0.15, 0.25)	0.23 (0.17, 0.30)
Age group				
6 to 59 months	79	15	0.19 (0.12, 0.29)	0.22 (0.13, 0.36)
5 to 17 years	81	18	0.22 (0.14, 0.33)	0.26 (0.17, 0.42)
18 to 45 years	80	14	0.18 (0.11, 0.28)	0.20 (0.12, 0.34)
Sex				
Male	114	29	0.25 (0.18, 0.34)	0.31 (0.22, 0.45)
Female	126	18	0.14 (0.09, 0.22)	0.16 (0.10, 0.25)
Area				
Area I	53	7	0.13 (0.06, 0.26)	0.14 (0.07, 0.30)
Area II	63	7	0.11 (0.05, 0.22)	0.12 (0.06, 0.25)
Area III	59	13	0.22 (0.13, 0.35)	0.26 (0.15, 0.45)
Area IV	65	20	0.31 (0.21, 0.43)	0.39 (0.25, 0.61)
TBS positive before enrollment[b]				
Yes	15	6	0.4 (0.17, 0.68)	0.51 (0.23, 1.12)
No	225	41	0.18(0.14, 0.24)	0.21 (0.15, 0.29)
Household SES				
Lower	68	14	0.21 (0.12, 0.32)	0.25 (0.15, 0.43)
Upper lower	31	12	0.39 (0.23, 0.58)	0.46 (0.26, 0.82)
Middle	51	9	0.18 (0.09, 0.31)	0.20 (0.10, 0.38)
Upper middle	43	9	0.21 (0.11, 0.36)	0.26 (0.14, 0.50)
Upper	47	3	0.06 (0.02, 0.19)	0.07 (0.02, 0.20)
Travel[c]				
Yes	18	5	0.28 (0.11, 0.55)	0.32 (0.13, 0.78)
No	221	42	0.19 (0.14, 0.25)	0.22 (0.16, 0.3)

[a] Rate per 24 weeks

[b] Malaria positive by [TBS at screening or between screening and enrollment (within 2 months before enrolled into the study), however all participants were given presumptive treatment and retested before the day of initiation of the study

[c] Yes, if a person traveled at least once at any time point during the study

interventions have variably impacted malaria prevalence and morbidity depending on the setting; importantly, however, nowhere have they interrupted transmission, a situation shared with many other settings in Africa [22–24]. The differences between locations were true for both males and females, with the former showing higher incidence rates in all locations.

Furthermore, significant local transmission remains despite the impact of the study itself, which, due to active surveillance and prompt treatment, could be expected to reduce the incidence of malaria infection in the participants beyond the effects of ongoing control measures, as has been observed in several sites in Africa [25, 26]. Hence, it is important that additional tools including vaccines be developed and implemented to fast track malaria control and elimination [8, 27].

The relatively similar incidences of both asymptomatic and symptomatic malaria among the age groups suggests that intensive control has shifted the age pattern of the risk of infection away from children compared to before the intervention [11], as seen elsewhere in Africa [28, 29]. This indicates that adults in Malabo have partially lost their acquired immunity, are at increased risk of malaria with or without symptoms, and will need additional protection when they visit areas of high transmission like mainland EG. The deployment of a malaria vaccine such as PfSPZ Vaccine aimed at protecting all age groups could be an effective additional tool for control and elimination of malaria in this setting.

Surprisingly, the results of this study indicate there was a significantly higher risk of malaria infection in males than in females, but no higher risk for other illness events including pneumonia and diarrhea. This sex difference in malaria infection, contrasting with similar studies in moderate transmission areas in Africa [30], extended to all age groups and thus, although partially explainable by gender-related sociocultural or occupational differences [22, 31], may reflect other factors as well. Animal and human studies suggest that gender-specific susceptibility to infection is related to hormonal differences [32, 33]. It remains to be seen if this difference will be confirmed in the large vaccine trial or more importantly if this translates into differences in protection or rates of adverse events related to vaccinations.

Travel has been documented to be an important contributing factor to the maintenance of local transmission in Malabo, and Bioko Island as a whole [34, 35]. However, no cases were categorized as travel-related though those individuals who had a history of travel tended to have more infections.

As expected, the incidence of malaria with symptoms was lower (13%) compared to asymptomatic infections (20%), likely due, at least in part, to intense follow up and prompt treatment [36] as the majority of the asymptomatic infections may have become symptomatic if untreated [37, 38]. Hence, the use of infection rather than infection plus symptoms as an end point for evaluation of malaria interventions is appropriate and safer [39]. Furthermore, the phase 3 evaluation of the pre-erythrocytic vaccine RTS,S/AS01 did not show any difference in the estimated efficacy between infection or clinical malaria with different parasitaemia density cut-offs.

Overall, there were few illnesses during follow up which is similar to what has been observed in other

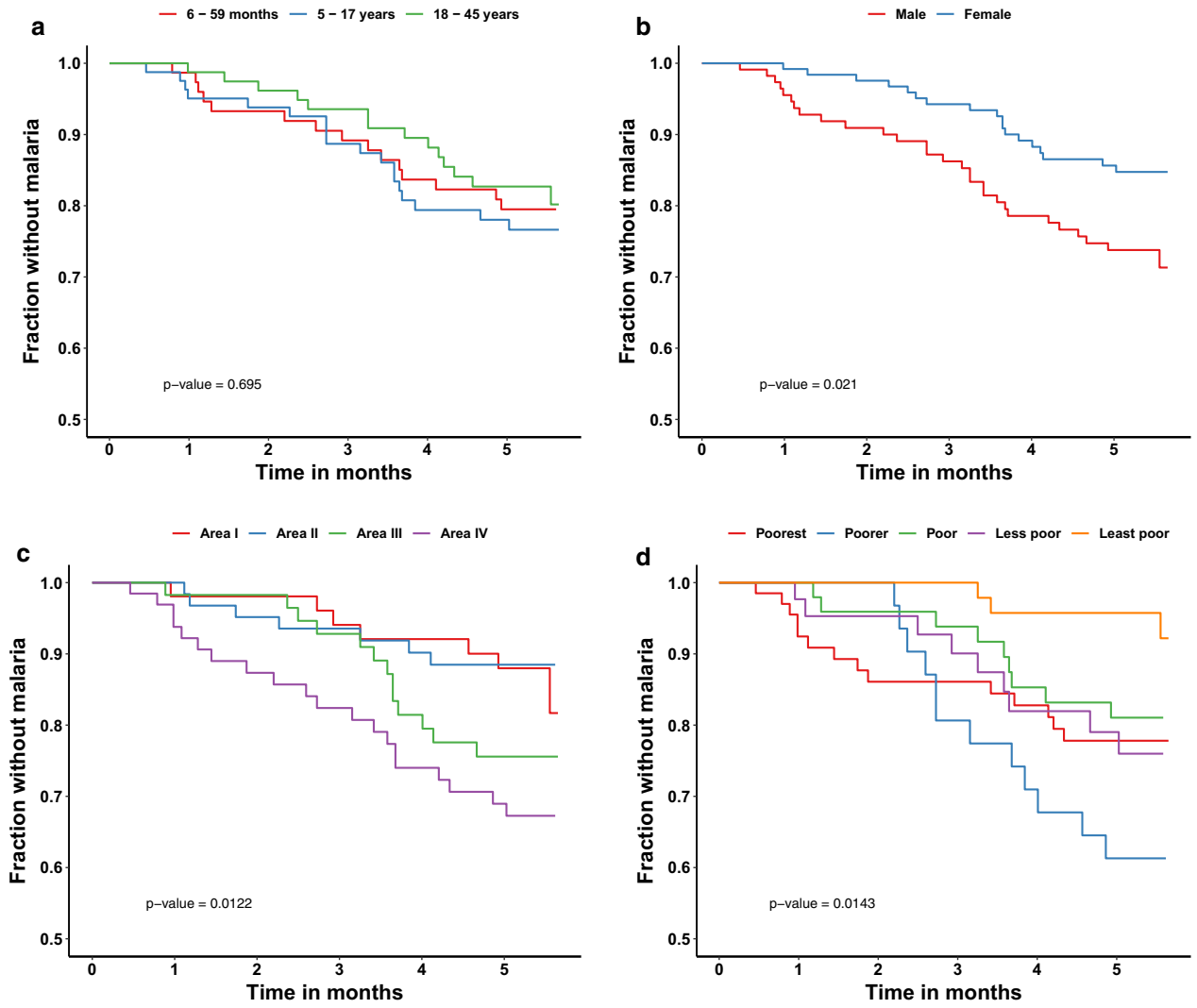

Fig. 3 Survival curves comparing time to first malaria infection over the 6 months of malaria incidence study follow-up on Bioko Island, Equatorial Guinea. **A** Incidence of first malaria infection by age group; **B** incidence of first malaria infection by gender; **C** incidence of first malaria infection by area; **D** incidence of first malaria infection by quantile of social economic status of the household

studies in Africa where good clinical care is provided as part of a study [25, 26]. As expected, most of the illness events were mild and concentrated in the younger children, with pneumonia and diarrhea being the common presentations [26].

This study was conducted to serve as a baseline for the conduct of a large safety and efficacy study of PfSPZ Vaccine that has so far shown promising results in EG and elsewhere [6, 7, 40]. The incidence across the age ranges of approximately 20% over 24 weeks and relatively good adherence to follow up of approximately 86% give confidence that a robust assessment of the safety and efficacy of PfSPZ Vaccine against natural infection can be conducted in Malabo. One caveat is that during the period during which the study was conducted, there was higher than average rainfall and an increase in health facility malaria cases of 44% across the island (unpublished BIMEP health Information system data); hence, the observed incidence may be on a higher side.

The study provided a good platform for capacity development, building the skills and confidence of the research team; the ability to conduct assessments of malaria incidence will be critical for tracking the success of future efforts to eliminate malaria from Bioko Island.

Table 3 Factors associated with first malaria infection among participants enrolled in malaria incidence study on Bioko Island, Equatorial Guinea (Cox regression, N = 240)

Variable	Crude hazard ratio (95% CI)	Adjusted hazard ratio (95% CI)[a]
Age group		
6 to 59 months	1	1
5 to 17 years	1.21 (0.61, 2.41)	1.31 (0.63, 2.76)
18 to 45 years	0.9 (0.43, 1.86)	1.09 (0.51, 2.33)
Sex		
Male	1	1
Female	0.5 (0.28, 0.91)*	0.39 (0.21, 0.72)*
Area		
Area I	1	1
Area II	0.83 (0.29, 2.37)	1.17 (0.4, 3.44)
Area III	1.84 (0.73, 4.60)	1.85 (0.71, 4.81)
Area IV	2.78 (1.17, 6.57)*	3.63 (1.5, 8.8)*
TBS positive before enrollment[b]		
Yes	1	1
No	0.41 (0.17, 0.97)*	0.25 (0.1, 0.68)*
Household SES		
Lower	1	1
Lower middle	1.82 (0.84, 3.94)	3.35 (1.46, 7.68)*
Middle	0.76 (0.33, 1.76)	1.06 (0.45, 2.52)
Upper middle	1.03 (0.44, 2.37)	1.56 (0.66, 3.69)
Upper	0.25 (0.07, 0.87)*	0.31 (0.09, 1.14)

* Wald test p value < 0.05

[a] Adjusted for all variables in the table

[b] Malaria positive at screening or between screening and enrollment (within 2 months before enrolled into the study); however all participants were given presumptive treatment and retested before the day of initiation of the study

Conclusion

Intensive malaria control efforts in Bioko Island have resulted in a large reduction in the prevalence of malaria; local transmission remains, however, and the risk of infection is now spread relatively evenly through all age groups, a finding which is consistent with areas that have changed from high to lower transmission areas. The remaining burden of malaria offers an opportunity to properly evaluate the efficacy and safety of PfSPZ Vaccine and establish the safety and efficacy data required to receive marketing authorization (licensure).

Table 4 Incidence rate of malaria with symptoms by TBS among participants enrolled in malaria incidence study on Bioko Island, Equatorial Guinea

	n	Events	Proportion positive (95% CI)	Incidence rate[a] (95% CI)
All clinical infections				
Total	240	31	0.13 (0.09, 0.18)	0.14 (0.09, 0.19)
First clinical infections				
Total	240	27	0.11 (0.08, 0.16)	0.13 (0.09, 0.19)
Age group				
6 to 59 months	79	12	0.15 (0.09, 0.25)	0.16 (0.09, 0.30)
5 to 17 years	81	7	0.09 (0.04, 0.17)	0.10 (0.05, 0.22)
18 to 45 years	80	8	0.10 (0.05, 0.19)	0.11 (0.06, 0.22)
Sex				
Male	114	14	0.12 (0.07, 0.19)	0.15 (0.09, 0.25)
Female	126	13	0.10 (0.06, 0.17)	0.11 (0.07, 0.20)
Area				
Area I	53	5	0.09 (0.04, 0.21)	0.10 (0.04, 0.24)
Area II	63	4	0.06 (0.02, 0.16)	0.06 (0.03, 0.18)
Area III	59	6	0.10 (0.04, 0.21)	0.12 (0.05, 0.27)
Area IV	65	12	0.18 (0.11, 0.30)	0.23 (0.13, 0.41)
TBS positive before enrollment[b]				
Yes	15	1	0.07(0.01, 0.41)	0.08 (0.01, 0.59)
No	225	26	0.12 (0.08, 0.16)	0.13 (0.09, 0.19)
Household SES2				
Lower	68	6	0.09 (0.04, 0.18)	0.10 (0.04, 0.24)
Upper lower	31	8	0.26(0.13, 0.45)	0.30 (0.15, 0.62)
Middle	51	6	0.12 (0.05, 0.24)	0.13 (0.06, 0.29)
Upper middle	43	5	0.12 (0.05, 0.26)	0.14 (0.06, 0.35)
Upper	47	2	0.04 (0.01, 0.16)	0.04 (0.01, 0.18)
Travel[c]				
Yes	18	3	0.17 (0.05, 0.51)	0.26 (0.08, 0.81)
No	221	24	0.11 (0.07, 0.15)	0.12 (0.08, 0.18)

[a] Rate per 24 weeks

[b] Malaria positive at screening or between screening and enrollment (within 2 months before enrolled into the study), however all participants were given presumptive treatment and retested before the day of initiation of the study

[c] Yes, if a person travels at least once at any time point during the study

Table 5 Other illnesses reported during the study among participants enrolled in malaria incidence study on Bioko Island, Equatorial Guinea (N = 240)

	n (%)	Age group		
		6 to 59 months n = 79	5 to 17 years n = 81	18 to 45 years n = 80
Total	108 (100)	63	17	28
Acute upper and lower respiratory infections	57 (52.78)	37	7	13
Gastroenteritis and diarrheal diseases	10 (9.26)	7	1	2
Infections of the skin and subcutaneous tissues	9 (8.33)	8	1	0
Injuries and open wound	7 (6.48)	1	3	3
Unspecified fever	6 (5.56)	4	0	2
Helminthiases	5 (4.63)	2	0	3
Allergic contact dermatitis	4 (3.70)	3	1	0
Bacterial infections	3 (2.78)	0	2	1
Others	7 (6.48)	1	2	4

Abbreviations

AL: Artemether/lumefantrine; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BIMCP: Bioko Island Malaria Control Programme; CHMI: Controlled human malaria infection; CI: Confidence interval; EDTA: Ethylenediaminetetraacetic acid; EG: Equatorial Guinea; EGMALEP: Equatorial Guinea Malaria Epidemiology Project; EGMOHSW: Equatorial Guinea Ministry of Health and Social Welfare; EKNZ: Ethics Committee of Northwestern and Central Switzerland; HIV: Human immunodeficiency virus; IHI IRB: Ifakara Health Institute Institutional Review Board; IHI: Ifakara Health Institute; IQR: Interquartile range; IRS: Insecticide residual spraying; ITN: Insecticide-treated nets; MCDI: Medical Care Development International; MUAC: Mid-upper arm circumference; MVPs: Mass vaccination programmes; NMCP: National Malaria Control Programme; PfSPZ: *Plasmodium falciparum* Sporozoite; PfSPZ-CVac: Chemo-attenuated sporozoite vaccine; RDT: Rapid diagnostic test; SHC: Sampaka Health Center; Swiss TPH: Swiss Tropical and Public Health Institute; TBS: Thick blood smear; VE: Vaccine efficacy; WHO: World Health Organization.

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Authors' contributions

VN, AH, AM drafted the protocol. VN, AH, AM, GB, MO, MD, EM, GN, BN, DO attended all volunteers' clinical visits. EN, MM, JR implemented and supervised all lab procedures. UK and SA analysed the data and produced the tables and figures. SA drafted the manuscript. All authors reviewed the protocol, the standard operating procedures, the data generated and the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

The study was reviewed and approved by the Equatorial Guinea National Ethics Committee (Comité Ethico National de Guinée Equatoriale), the Ifakara Health Institute Institutional Review Board (IHI IRB), and the Ethics Committee of Northwestern and Central Switzerland (EKNZ).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

Curriculum Vitae

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CURRENT POSITIONS	Principal Investigator, Internal Medicine Research Physician and Clinical Epidemiologist stationed at the Bagamoyo Clinical Trials Unit and BIMEP CRC-Bioko Island, with primary responsibility on the assessment of Safety and Efficacy of participants in clinical trials for vaccines, drugs and human challenge trials

Professional Experience and Activities

INTERNAL MEDICINE PHYSICIAN SCIENTIST AND CLINICAL EPIDEMIOLOGIST: 2013 to Present: Ifakara Health Institute.

- Supervising project specific Clinical Sciences and Medical Operations at clinical trials sites:
  - IHI-Bagamoyo Clinical Trials Facility, Bagamoyo
  - IHI Clinical Research Centres and Satellite sites (Mwananyamala, Amana and Temeke hospitals), Dar es salaam – Tanzania
  - Bioko Island Malaria Elimination Project (BIMEP) Clinical Research Centre, Malabo – Equatorial Gui

2009-2012:

- Doctor of Medicine; general practitioner at Bombo Regional Hospital
- Department of Internal Medicine, Cardiology Unit Shanghai Tenth Peoples Hospital Affiliated to Tongji University

2008-2009: CLINICAL RESEARCH INVESTIGATOR at Muhas-Havard Research Project

2007-2008: MEDICAL INTERNSHIP at St Gasper Hospital – Itigi, Singida {Internal Medicine & Obstetrics/Gynecology} Muhimbili National Hospital – Dar Es Salaam {Surgery, Psychiatry & Paediatrics}

Major Responsibilities

- Serving as Principal Investigator, Internal Medicine Research Physician, and Clinical Epidemiologist stationed at the Bagamoyo Clinical Trials Unit and BIMEP CRC-Bioko Island, with a primary focus on assessing the Safety and Efficacy of participants in clinical trials for vaccines, drugs, and human challenge trials.
- Providing supervision for study-specific clinical sciences and medical operations, including designing clinical studies, offering clinical and scientific input throughout trial implementation, and managing safety reporting for study participants.
- Overseeing and supporting projects from setup to implementation and closeout phases.
- Engaging in medical writing tasks such as developing clinical trial protocols, manuscripts, and documents necessary for Clinical Trial Authorization (CTA) applications.
- Leading and supporting the development of concept notes, program development, grant applications, and project leadership.
- Collaborating with ethical and regulatory authorities for clinical studies requiring a Clinical Trial Authorization (CTA), including Phase I trials in healthy and patient volunteers, as well as later phase trials (i.e., Phase II to IV).
- Interacting with local stakeholders on matters concerning healthcare systems and health intervention policies.
- Assisting in key institutional administrative activities.
- Supporting training, capacity building, and skill transfer in Clinical Research.
- Conducting training sessions on advanced diagnostic approaches such as performing and interpreting ECGs.
- Serving as a member of the Board of Trustees for RSTMH.
- Participating as a member of the Policy and Advocacy Committee at RSTMH.

Major Research Projects and Roles

Description	Role
• A randomized, controlled clinical trial to assess and compare the immunogenicity, safety and reactogenicity of the bivalent Omicron BA.4/BA.5 adapted, and the original Wuhan-Hu-1-strain, BNT162b2 COVID-19 vaccine formulations in healthy unvaccinated East African adults	**Principal Investigator**
• A randomized, open-label, parallel design, phase I study to evaluate the pharmacokinetics of Piperaquine oral dispersible granule formulation when administered as a single dose in the fasted and fed state compared to Piperaquine hard tablets in healthy adult participants	**Project Leader**
• A Phase 1, Bioavailability Study to Investigate the Pharmacokinetics, Safety and Tolerability of an Oxfendazole Tablet Formulation in a Randomized, Double-Blind, Placebo-Controlled Design after Single and Multiple Oral Dosing in Healthy Adult Volunteers: **NCT04920292**	**Principal Investigator**
• Clinical Trial to Evaluate the Safety and Immunogenicity in Age De-Escalation of Direct Venous Inoculation of a Plasmodium falciparum Sporozoite Vaccine in Tanzanian Adults, Children, and Infants. **NCT02613520**	**Principal Investigator**
• Clinical Trial to Evaluate the Safety, Immunogenicity and Efficacy of Direct Venous Inoculation of Plasmodium falciparum Sporozoite Vaccine (PfSPZ Vaccine) in HIV negative and HIV positive Tanzanian Adults. **NCT03420053**	**Principal Investigator**
• Randomized, Double-Blind, Placebo-Controlled, Regimen Optimization Study of a Radiation-Attenuated Plasmodium falciparum (Pf) Sporozoite Vaccine (PfSPZ Vaccine) In Equatoguinean Adults. **NCT03590340**	**Principal Investigator**
• Pilot Study to Optimize Recruitment and Screening Procedures for Future Clinical Trials and to Create a Registry of Potential Research Participants on Bioko Island, Equatorial Guinea	**Co-Principal Investigator**
• A randomized, open label, two-part, parallel-group, phase I study to evaluate the pharmacokinetics of Piperaquine oral dispersible Granules Formulation compared to Piperaquine hard Tablets administered as a single dose in fasting condition (Part 1) and of Piperaquine oral dispersible Granules Formulation administered as single dose in various fed states (Part 2) in healthy adult participants	**Project Leader**
• Phase IV Open Label Study of Fixed Artemisinin / Naphthoquine (Arco®) Therapy to Determine Safety, Tolerability, Pharmacokinetics and Efficacy in Adults and Children with Uncomplicated P. falciparum Malaria in Tanzania. **NCT01930331**: **2013–2014**	**Project Leader**
• The Candidate Blood Stage Malaria Vaccine P27A Induces a Robust Humoral Response in a Fast Track to the Field Phase I Trial in Exposed and Non-Exposed Volunteers. **NCT01949909**	**Project Leader**
• Safety, immunogenicity and protective efficacy against controlled human malaria infection of PfSPZ Vaccine in Tanzanian adults. **NCT02132299**	**Project Leader**
• An open-label, multicentre, randomised, adaptive platform trial of the safety and efficacy of several therapies, including antiviral therapies, versus control in mild / moderate cases of COVID-19	**Project Leader**
• Pilot Study to Test Recruitment and Screening Procedures to be Performed in Upcoming Clinical Trials at Bagamoyo Clinical Trial Unit.	**Project Leader**
• Recruitment and Screening Procedures for Upcoming Malaria vaccine and Challenge Studies to be conducted at The Newly Established Phase One Clinical Trial Unit, at Bagamoyo Branch, Ifakara Health Institute	**Project Leader**

Publications

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