Heterogeneity in *Plasmodium falciparum* whole sporozoite vaccine induced humoral immune responses and protection in African volunteers: The role of age, human pegivirus and human immunodeficiency virus co-infections

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Summary

Malaria, a vector borne disease caused by *Plasmodium* species remains a major public health problem especially in sub-Saharan Africa. In 2018 alone, there were an estimated 228 million clinical cases and 405,000 deaths attributed to malaria. New tools such as efficacious vaccines, better drugs and diagnostics are needed to supplement the current malaria control tools that rest mainly on vector control measures. Clinical trials in malaria naïve volunteers have demonstrated high level of sterile vaccine induced protection in healthy individuals immunized with live, metabolically active, irradiation attenuated purified sporozoites (PfSPZ Vaccine) or live non-attenuated purified sporozoites given under chloroquine chemoprophylaxis (PfSPZ-CVac). Immunogenicity and protective efficacy against malaria induced by these whole sporozoite based vaccines varies widely between European/US versus African volunteers. Interestingly, variations in protection and immunogenicity of these malaria vaccines have been observed among African volunteers residing in different malaria endemic regions of East and West Africa. These different outcomes could be linked to levels of malaria pre-exposure and co-infections at the time of vaccination. Until recently safety, tolerability, immunogenicity and protective efficacy of PfSPZ Vaccine in immunocompromised populations including individuals infected with the Human Immunodeficiency Virus (HIV) was unknown. Given the high geographical overlap of HIV and malaria, an effective malaria vaccine deployed in this population could possibly support the long-term objective of regional elimination of malaria by using mass vaccination. Furthermore, the role of asymptomatic, under researched, highly prevalent viruses like Human Pegivirus (HPgV-1) circulating in Sub-Saharan Africa on malaria pathogenesis and vaccination outcomes remains elusive.

The aims of this PhD thesis include: 1) Evaluate the safety, tolerability, efficacy, and immunogenicity of the PfSPZ Vaccine in different populations and age groups residing in malaria endemic countries. 2) Compare the safety, tolerability, efficacy, and immunogenicity

of two different whole sporozoite based vaccination approaches, PfSPZ Vaccine and PfSPZ-CVac in Equatorial Guinean adults. 3) The unbiased assessment of PfSPZ Vaccine induced humoral immunity using protein microarrays probed with serum samples of HIV positive and HIV negative volunteers to understand immune status before vaccination, immuno-dominance of vaccine induced antibody targets, and distinct antibody profiles that might be associated with vaccine-induced protection. 4) Investigate the interaction of chronic HPgV-1 co-infection on PfSPZ Vaccine induced humoral immunity and protection against homologous CHMI. These aims are structured around 6 manuscripts presented in this PhD thesis.

Manuscript 1-3: Safety, immunogenicity, and efficacy of radiation attenuated whole sporozoite vaccine (PfSPZ Vaccine) in African populations of different ages

In this chapter, we include the outcomes of clinical trials conducted in Bagamoyo, Tanzania. These trials for the first time i) evaluated the safety, immunogenicity and efficacy against homologous CHMI of irradiation attenuated purified *Plasmodium falciparum* sporozoites (PfSPZ Vaccine) in adult volunteers; ii) tested increasing dosages of PfSPZ Vaccine in different age groups including adults, adolescents, children and infants. We found PfSPZ vaccine to be safe and well tolerated and that vaccine inoculation by intravenous inoculation is well accepted even in younger age groups. Protective efficacy varied in the different trials leading to the identification of a vaccine regimen of $9x10^5$PfSPZ per dose as suitable for further development. PfSPZ Vaccine induce immune responses, both cellular and humoral, were age dependent with infants mounting no measurable malaria specific cellular immunity in peripheral blood. Surprisingly, compared to other age groups, older children and adolescents mounted higher cellular and humoral immune responses. These findings are relevant for further optimization of PfSPZ vaccine regimen that might need to be adapted to different age groups to optimize vaccine induced protection. As an extension of these trials, we have compared

PfSPZ Vaccine safety, immunogenicity and efficacy in HIV positive versus HIV negative volunteers. We observed marked differences in PfSPZ Vaccine induced efficacy between HIV positive (0%) and HIV negative individuals (80%) undergoing homologous CHMI (manuscript in preparation).

Manuscript 4: Immunogenicity and protective efficacy of radiation-attenuated and chemo-attenuated PfSPZ vaccines in Equatoguinean adults

This work describes the outcome of a first time side-by-side comparison of two whole sporozoite based vaccine approaches (PfSPZ Vaccine and PfSPZCVac) in malaria pre-exposed individuals of Equatorial Guinea. We evaluated PfSPZ Vaccine dosages ($2.7\ X10^6$) given three times at 8-week interval and PfSPZ CVac dose ($1X10^5$) given three times at 4 weeks interval. Homologous CHMI was employed for assessment of vaccine efficacy. Both approaches were safe and well tolerated in malaria pre-exposed individuals but the immunogenicity and protective efficacy differed. Vaccine efficacy was lower in the PfSPZ Vaccine group (27%) compared to the PfSPZ CVac group (55%), despite induction of about 2.9 times higher antibody titres against the circumsporozoite protein in the PfSPZ Vaccine group prior to CHMI. These results highlight the potential involvement of different protective immune mechanisms induced by each of the two whole sporozoite vaccines approaches and the effect of malaria pre-exposure on PfSPZ CVac vaccine induced efficacy in comparison to malaria naïve volunteers. We show that induction of high antibody titres against the circumsporozoite protein does not correlate with protection since no difference was observed between CHMI protected and non-protected volunteers.

Manuscript 5: HIV-1 positive and HIV-1 negative Tanzanian adults undergoing whole irradiation attenuated *Plasmodium falciparum* sporozoite vaccination mount antibody responses targeting the circumsporozoite protein and merozoite surface protein 5

In this manuscript, we investigated antibody profiles binding to 262 pre-selected antigens of Pf before and after vaccination as well as after homologus CHMI. We aimed to identify antibody profiles that might explain the observed poor vaccine induced protection in HIV positive individuals. We found a lower - albeit not statistically significant - antigen breadth in HIV positive volunteers at baseline before first vaccine inoculation. Immunization with PfSPZ Vaccine induced IgG and IgM isotypes specific for the Merozoite surface protein 5 (PfMSP 5) and the circumsporozoite protein (PfCSP) regardless of HIV infection status. Interestingly, volunteers displayed a highly personalized IgG and IgM immune profiles targeting Pf antigens before vaccination and these remained unchanged after PfSPZ vaccination confirming our previous results of antigenic imprinting in malaria.

Manuscript 6: Role of Pegivirus infections in whole *Plasmodium falciparum* sporozoite vaccine induced humoral immunity and controlled human malaria infections in African volunteers

In this study, we wanted to understand the role of human pegivirus infections in East and Western African adult volunteers and its impact on PfSPZ Vaccine induced humoral immune responses and homologous CHMI. We found HPgV-1 to be highly prevalent in our volunteers (29.2%) with circulating genotypes 1, 2 and 5 as described in other African settings. HPgV-1 infection did not alter PfSPZ vaccine induced antibody responses and parasite multiplication rates during CHMI. However, a higher proportion of individuals were protected against homologous CHMI that had ongoing, active human pegivirus infections. Significantly higher serum concentrations of IL-2 and IL-17A were measured in HPgV-1 positive volunteers likely indicating chronic activation of the immune system. CHMI was safe and well tolerated in HPgV-1 positive individuals since the viremia did not change upon acute asexual blood stage parasitemia. These results highlight the potential impact of chronic, asymptomatic viral infections on PfSPZ vaccine efficacy that needs confirmation in larger cohorts and in field studies of naturally occurring malaria infections.

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"Faith is taking the first step even when you don’t see the whole staircase- MLK"

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LIST OF ABRREVIATIONS

BRTC	Bagamoyo Research and Training Centre
BSPZV1	Bagamoyo Sporozoite Vaccine Trial #1
BSPZV2	Bagamoyo Sporozoite Vaccine Trial #2
BSPZV3a	Bagamoyo Sporozoite Vaccine Trial # 3
DVI	Direct Venous Inoculation
EA	Early antigen (EBV)
EBV	Epstein-Barr Virus
EG	Equatorial Guinea/Equato Guinean
CMV	Cytomegalovirus
EGSPZV1	Equatorial Guinea Sporozoite Vaccine Trial #1
EGSPZV2	Equatorial Guinea Sporozoite Vaccine Trial #2
ELISA	Enzyme-linked Immunosorbent Assay
ELISpot	Enzyme-linked Immunosorbent Spot
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HSA	Human Serum Albumin
ICS	Intracellular Cytokine Staining
IFA	Immunofluorecence Assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFN	Interferon
IHI	Ifakara Health Institute (Bagamoyo, Tanzania)
IL	Interleukin
IRS	Indoor Residual Spraying
ISI	Inhibition of Sporozoite Invasion of Hepatocytes
IV	Intravenous(ly)
MSP	Merozoite Surface Protein
MVPs	Mass Vaccination Programs
NIH	National Institutes of Health (Bethesda, USA)
NIMR	National Institute for Medical Research (Dar-es-Salaam, Tanzania)
Pf	Plasmodium falciparum
PfCSP	Plasmodium falciparum Circumsporozoite Protein
PfLSA	Plasmodium falciparum Liver Stage Antigen

PfMSP	Plasmodium falciparum Merozoite surface protein
PfSPZ	Plasmodium falciparum Sporozoite(s)
PfSPZ-CVac	PfSPZ Chemoprophylaxis Vaccine (PfSPZ Challenge administered in conjunction with antimalarial chemoprophylaxis)
PfSPZ Challenge	live, fully infectious, aseptic, purified, cryopreserved *Plasmodium falciparum* sporozoites
PfSPZ Vaccine	live, radiation attenuated, aseptic, purified, cryopreserved *Plasmodium falciparum* sporozoites
qPCR	Quantitative Polymerase Chain Reaction
RTS,S	A recombinant protein subunit vaccine that includes part of the repeat region of the Plasmodium falciparum CSP (R), part of the region of CSP that includes thymus-derived lymphocyte epitopes (T), and the surface antigen of hepatitis B (S)
SPZ	Sporozoite(s)
HPgV-1	Human pegivirus-1
HPgV-2	Human pegivirus-2
SwissTPH	Swiss Tropical and Public Health Institute (Basel, Switzerland)
TB	Tuberculosis
Treg	Regulatory T cell

Chapter 1

General Introduction

1.1 Malaria epidemiology, biology and control strategies

1.1.1 Malaria epidemiology and disease burden

Malaria, a vector borne disease caused by apicomplexan parasites belonging to the genus *Plasmodium* presents still a major public health problem, particularly in sub-Saharan Africa [1]. In 2018, an estimated 228 million cases and 405,000 deaths were due to malaria [2] and no reduction in the number of the incident cases has been observed since 2014. *Plasmodium spp* have a wide range of human and animal host [1], [3], [4]. Infections in humans are attributed to six species including *Plasmodium falciparum, P. vivax, P. malariae*, *P. ovale curtisi, P. ovale wallikeri* and the zoonotic parasite species *P. knowlesi* [1], [3], [4]. Of these, *P. falciparum* is the most virulent and accounts for most deaths, especially in primigravidae women and children under five years of age [2], [5]. The distribution of malaria species varies globally - depending on geographical locations of human reservoir and climatic conditions of the transmitting vector and the parasite [6]. For example, *P. falciparum* infections are predominantly found in sub-Saharan Africa whereas *P. vivax* is prevalent in the Americas (Central and South), South East Asia and parts of Africa [1], [4], [5]. For a long time, studies suggested that *P. vivax* is essentially absent in the African region due to the lack of the Duffy receptor expressed on the surface of red blood cells (RBCs) which aids the invasion of *P. vivax.* Recently, *P. vivax* infections have increasingly been documented in individuals negative for the Duffy receptor, suggesting presence of alternative parasite invasion mechanisms into RBC [7]. *P. malariae* and *P. ovale* are distributed primarily in West Africa and South East Asia, respectively. These two species are not well studied and hence their geographical distribution is likely to be underestimated. *P. knowlesi* is a zoonotic parasite associated with malaria cases in South East Asia, particularly in Malaysian Borneo [8]. Furthermore, reports have demonstrated zoonotic human infections with other simian parasites such as *P. cynomolgi* [9], [10] and *P. simium* [11] in South East Asia and South America, respectively. These reports

clearly indicate the need for extending epidemiological and clinical investigations of the presence of *Plasmodium spp*. beyond the currently dominating *P. falciparum* and *P. vivax* - if the aim of malaria elimination is to be achieved [2].

Economically, malaria is linked to marked loss of productivity, both at individual and population level, thus impeding economic growth and development of affected societies [12]. Children and pregnant women are the most at risk groups for *P. falciparum* infection in endemic settings, but malaria also poses an increased risk to individuals living with co-infections caused by the human immunodeficiency virus (HIV). These individuals often show severe forms of malaria and therefore it is important to monitor them constantly for malarial parasitaemia, CD 4+ T-cells counts and HIV RNA viral loads to ensure improved outcomes [13], [14].

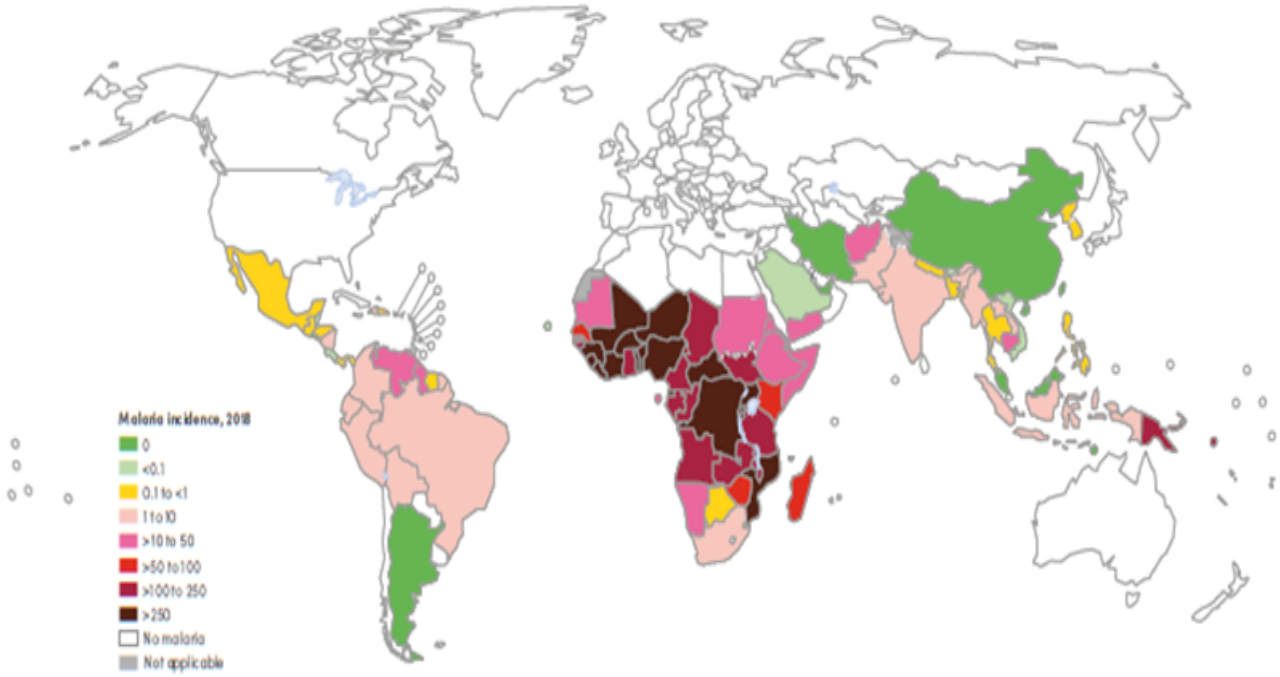

Figure 1 Malaria case incidence rate (cases per 1000 population risk) by country, 2018[2]

1.1.2 Malaria life cycle

Understanding the biology of etiological agents of infectious diseases is important for designing and implementation of appropriate control strategies, including vaccines and drugs. However, to some extent this has proven to be difficult for *Plasmodium spp*, which are known to have complex life cycles alternating between the human and mosquito hosts [1], [3], [4]. The human part of the life cycle begins when a female *Anopheles* mosquito injects sporozoites into the dermis of the human host during a blood meal. The sporozoites glide through the skin epidermis, reach lymph and blood vessels and finally access the blood stream to end up in the liver. Once in the liver, sporozoites traverse through several hepatocytes to finally settle in the liver parenchyma cell making use of the human surface proteins (heparan sulfate proteoglycan-HSPG, tetraspanin membrane cluster of differentiation 81-CD 81, and transmembrane receptor ephrin A2-EphA2). Inside the liver cell, the parasites form a parasitophorous vacuole [15]. This structure is crucial for accommodating asexual reproduction leading to a high numbers of merozoites developing during the late liver schizont stage [15]. Important to this process of liver cell adhesion and invasion is the circumsporozite protein (CSP), the pre-dominant protein on the surface of sporozoites that interacts with the heparin sulphate proteoglycans sugar residues found on the surface of hepatocytes [15]. The duration of the pre-erythrocytic developmental stage is dependent on the malaria parasite. *P. falciparum* and *P. vivax* usually

have a pre-erythrocytic stage that lasts 5.5-7 days and 14 days, respectively. In addition, *P. vivax* and *P. ovale* have the ability to form

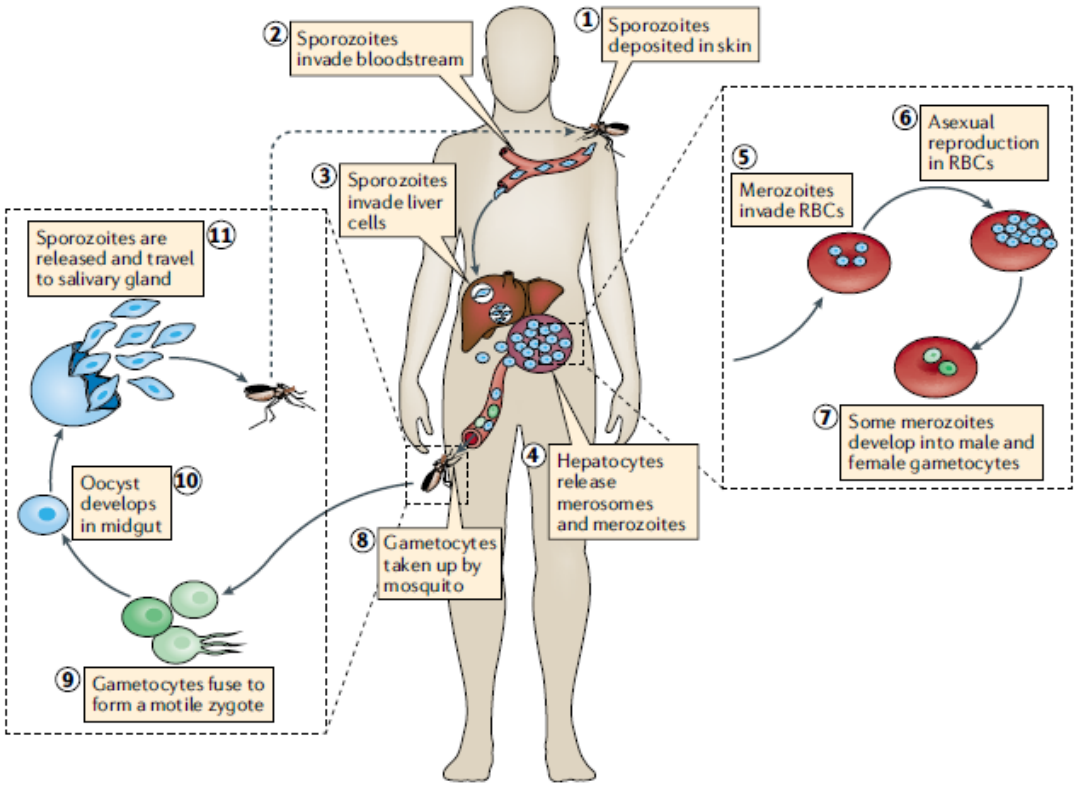

Fig 2. Plasmodium life cycle in human and mosquito hosts ([16])

dormant liver stage parasites (hypnozoites) that can potentially reactivate months or years after the primary infection and give rise to asexual blood stage infections [15]. The liver merozoites are released from the infected liver parenchyma cells into the blood stream in form of parasite-filled vesicles (merosomes) thus allowing the transition to the asexual blood erythrocytic cycle [15].

Merozoite invasion of RBC occurs in a multi-step process, which involves low affinity attachment of the parasite and its re-orientation to the erythrocyte surface, followed by the creation of tight junctions to allow parasites to firmly attach on the surface. The invasion of the

RBC follows distinct steps with release of parasite proteins that reside in the apicomplexan organelles [17]. Several merozoites proteins localized in distinct parasite organelles and surface receptors on RBC facilitate this process. The surface proteins such as merozoite surface proteins (eg MSP1) allow the initial attachment of merozoites on the surface of RBC whereas erythrocyte binding ligands and reticulocyte binding proteins located in the microneme and rhoptry respectively, interact directly with the erythrocyte membrane receptors to attach the parasite firmly [17]. An example of proteins include the rhoptry protein (RON2) and apical membrane protein 1 (AMA-1) which interact to form tight junction crucial for the effective invasion of mature RBCs [18]. Merozoite`s actin-myosin movement causes invagination of the RBC membrane leading to the formation of a parasitophorous vacuole [17]. Detachment of parasitophorous vacuole from RBC membrane follows and facilitates parasite`s entry into the host cell. Once inside the RBC, the merozoite undergoes a series of developmental steps resulting in the emergence of ring, trophozoite and schizont stages [1], [3], [4]. A single schizont can harbor up to 32 merozoites (depending on the malaria species) and when schizonts rupture, these merozoites are released and rapidly infect new RBC to continue the asexual blood stage cycle involving rounds of invasion, intracellular multiplication and rupture. Due to environmental signals such as temperature, pH and drug treatment a proportion of merozoites transforms to sexual forms known as male and female gametocytes [19], [20]. The first developmental stage that is functionally different from an asexual parasite is the sexually committed schizont and is mainly driven by expression of the Apatella 2 gene (AP2-G)which is under tight control of heterochromatin 1[19], [21] Five morphologically distinct gametocyte stages exists in *P. falciparum*. The stage I gametocyte resembles the trophozoite, stage II has the shape of a lemon, stage III is D shaped, stage IV looks like a banana and stage V mirrors a sausage [19], [22] In order to escape immune recognition, gametocyte stage I-IV reside in the

bone marrow [22], [23]. Gametocyte stage V re-enter the peripheral blood circulation where they can be ingested by a female Anopheles mosquito during the blood meal [19], [22], [23]

In the mosquito midgut, male (microgamete) and female (macrogamete) gametocytes fuse to form a zygote that transforms into a motile parasite form named ookinete. The ookinete traverse the midgut epithelium to the basal lamina. Here, it develops into oocysts that develop further into mature sporozoites. The release of these sporozoites occurs when the oocyst ruptures and subsequently the sporozoites navigate to the salivary glands of the mosquito. The sporozoites are then injected during the blood-feeding mosquito into the human host [1], [3], [4].

1.1.3 Malaria pathogenesis

P. falciparum infection can present as complicated, uncomplicated and asymptomatic malaria and it is now widely accepted that the severity of malaria infection relies on factors like the infecting species, hosts immune status, age and pregnancy status [3]. Despite the fact that many of the mechanisms involved in malaria pathogenesis remain unknown, evidence suggests that much of the malaria pathology is driven by inflammation, anemia and microvascular obstruction [24], [25]. Complicated and uncomplicated malaria are accompanied with signs and symptoms of malaria plus detection of malaria parasites in peripheral blood. Non-specific signs such as headache, nausea, vomiting, diarrhea, fatigue, fever, chills and mild anemia characterize uncomplicated malaria [24]. These symptoms are usually a result of the large amount of parasites and parasite associated products produced during the erythrocytic stage, which subsequently induce the onset of pro-inflammatory responses [24] The duration of the symptoms differs and is influenced by hosts immunity as well as the length of erythrocytic cycle, which varies according to the infecting *Plasmodium spp.* For *P. falciparum* this cycle lasts for about 48 hours [1]. *P. falciparum* infections result in endothelial cell activation and cytoadherence of parasite infected RBC to small blood vessels (named sequestration)

consequently leading to microvascular obstruction. Microvascular obstruction can also arise from infected RBCs binding onto uninfected RBCs, a process known as rosetting. This may result into complicated malaria if obstruction occurs in the brain, kidneys or intestines [24]. Neurological manifestations such as seizures, agitation, psychosis, impaired consciousness and coma may occur in cerebral malaria only [24]. Other symptoms associated with complicated malaria are severe anaemia, metabolic acidosis, pulmonary distress, multi-organ failure and sometimes death [24]. These processes of sequestration is mediated by parasite-exported proteins located on the surface of erythrocytes including the *P. falciparum* membrane protein 1 (PfEMP-1) family [24], [26]. A certain subset of this protein family, known as VAR2CSA is expressed on the surface of infected RBC that preferably sequester into the placenta by binding to chondroitin sulphate leading to the *P. falciparum* malaria associated pathology in pregnancy

[27]. Malaria in pregnancy results into low birth weights, pre-term labor, stillbirths as well as maternal death [24], [25]

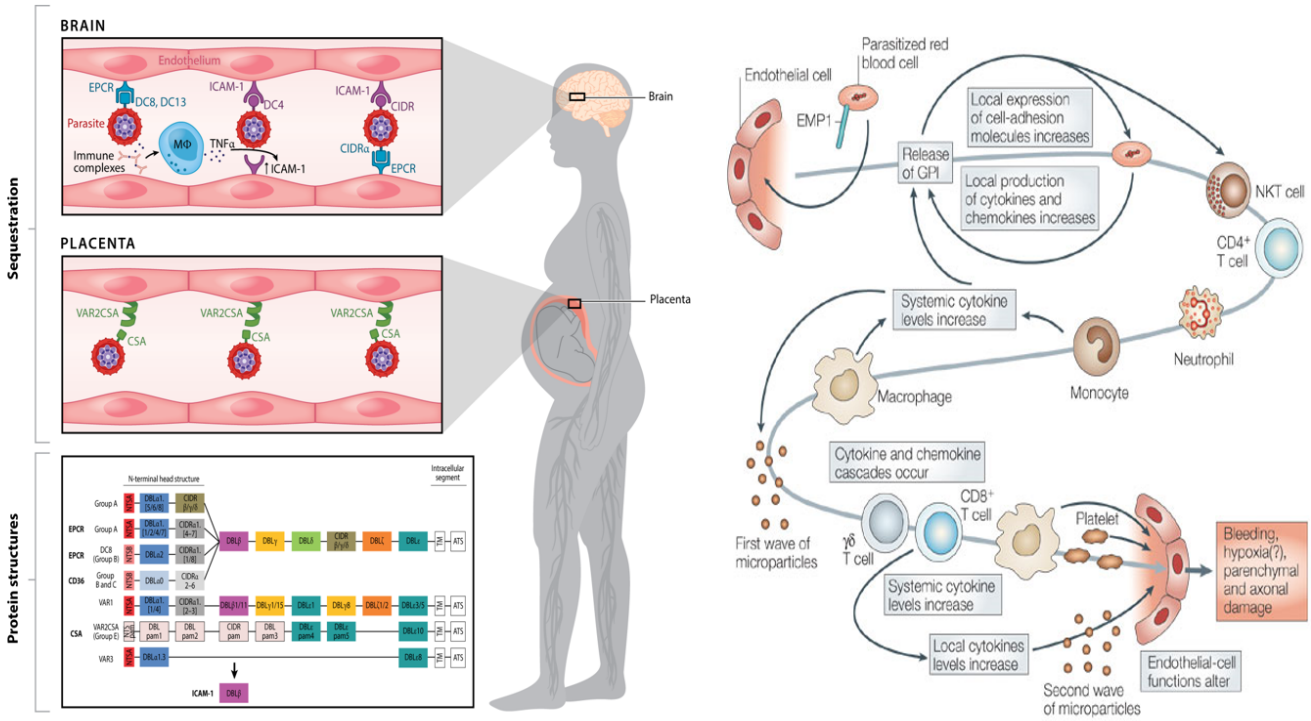

Figure 3: Pathophysiology of malaria: Left: Different receptor- ligand interaction mediating sequestration of infected RBC in brain and placenta [24] Right: Parasite products and immunological processes contributing to malaria-induced inflammation [28]

1.1.4 Malaria diagnosis

Rapid, robust, sensitive and specific malaria diagnosis is crucial for both, disease management at the individual level and malaria surveillance programs at the population level. Current malaria diagnosis is based on the usage of a range of methods such as thick and thin blood smears read under a microscope, rapid diagnostic tests (RDT) and molecular methods [29]. Traditionally, microscopy has been widely used as a gold standard for malaria diagnosis.

Microscopy is a well-established method with capacity to distinguish the malaria species and estimate parasite load. However, it lacks sensitivity (lower limit of detection ranges between 20 to 50 parasites/ul), it is labor and time intensive and requires a well-trained microscopist and maintained microscope [30] As an alternative, RDT as tools for diagnosis or confirmation, particularly in resource-limited settings, have been used increasingly [31], [32]. RDTs are based on detection of parasite proteins expressed during the erythrocytic stage, and include *P. falciparum* Histidine rich protein 2 (*Pf*HRP2), lactate dehydrogenase (*Pf*LDH) or p-aldolase antigens (*Pf*Aldolase) [33], [34]. These tests provide results within 5-20 minutes and allow treatment measures to be taken promptly at the health care centre. In contrast, RDTs have some drawbacks in that infections with low parasitaemia (i.e. below the lower limit of detection of 100 parasites /microliter of blood for the PfHRP2 based RDT) or parasite strains with *hrp2* and/or *hrp3* gene deletions are potentially missed [33], [35]. So far, parasites carrying these gene deletions have been documented in Asia, South America and sub-Saharan Africa [36]–[39]. Moreover, non-falciparum infections are often missed by RDTs based on their usually low parasitemia levels [35]. Therefore, there is a need for development of better diagnostic tools to ensure that all infections are detected, particularly those in low transmission settings [40], [41]. If stored properly, RDTs can potentially act as biobank for epidemiological characterization of other blood borne infectious diseases present in malaria endemic regions [42]. Thus, using these RDTs could provide additional information on the dynamics of co-infections in malaria endemic areas and their potential impact on the epidemiology of *Plasmodium spp* [43], [42].

Unquestionably, the introduction of highly sensitive and specific nucleic acid based amplification methods such as polymerase chain reaction (PCR) have revolutionized the malaria diagnostic field [44], [45]. PCR based diagnostic methods allow for the identification of *Plasmodium* spp at low density as well as the quantification of parasite load [44], [45].

Nevertheless, these molecular based methods have limitations of higher running expenses, infrastructure requirements and the dependency on well-trained laboratory personnel to conduct the analysis and interpretation of results [45]. Currently, some of these setbacks are addressed by on-going technological advancements aiming at adapting simpler molecular methods for field studies like loop isothermal mediated amplification (LAMP) and novel detection methods for PCR [46], [47]. It is envisioned that these methods will allow extensive deployment of molecular tools even in resource limited settings at reasonable costs, easiness of use and high throughput [47]. Additional information generated based on nucleic acid amplification is genotyping of parasite strains and the identification of strains carrying drug-resistance markers [48], [49].

1.1.5 Malaria control strategies

A mix of control intervention strategies based on vector control, insecticide treated bed nets and rapid diagnosis and prompt treatment based on Artemisinin combination therapies (ACT) are linked to the recent decline of clinical malaria in endemic areas [50]. However, the success of these tools is under threat with emergence of drug and insecticide resistant malaria parasites and vectors, respectively [50]. Therefore, alternative strategies to supplement current malaria control measures are needed, more so if the goal of malaria elimination is to be achieved [51]. It is envisioned that one of the most cost-effective solution would be an integration of effective and long-lasting malaria vaccines to the current control tools [52].

1.2 Immunity to malaria

1.2.1 Immune response during the pre erythrocytic response

Although immune responses to malaria infections in the field and under controlled infection conditions in humans have been studied extensively, mechanisms driving sterile protection in malaria infections are only incompletely understood [53]. The understanding of malaria specific immunity is compounded by the highly complex nature of the *Plasmodium* life cycle, lack of an animal model for P. falciparum that is easily available and the limited access to tissue resident immune cells in humans. It is likely that protective immunity to malaria involves complex interactions of a diverse array of host and parasite proteins and metabolites at different stages of life cycle.

The inoculation of sporozoites in the skin during natural infections induces dendritic cell phagocytosis, which results into priming of T and B cells in the skin draining lymph nodes [25]. Sporozoites that manage to emerge into the blood are prevented from liver invasion by specific antibodies, through various antibody-mediated mechanisms [54]. A fraction of sporozoites evades these initial immune responses and migrates to the liver where they infect hepatocytes. Here, the CD4+ T-cells, CD8+ T-cells, γδ T cells and natural killer cells are thought to be involved in the elimination of liver stage parasites, primarily through the release of IFN-γ. Macrophage-generated reactive oxygen species like nitric oxide (NO) are thought to be important in infection control of this intracellular stage [25], [55]. The pre-erythrocytic stages in skin and liver is clinically silent and the generated immune responses are usually insufficient to provide sterile protection against invading sporozoites in the field [25], [55]. This has been linked to low amount of sporozoites injected in the skin by the mosquito and immune tolerance resulting from presence of high frequency of T regulatory cells in the skin [25], [56]. The T regs in particular have also been implicated to contribute to poor expression

of MHC class II and CD86 molecules on the surface of dendritic cells, potentially negatively influencing their ability to initiate immune responses [25], [55]. The disruption of the respiratory burst in the specialized macrophages found in the liver sinusoids, known as Kupfer cells, has also been suggested as a factor in the observed poor pre-erythrocytic immunity [57].

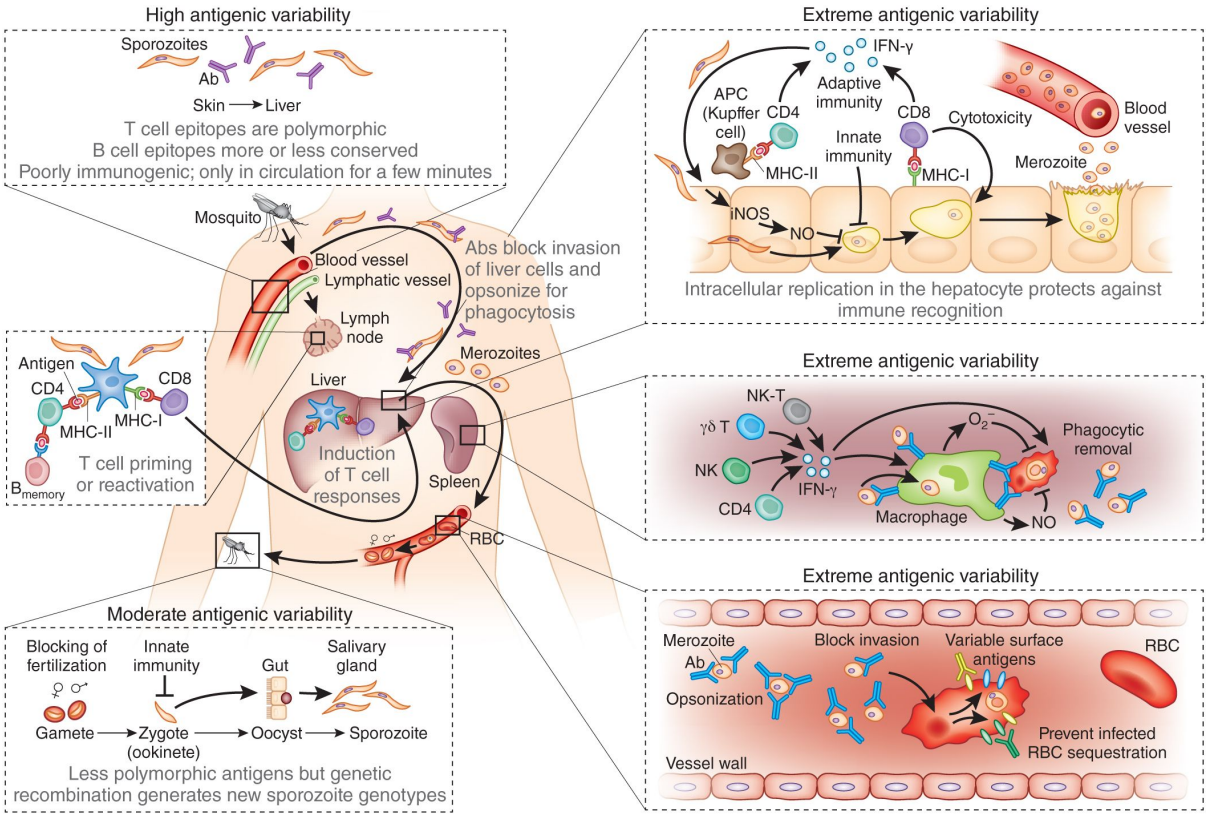

Fig. 4: Overview of the array of immune effector mechanisms involved in immunity against malaria at different stages of the Plasmodium life cycle (Figure adapted from [58])

1.2.2 Immune response during blood stage infection

Immune responses against the asexual blood stage parasites are induced primarily in the spleen. The spleen is also crucial for the removal of aged and infected RBCs [59]. A broad range of immune effector mechanisms for the control of asexual blood parasites, including production of neutralizing antibody and cellular responses, have been described [25], [54]. The onset of

inflammation occurs when parasite proteins and *P. falciparum* pathogen associated molecular pattern molecules (PAMPs) bind to various host receptors in the microvascular endothelium and tissues [25], [60]. This process is associated with several parasite protein families including members of the *Pf*EMP-1 protein family, which binds to host receptors such as endothelial cell protein C receptor (EPCR), intercellular adhesion molecule 1 (ICAM1) and cluster of differentiation 36 (CD 36) found in the brain, as well as placental chondroitin sulphate (CSA) (Figure 3). The *Pf*EMP1 mediates interactions with these host receptors through its Duffy-binding ligand (DBL) and the cysteine rich interdomain regions (CIDR) which exists in multiple subclusters. Recombinants of DBL and CIDR forms structures known as the disc cassettes [61]. *Pf*EMP1 variant -VAR2CSA is associated with pregnant malaria whereas the disc cassettes 4, 8, 13 mediates sequestration of infected erythrocytes in the brain. The Merozoite surface protein (MSP-1) also induces inflammation through binding to the S100 protein family that are released from damaged RBCs [25], [60], [62]. The *P.falciparum* PAMPs implicated in inflammation process are haemozoin, glycosylphosphatidylinositol (GPI) anchors and CpG containing DNA motifs [63]–[65]. These PAMPS are known to bind on node like receptor protein 3 (NLRP3 inflamasome), toll like receptor 2 and 9, respectively [25], [65]. Collectively, the interactions results into release of cytokines and chemokines such as IL-6, IL-1β, IL-8, Il-12p70, IFN-γ and TNF-α which facilitate a pro-inflammatory microenvironment [25], [66]. These cytokines and chemokines are crucial in inter-cellular communication, cellular activation and control of parasite growth through induction of nitrogen oxide, inducible nitrogen oxide, super oxide radicles and hydrogen peroxide amongst other mechanisms [66], [67]. Excessive *Plasmodium*-induced inflammation can be detrimental especially in malaria naïve populations leading to severe anaemia, pulmonary distress, metabolic acidosis, multi-organ failure and death [25], [68], [69].

Antibody mediated control of blood stage parasites has been appreciated for a long time, supported by passive transfer experiments of serum from malaria-immune adults into malaria infected children that resulted in a lowered malaria parasitaemia and reduced disease severity [70]. Antibodies mediate their biological functions through a suit of effector mechanisms including antibody dependent cellular inhibition (ADCI), antibody dependent respiratory burst (ADRB), antibody dependent cellular cytotoxicity (ADCC), opsonic phagocytosis and antibody mediated-complement lysis in combination with phagocytic cells such as monocytes and neutrophils [54]. The latter process is also particularly important for the lysis of sexual parasite forms hence preventing parasite transmission to the mosquito [54] Additionally, antibodies act on infected erythrocytes to prevent rosetting, adhesion to the endothelial cells, inhibition of iRBC rupture and parasite egress [54]. These processes are mediated by antibody blockade of different plasmodium variant surface antigens expressed on iRBC preventing binding to their corresponding host receptors in the vascular endothelium. It is believed that antibodies directed towards *Pf*EMP1- one of the most polymorphic *P. falciparum* antigens expressed in placenta (VAR2CSA) and brain (DC 4, 8,13, CIDR) [54], [71] - block interaction with CSA, ECPR and ICAM-1 receptors, respectively[54].Interestingly, some of these antibodies have further been linked with protection from severe and symptomatic malaria [71]–[73]. The innate immune cells such as monocytes, macrophages, mast cells, neutrophils, natural killer T cells (NKT) and natural killer cells (NK) are important during this stage as they sense blood stage infection, promote inflammation, and inhibit parasite growth [25], [74]. They further shape the immune response by coordinating the size and type of adaptive immune cell responses including the CD4+ T cells. CD4+ T-cells eliminate parasite-infected cells through the release of IFN-γ [16]. A subset of CD4+ T-cells known as T follicular cells (TFH) are critical for providing B cell help in the germinal centers of lymphoid organs [16]. It is thought

that dysregulation in the development and function of T follicular helper cells negatively impact the quality of antibody responses against malaria [75].

1.2.3 Naturally acquired immunity to malaria

In malaria endemic countries, naturally, acquired immunity to malaria develops slowly, strain specific, unstable and dependent on age and malaria transmission intensity [75]. Infants and children under 5 years of age carry the highest risk of clinical malaria, marked by high parasitaemia and morbidity [25], [76]. This risk of malaria related deaths is reduced in adolescents, and protection against clinical malaria is attained in adulthood [25], [76], [77]. However, naturally acquired protection is not sterile and short-lived and can even be lost in individuals that move to areas with low or no malaria transmission [25], [76], [77]. In a stark contrast, immunity to severe non-cerebral malaria is often acquired after one or two malaria infections [78], possibly involving different array of effector mechanisms [77].

1.2.4 Poor acquisition of antibody response amongst individuals in malaria endemic countries

It is well established that humoral immunity generated by memory B cells and long-lived plasma cells are crucial for development of immunological memory resulting in enhanced host response against invading pathogens [79], [80]. Humoral responses develop at specialized structures known as germinal centers (GC) found within secondary lymphoid organs [79], [80]. The quality of generated antibodies depends on the location where B cell-antigen interactions occur and the duration of cognate help received from TfH cells to B cells. The key molecules mediating this process include CD 40 and CD 40 ligand (CD40L) expressed on the B cells and TfH cells, respectively [81]. Other critical molecules on T follicular cell surface are Signaling lymphocytic activation molecule associated protein (SAP), Programmed cell death protein-1 (PD-1), Inducible T cell co-stimulator (ICOS) , T cell receptor (TCR) and CD28. These bind

to corresponding receptors found on B cells including SLAM family receptors, Programmed cell death protein 1-ligand (PD-1L), Inducible T cell co-stimulator ligand (ICOS-L), Major histocompatibility class II molecules (MHCII) and CD 80/86, respectively [81]. Antigen binding to B cell receptors (BCR) of cells located outside GC (extrafollicular zone) results in development of short-lived plasma cells, which produce antibodies that provide the initial protection against pathogens [79], [80]. Some of these activated antigen specific-B cells migrate into GC (intrafollicular zone) where they receive help from TfH cells characterized by surface expressed markers CXCR5, PD-1, ICOS and low CCR7) [80] . Within the GC, B cells proliferate rapidly and simultaneously undergo somatic mutation of the BCR leading to development of class switched and affinity matured memory B cells (MBC) or long lived plasma cells (LLPC) [80]. While both these type of cells are known to persist in the absence of infection, memory B cells respond faster to secondary infection compared to naïve cells and have higher antigen affinity whereas the LLPC constantly produce antibodies [80]. Other factors influencing the quality and quantity of humoral immunity include the cytokine microenvironment in the GC (IL-6, IL-21 and IFN-γ), T and B cell receptor signaling as well as transcription factors [82]–[84].

Continuous exposure to asexual blood stage *P. falciparum* has been implicated in dysregulation of B cell responses within the GC thus resulting into defective humoral immune responses [85], [86]. *Plasmodium* spp are thought to induce these changes through various mechanisms such as impairment of different B cell developmental stages, poor circulation of B and T cells activation factors and inhibition of T follicular cell differentiation.[85]–[87]

One of these interactions is the interaction of the *Pf*EMP1-cysteine-interdomain region 1α (CIDRα) with the antigen binding fragment (Fab) of Ig expressed on surface of memory B cells. This interaction results into T-independent polyclonal activation of B cells accompanied with high cytokine release, leading to B cell exhaustion and B cell dysfunction [85], [88]. B

cell exhaustion is thought to be due to constant stimulation of signaling pathways like the mitogen activated protein kinase, MAPK and nuclear factor kβ. Although these pathways are activated by both BCR crosslinking and *Pf*EMP1-CIDRα interaction with the memory B cell [85]; the latter does not seem to activate other downstream signaling pathways such as lyn or phospho-tyrosine activation, thus leading to persistent B cell activation and exhaustion [89]. The impaired B cells responses are observed even after strong, non-antigen specific activation through TLR 7 and 9 [85], [89]. In contrast, the *Pf*EMP-1 variant VAR2CSA expressed on iRBC during malaria in pregnancy lacks this specific CIDRα domain and presumably thus allowing the rapid induction of VAR2CSA-specific antibodies that confer protection in subsequent pregnancies [85], [90]. *P. falciparum* is known to modulate the expression of the B cell activation factor (BAFF) - a cytokine important for B cell activation [91]. Reports from murine studies have shown that malaria infections drive low expression of BAFF on follicular dendritic cells, potentially influencing poor antigen presentation [92]. However, this seems to be different in humans as excessive expression BAFF on the human monocytes have been reported. Consequently, the excessive parasite induced BAFF expression on monocyte is believed play a role in the activation of naïve B cells (PMID: 21849293) (PMID: 20804716) [93], [94]. Increased levels of BAFF further affects expression of the BAFF receptor found on the surface of B cells leading to impaired B cell survival [92], [93]. Taken together, dysregulation in BAFF expression and release of its secreted form likely play a role in severity of malaria and therefore requires further investigation [93]. Other known cellular sources for BAFF include macrophages, neutrophils and T cells [85], [95]

Another mechanism employed by *Plasmodium* spp is disruption of B cell development altering frequencies of transitional and marginal B cells in both murine models and humans [96], [97]. In humans, these transitional and marginal B cells express the surface markers $CD10^{+}$ $CD19^{+}$ $CD\ 27^{-}$(transitional) and high IgD^{+} $CD\ 27^{+}$ (marginal), respectively [96], [98]. *P. falciparum*

infections induces expansion of the transitional B cells consequently affecting the differentiation of memory B cells. Moreover, it has been implicated in the deletion of marginal B cells and macrophages in secondary lymphoid organs such as the spleen [85], [86], [99].

Even more interesting is the fact that *P. falciparum* infections drives the induction of a subpopulation of memory B cells known as atypical memory B cells (aMBCs) [100], [101]. aMBCs are characterized by absence of classical B cell markers such as CD 21 and CD 27 and expression of transcriptional factor T-bet [87], [102]–[104]. aMBC also show poor proliferation, reduced B cell signaling, increased expression of inhibitory receptors including FcRL5 and impaired cytokine release like IL-6 and IL-8 and reduced antibody production in vitro [85], [87], [105]. Other studies have linked aMBCs with secretion of neutralizing, functionally active antibodies against malaria parasites [85], [106] . It is thought that these cells contribute to the slow and suboptimal humoral immunity against *Plasmodium* infections similarly observed in chronic viral infections like HIV [101]. The exact mechanisms driving their expansion in malaria are not fully understood. [85], [102], [103], [107]. The induction of aMBC is linked to the extent of malaria exposure and parasitaemia levels with high frequencies of aMBC seen in individuals extensively exposed to malaria infection [108]

As mentioned earlier, T cell help is critical for survival and differentiation of B cells [16]. Thus, dysfunctional interactions in the T-B cognate help within the GC results into production of aberrant quantity and quality of antibodies [107], [109] . Malaria likely contributes to this dysfunction by driving the development of TfH cells with a phenotype that carries $CXCR3^{+}$ $PD\text{-}1^{+}$ [109]. Although the high frequency of these cells have been well demonstrated in Malian and Brazilian individuals infected with *P. falciparum* and *P. vivax*, respectively, their contribution to malaria immunity remains to be investigated closer [109], [110]. Malaria infections in endemic countries have also been associated to the induction of T follicular regulatory cells (Tfr) which are thought of downregulating the cognate functions of TfH and

consequently impair B cell functions [16], [75], [111]. So far, much of the information on malaria-induced changes in GC biology has relied on mouse studies or the characterization of cells in the peripheral blood compartment. Recent success in isolation of lymph node biopsies conducted within malaria endemic countries [112] provides an excellent opportunity to investigate not only malaria-induced GC modulations but also the impact of co-infections and vaccine induced responses on immune responses ongoing in GC.

1.3 Malaria and other coinfections

Co-infections involves complex host and parasite interactions that potentially modulate immune responses, evolution of pathogens involved, and further pose challenge to diagnosis and treatments [113]. Various studies have attempted to dissect the interplay of bacteria, parasitic, and viral infections in malaria endemic countries [113]. However, these investigations are often difficult and with conflicting findings [113]. *Plasmodium* infections in particular are thought to drive emergence of invasive bacteria diseases such as salmonella enterica (serotype typhi and non-typhoid), staphylococcus aureus, staphylococcus pneumonia and haemophilus influenza [114]. This is thought to be through functional impairment of the polymorphonuclear cells critical for the control of bacterial invasion [114]. Interestingly, reduction in the prevalence of some bacterial infections including pneumonia and gastroenteritis has been reported in east african children immunized with advanced malaria vaccine- the RTS, S; indicating further the potential role played by malaria in these diseases [115]. Also, the prevalence of *Plasmodium* infections and soil-transmitted helminthes including Enterobias vermicularis, hookworm and strongloides stercoralis amongst children in endemic countries is linked to age, with more of infections seen in older children than infants [116]. Surprisingly, while hookworm tended to be more associated with malaria, E. vermicularis conferred protection to clinical malaria [116]. Low hemoglobin levels and

anaemia characterizes triple infections with *P.falciparum*, *Schistosoma haematobium* and *Schistosoma mansoni* in school and pre-school children consequently impairing children`s development and learning abilities [117]. Whilst on one hand co-occurrence of malaria and other vector (dengue and chikungunya) [118] and non-vector borne viral infections (Epstein bar virus, respiratory viruses, hepatitis C and B) has been extensively investigated [118]. On the other, the interaction of malaria with many of the emerging (SARS-COV2) viruses is still unknown [119]. More investigations in the context of co-infections and other non-communicable diseases across different populations are rather inevitable, as the understanding of mechanisms involved will enable implementation of proper therapeutic strategies as well as designing of effective vaccines. In this regard, part of this sought to particularly contribute to the improved understanding of the interactions between malaria and chronic viral infections-HIV and human pegivirus (HPgV-1).

1.3.1 Malaria and Human Immunodeficiency virus co-infections

HIV and malaria are both of great public health relevance in sub-Saharan Africa where both infectious diseases have largely overlapping geographic distributions [120]. Complex biological relationships exist during HIV and malaria co-infections [121]. It is thought that HIV influences the pathology of malaria by negatively affecting clinical presentation and some antimalarial drug treatment response [121], [122]. Conversely, malaria infection influences HIV pathogenesis by transiently increasing viral loads and CD 4 T cell depletion through induction immune cells activation and excessive pro-inflammatory cytokine release. [123]. Malaria-induced activation of CD4+ CD45RO+ T-cells and antigen presenting cells result in the release of pro-inflammatory cytokines such as TNF-α and IL 6, an immune activations status that is considered as suitable condition for increased HIV viral replication and thereby increasing the reservoir of HIV infected cells [123], [124]. Increase in viral replication likely

favours HIV transmission in case of lack of antiretroviral (ART) treatment [124]. Monitoring of HIV disease status involves periodic measurements of CD4+ T cell counts and the plasma viral load under ART treatment. These parameters are critical in predicting treatment failure or disease progression [125], [126]

1.3.2 Pegivirus and immunosuppression

Human pegivirus (HPgV), is a member of the Flaviridae viral family and one of the most prevalent viruses that constitutes the human virome [127]–[129]. Similar to HIV and Malaria, HPgV is predominant in Africa where an estimated 18-28% of its 750 million global infections occur [130], (Reviewed in Chivero and Stapleton) [131]. HPgV is thought to be generally non-pathogenic and causes most likely persistent infections in human immune cells (T and B cells, monocytes, and natural killer cells) [131]. Some studies have associated this virus even with increased risk of developing non-Hodgkin Lymphoma [132]. Interestingly, studies have linked HPgV co-infections to survival advantage of humans in presence of ongoing HIV and Ebola virus infections [133], [134]. It has been hypothesized that this favourable outcome is based on immune modulatory properties of the HPgV infection [131]. HPgV-induced immune modulatory mechanisms possibly include secretion of suppressive cytokines (TGF-β and IL10), induction of double negative and naïve T cells instead of memory T cells, inhibition of T cell activation through reduced expression of activation markers (CD69, CD25 and CD38), reduction of T cell proliferation and cytotoxicity (reviewed in Chivero and Stapleton[131]). Critical to antibody production are B cells and they seem to be impaired in presence of HPgV infection based on reduced expression of activation markers like CD86 [131]. Natural killer cells whose main function is early clearance of viral and tumorous particles prior to the activation of adaptive immune system are also impeded - likely through reduced expression of IL-12 induced IFN-ɣ [135]. IL-12 is essential for facilitating release of IFN-ɣ from the NK

and T helper-1 cells consequently supporting the containment of infectious diseases [136]. In summary, HPgV causes chronic, asymptomatic infections in humans most likely facilitated by significant immunomodulation. So far, the effects of HPgV infection on vaccination outcomes are largely unknown. Therefore, we have included in this thesis *(Chapter 5)* the investigation of HPgV infection status on experimental malaria vaccine outcomes in our cohorts from East- and West Africa.

1.4 Malaria vaccines

1.4.1 The need for malaria vaccines

An effective and efficacious malaria vaccine would not only significantly reduce morbidity and mortality attributed to malaria [52], but also prevent re-emergence of malaria in areas where it has been eliminated and help to curb the spread of drug-resistant malaria parasites [52]. Efforts to develop a malaria vaccine have been impeded by the parasite`s complex life cycle [30] in combination with a poor understanding of the protective mechanisms mediating naturally acquired immunity [138]. Currently, several malaria vaccine candidates targeting different parasite life stages are at various stages of clinical development (Fig.5). These include, pre-erythrocytic, erythrocytic and transmission blocking vaccines [139]. Pre-erythrocytic vaccines should primarily induce antibodies and CD8+ T cells that aim to prevent sporozoite movement through the skin and blood, infection of hepatocytes and to eliminate parasite-infected hepatocytes [139].Vaccines targeting the asexual blood stages function by boosting humoral response to inhibit parasite invasion of erythrocytes, therefore preventing mainly malaria disease by reducing the parasite burden [139]. Transmission-blocking vaccines, which induce antibodies in the human host, are designed to prevent the invasion and development of *Plasmodium* parasites within the mosquito vector consequently reducing malaria transmission [139]. In the face of non-sterile naturally acquired immunity, all malaria vaccine development

programs aim at inducing anti-parasite responses that are superior to naturally acquired immunity [139]. A number of different strategies and approaches are followed currently in the scientific community [140].

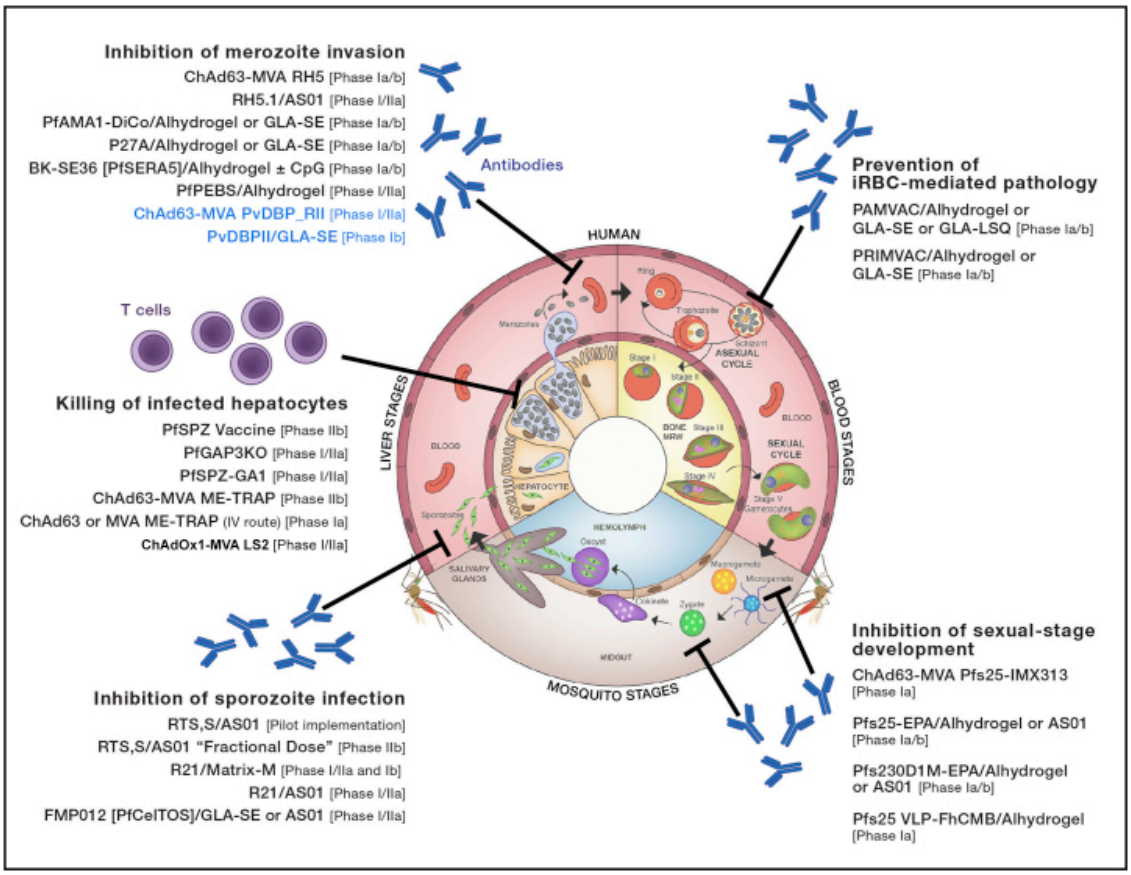

Figure 5: Malaria vaccine candidates in clinical development and targeted parasite stages [140]

1.4.2 Sub-unit malaria vaccines

RTS, S/AS01 is the most advanced subunit malaria vaccine and is based on the P. falciparum circumsporozoite protein (amino acids 207 – 395 derived from the NF54 strain of P. falciparum including 19 copies of the NANP central repeat region and the C-terminal domain expressed as recombinant fusion protein with the hepatitis B surface antigen. This fusion protein is combined with free recombinant hepatitis B particles, named S particles, that spontaneously form virus-like particles formulated with the adjuvant AS01 [140]. A phase III clinical trial

conducted in eleven African centres showed protection of 25.9% and 36.3% (18.3% and 28.3% without booster dose) against clinical malaria in infants and children, respectively [141].The main mechanisms of vaccine induced protection has been based on induction of high levels of antibodies against CSP and CD4+ T-cells producing INF-γ [142], [143]. One possible reason why subunit vaccines might not provide high levels of durable protection in the field rest on the lack of strain transcending immunity and the fact that protective antibody titers wane rapidly within the first six months after vaccination [144], [145]. Clearly, for this to be overcome, several antigens spanning different life cycle stages and more conserved epitopes across species need to be incorporated to the current vaccine designs [146], [147].

1.4.3 Whole malaria sporozoites as immunogens

In contrast to subunit vaccines, deployment of vaccines based on whole, metabolically active malaria sporozoites and asexual blood stages exposes the immune system to a wide range of antigens, thus assumed to lead to broader and more potent immune responses (reviewed in [148], [149]). Whole sporozoite based experimental malaria vaccines have been studied in avians [150], mice [151], non-human primates [152], [153] and humans [154]. These seminal studies have demonstrated that sterile protection against malaria is possible to induce when larger numbers of irradiation attenuated *P. falciparum* sporozoite are administered either intravenously or through bites of infected mosquitoes [151], [153]. However, whole sporozoite based vaccine approaches did not advance further at that time due to inability to produce sporozoites in mass numbers, the lack of sporozoite preservation technologies and the delivery of sporozoites to humans independent of infected mosquitoes [155]–[157]. Sanaria Inc., an US American Biotech Company, has developed a novel, injectable whole purified sporozoite based vaccine approach that is currently tested in clinical trials in Europe, US and Sub-Saharan Africa [155], [157]. Attenuation of sporozoites is achieved by radiation-attenuation which utilizes

gamma radiation to cause non-specific DNA breaks within parasites [149]. Consequently, these parasites can infect hepatocytes but arrest early during liver stage development [149]. The early arrest of parasite growth in the hepatocyte results most likely in short exposure of the immune system to liver stage antigens, hence limited immune responses are induced [158]. Alternative attenuation methods of infectious sporozoites are (ii) chemical attenuation by cultivation of whole parasites with DNA binding drugs like Centanamycin [149] (reviewed in [159]). Chemically attenuated parasites show complete liver stage development with induction of immune responses similar to radiation attenuated parasites [160]. iii) genetic attenuation based on the deletion of gene combinations that are supporting parasite liver stage development [161]. In animal models, a triple knock out parasite developed up to late liver stages and induced robust sterilizing immunity [162]. This vaccination approach has also been tested in human volunteers and proven to be safe and well tolerated [163]. Further to this, direct intravenous administration double knockout plasmodium parasites (PfSPZ-GA1) has also been evaluated for the first time in dutch volunteers. It induces robust humoral and cellular responses volunteers however the efficacy is low [164] iv) vaccination under chemoprophylaxis whereby live non-attenuated sporozoites are administered together with the anti-malarial drug chloroquine targeting asexual blood stage parasites that are eventually appearing from the liver (PfSPZ-CVac) [165], [166]. It is assumed that during this vaccination procedure the human immune system is exposed to a high load and over a longer time period to a broad range of antigens expressed during the liver stage initiating robust cellular and humoral immunity (reviewed in [167]).

1.4.4 Radiation attenuated malaria sporozoite vaccine induced protection: PfSPZ-Vaccine

The most advanced whole sporozoite based vaccine candidate is PfSPZ-Vaccine based on radiation attenuated sporozoites [149]. Proof of concept for efficacy of PfSPZ-Vaccine was shown in 2011 at the Vaccine Research Center (NIH, USA) in malaria naïve volunteers. 100% protection was observed in 9 out of 9 volunteers against a homologous mosquito based challenge at the highest dose of sporozoites applied [168]. Here, PfSPZ-Vaccine was used for the first time intravenously and a PfSPZ-Vaccine dose dependency of protection was observed [168]. Next, PfSPZ vaccine was tested in malaria pre-exposed volunteers in several sub-Saharan African countries [155]. In Bagamoyo, during the BSPZV1 study (NCT02132299), repeat intravenous inoculation of either $1.35x10^5$ or $2.7\ X10^5$ PfSPZ-Vaccine in healthy male Tanzanian adults was tested [169]. Volunteers were challenged with 3200 fully infectious homologous sporozoites intravenously (PfSPZ Challenge) three weeks past last vaccination, also called controlled human malaria infection (CHMI) [169]. 1 out of 18 (6%) individuals in the $1.35x10^5$ dose group and 4 out of 20 (20%) individuals in the 2.7×10^5 dose group were sterile protected [169]. This level of protection was considerably lower than the one observed in malaria naïve volunteers that had undergone a similar vaccination regimen [170]. Based on the excellent safety profile, a follow up study was conducted from 2015 to 2017 in Bagamoyo to evaluate PfSPZ-Vaccine in adults, adolescent, children and infants (BSPZV2 study NCT02613520 [171], [172],. These individuals were immunized three times with different doses of PfSPZ-Vaccine including 1.8×10^6, 9×10^5, 4.5×10^5 or 2.7×10^5. Interestingly, sterile protection against homologous CHMI was achieved in adult 5 out of 5 volunteers (100%) in the 9×10^5 dose group following CHMI at 3 or 11 weeks after last vaccination. However, vaccine efficacy decreased with increase of dosage to 1.8×10^6 where only 2 out of 6 (33%) individuals became protected [172] In 2018, the safety, tolerability, immunogenicity and efficacy of PfSPZ-Vaccine in HIV-positive individuals who are under ART was evaluated. The

PfSPZ-Vaccine was compared to HIV-negative volunteers of same age (BSPZV3a study, NCT03420053) (Manuscript in preparation). Investigation of HIV viral dynamics before, during and after PfSPZ-Vaccine inoculation are important to assess, particularly in Sub-Saharan Africa. Similarly, to the BSPZV1 study, the HIV-positive and HIV-negative volunteers were challenged with homologous CHMI three weeks past last vaccination to assess vaccine efficacy. The outcome was striking in that the sterile protection in HIV-negative and HIV positive volunteers were 80 % and 0 %, respectively.

1.4.5 Non-attenuated P. falciparum sporozoites under chemoprophylaxis as vaccine approach: PfSPZ-CVac

PfSPZ-CVac has been tested successfully in malaria naïve volunteers in Tübingen, Germany, and has been shown to be safe, well tolerated and able to induce sterilizing protection in 9 out of 9 volunteers (100%) [165]. In Equatorial Guinea, we have conducted the first PfSPZ-CVac study in Sub-Saharan Africa enabling the site-by-site comparison between PfSPZ-Vaccine and PfSPZ-CVac. This study is labelled as EGSPZV2 (NCT02859350) and was conducted in 2017 in Malabo on Bioko Island (Jongo etal., manuscript in press). In this study three doses of 2.7 X 10^6 PfSPZ Vaccine and 1 X 10^5 fully infectious non-irradiated sporozoites given under chloroquine chemo-prophylaxis (PfSPZ-CVAC) were administered intravenously in adult volunteers. These individuals underwent homologous CHMI at 14 and 15 weeks past last vaccination to assess vaccine efficacy. 6 out of 16 (27%) and 8 out of 13 (55%) individuals were malaria negative in the PfSPZ vaccine and PfSPZ CVAC groups, respectively (Jongo etal., manuscript in press.). The observed lower vaccine efficacy in the PfSPZ vaccine group mirrors, the findings of other studies in endemic countries which saw reduction in vaccine efficacy following immunization with higher dosages of irradiated sporozoites [173]. This is contrary to the outcome of the PfSPZ CVAC study conducted in malaria pre-exposed volunteers (Jongo etal., manuscript in press.) Interestingly, lower CSP-antibody titers were

observed in individuals in the PfSPZ –CVAC group despite the higher number of protected volunteers (Manuscript submitted). This observation highlights the need to further characterize correlates of vaccine induced immunity and malaria protection to understand the relative contribution of humoral versus cellular immunity.

1.4.6 Immune response to whole sporozoite based malaria vaccines

Immunization with PfSPZ-Vaccine in healthy population induces mainly antibodies targeting CSP [174], [175] (reviewed in [158]). These antibodies are thought to be involved in preventing sporozoite motility and infection of hepatocytes [174], [175]. Moreover CD4+ and CD8+ T-cells producing any combination of IFN-Ɣ; TNF-α and IL-2, are likely to be involved in vaccine induced protection [165], [176]. The contribution of CD8+ T-cells possibly exceeds that of CD4+ T-cells as demonstrated in T cell depletion assays in animals models [177] [158].

1.5 Impact of co-infections and innate immune activation on vaccine induced responses

A broad range of different vaccine studies have reported differences in vaccine induced responses among populations of different age and ethnicity [178], [179]. Similar observations have also been reported in PfSPZ-Vaccine studies [180]. Most strikingly, comparisons of CSP antibody serum titers measured by the identical enzyme-linked immunosorbent assays (ELISA) show significantly lower titres in malaria pre-exposed volunteers when compared to malaria naive subjects treated with comparable vaccine regimen [176], [181]–[183]. Intriguingly, higher antibody titres in Tanzanians compared to Malian and Equatorial Guineans became evident that have been inoculated with PfSPZ Vaccine (Figure 6).

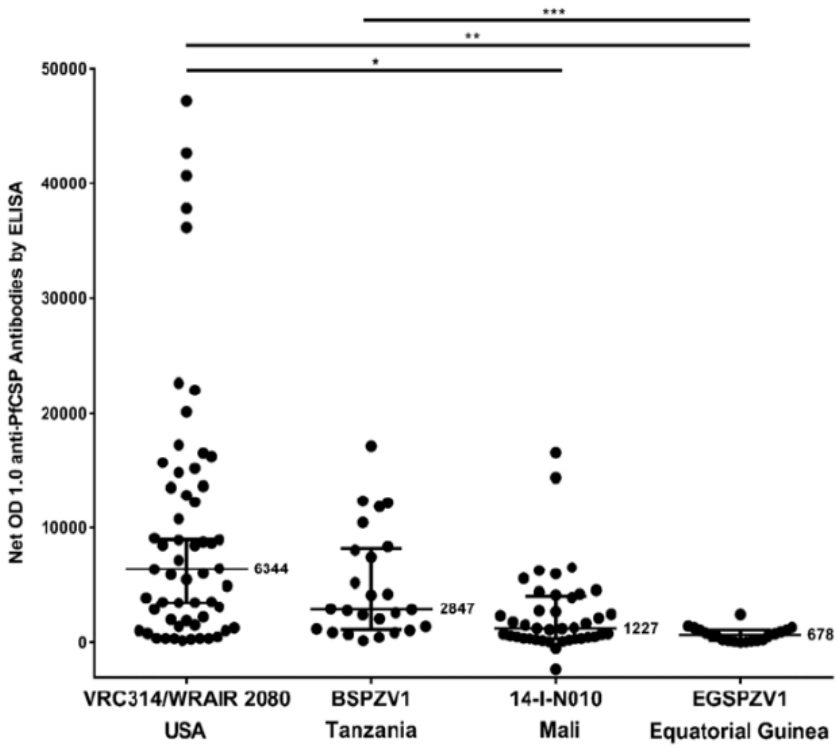

Figure 6: Comparison of anti-*Plasmodium falciparum* circumsporozoite protein (PfCSP) antibody responses in four different clinical trials in which the same dose of PfSPZ Vaccine was administered. Antibody responses measured by enzyme-linked immunosorbent assay (ELISA) are reported as the net OD 1.0. The OD 1.0 is the serum dilution at which the optical density is 1.0, and net OD 1.0 is the difference between the post- and pre-immunization OD 1.0. In the USA (VRC314/WRAIR 2080) (most subjects), Mali and Tanzania vaccinations were at 0, 4, and 8 weeks. In Equatorial Guinea (EG), vaccinations were at 0, 8, and 16 weeks. In the United States, Mali, and EG, sera were drawn at 2 weeks after the last vaccination. In Tanzania, sera were drawn at 4 weeks after the last vaccination. Bars with asterisks indicate the statistical significance, as determined by a Kruskal–Wallis test followed by a Dunn's multiple comparisons test (P = * < 0.0001; ** < 0.0001; *** 0.0008 (Adapted from Oluto et al., 2018)

Underlying reasons for this difference in humoral immunity are currently unclear. Chronic viral (including HIV), bacterial or parasitic infections can induce systemic inflammation consequently affecting humoral immunity to a range of vaccines [184]–[186]. Investigations

of impact of immune activation and co-infections in the context of whole sporozoite based vaccines within and between malaria endemic populations do not exist. More information is clearly needed to elucidate mechanisms involved in the observed variation of vaccine immunogenicity in different populations.

1.6 Controlled human malaria infection

Controlled human malaria infection (CHMI) involves the deliberate infection of human subjects with malaria parasites by mosquito bite or injection (intradermal, intramuscularly, subcutaneous or intravenous) in a controlled environment [187]. Notably, while sporozoites have been used extensively in malaria infection studies [187], the asexual blood stage parasites are also utilized increasingly [188]–[190].

In the past, malaria infections were used as therapy to treat neurosyphilis [191] and HIV [192]. The neurosyphilis treatment in particular involved the use of wide range of *Plasmodia* parasite species including *P. vivax, P. malariae P. ovale and P. falciparum* [187], [193]. Early studies have also documented the use of malaria infection for the evaluation of anti-malarial drug effects in non-immune individuals [194]. Malaria infections conducted in these early years were challenging and employed inadequate research methods. However, they were instrumental in providing wealth of information on natural course of malaria infection and the life cycle [187], [193] . In later years, malaria infection models were strengthened by the ability to culture *P. falciparum* and the generation of gametocytes in vitro to infect *Anopheles* [195], [196] as well as existence of laboratory reared *Anopheles* mosquito that could be used to challenge volunteers [197]. Using CHMI has the clear advantage that costs associated with the conduct of phase II field studies can be substantially reduced whilst obtaining very early in the clinical development path robust information on vaccine efficacy in smaller numbers of volunteers [187].

Sanaria Inc. has developed a method to produce GMP compliant aseptic, cryopreserved fully infectious sporozoites (PfSPZ-Challenge) circumventing the need for the use of mosquito bites in evaluating the efficacy of malaria vaccine candidates [198]. Consequently, CHMI studies by needle and syringe are increasingly used to evaluate the efficacy of malaria vaccines candidates in malaria pre-exposed populations [199]. Development of PfSPZ-Challenge has enabled vaccine efficacy studies to be conducted in malaria endemic countries where the target population of a malaria vaccine resides [200].

The first trial in which the PfSPZ-Challenge was administered by needle and syringe was conducted in Dutch volunteers [199]. Further optimization of the dose and route of administration were tested across different populations including in individuals residing in malaria endemic countries [199], [201], [202]. Direct venous inoculation of 3200 sporozoites resulted to 100 % of infection of volunteers and this dose is currently used as standard in homologous CHMI studies conducted [203].

A range of different *P. falciparum* strains have been employed in CHMI studies, including strain NF54, NF166.C8, 7G8 and NF135.C10 [48]. These strains have been evaluated in homologous (similar to vaccine strain) and heterologous (different from vaccine strain) challenge models following immunization with PfSPZ-Vaccine [48], [204]. There is a need include strains endemic to distinct malaria regions as this will help to accelerate the assessment of cross strain immunity [48]

Identification of malaria vaccine-induced immune responses and how they potentially correlate or mediate vaccine induced protection is of highest priority in malaria vaccine development [205]. However, to date no definite biomarker of PfSPZ-Vaccine induced protection has been identified. CHMI is therefore a unique platform for the investigation of host parasite interactions in a comprehensive manner under a highly controlled setting [206]. Apart from

testing vaccine induced protection, CHMI studies are also ideal to provide insight into the natural host-pathogen interaction [207] and human immune responses [208], [209]

CHMI studies provides a unique platform for investigating the interaction of malaria infection with chronic co-infections like HIV that are highly prevalent in malaria endemic regions. Apart from its role in clinical vaccine development, CHMI also forms an important tool for testing malaria drug efficacy [210] and evaluating new diagnostic tools [211]. Conclusively, CHMI has a wide array of applications in malaria research and product development. It is important to note that there are also well-established controlled human infection models for a wide range of bacterial, viral and parasite infectious diseases such as *Salmonella enterica* serovar typhi, Vibrio cholera, Influenza, Rhinovirus, Respiratory syncytial virus, Norovirus, Dengue virus and Hookworm and *Schistosoma mansoni* infections, respectively [212].

1.7 Aims of the thesis

Overall objective:

Sustainable malaria control efforts with a continuous strive for local malaria elimination will most likely need a combination of interventions including vector control, prompt testing and safe treatment of malaria infected people and the development of a malaria vaccine that is safe and protective in all age groups and irrespective of their HIV infection status. The overall objective of the PhD work presented is to contribute to the evaluation of the safety, immunogenicity and efficacy against homologous CHMI of whole, purified P. falciparum sporozoite based malaria vaccination approaches in different population and age groups residing in malaria endemic countries. In addition, we investigated the potential role of chronic viral infections on vaccine-induced protection against homologous CHMI. This has been approached by addressing the following specific aims:

Aim 1: To evaluate the safety, tolerability, efficacy, and immunogenicity of the PfSPZ Vaccine in different dosing and vaccination regimens in Tanzanian volunteers of different ages.

Aim 2: To compare the safety, tolerability, efficacy, and immunogenicity of two different whole sporozoite based vaccination approaches, PfSPZ Vaccine and PfSPZ-CVac, in adult Equatorial Guinean volunteers.

Aim 3: To use *P. falciparum* protein microarray analyses to profile PfSPZ-Vaccine induced humoral immunity in HIV positive and HIV negative adult volunteers to understand immune status before vaccination, immune-dominance of vaccine induced antibody targets, and distinct antibody profiles that might be associated with vaccine-induced protection.

Aim 4: To investigating the interaction of chronic HPgV co-infection on PfSPZ Vaccine induced humoral immunity and protection against homologous CHMI.

Chapter 2

Safety, immunogenicity, and efficacy of radiation attenuated *Plasmodium falciparum* whole sporozoite vaccine (PfSPZ Vaccine) in African populations of different ages

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This chapter contains the following publications:

- Jongo SA, Shekalaghe SA, Church LWP, Ruben AJ, Schindler T, Zenklusen I, Rutishauser T, Rothen J, **Tumbo A,** Mkindi C, Mpina M, Mtoro AT, Ishizuka AS, Kassim KR, Milando FA, Qassim M, Juma OA, Mwakasungula S, Simon B, James ER, Abebe Y, Kc N, Chakravarty S, Saverino E, Bakari BM, Billingsley PF, Seder RA, Daubenberger C, Sim BKL, Richie TL, Tanner M, Abdulla S, Hoffman SL. Safety, Immunogenicity, and Protective Efficacy against Controlled Human Malaria Infection of *Plasmodium falciparum* Sporozoite Vaccine in Tanzanian Adults. Am J Trop Med Hyg. 2018 Aug;99(2):338-349. doi: 10.4269/ajtmh.17-1014.

- Jongo SA, Church LWP, Mtoro AT, Chakravarty S, Ruben AJ, Swanson PA, Kassim KR, Mpina M**, Tumbo AM,** Milando FA, Qassim M, Juma OA, Bakari BM, Simon B, James ER, Abebe Y, Kc N, Saverino E, Gondwe L, Studer F, Fink M, Cosi G, El-Khorazaty J, Styers D, Seder RA, Schindler T, Billingsley PF, Daubenberger C, Sim BKL, Tanner M, Richie TL, Abdulla S, Hoffman SL. 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. **Am J Trop Med Hyg. 2019 Jun;100(6):1433-1444. doi: 10.4269/ajtmh.18-0835**

- Jongo SA, Church LWP, Mtoro AT, Chakravarty S, Ruben AJ, Swanson PA, Kassim KR, Mpina M, **Tumbo AM,** Milando FA, Qassim M, Juma OA, Bakari BM, Simon B, James ER, Abebe Y, Kc N, Saverino E, Fink M, Cosi G, Gondwe L, Studer F, Styers D, Seder RA, Schindler T, Billingsley PF, Daubenberger C, Sim BKL, Tanner M, Richie TL, Abdulla S, Hoffman SL. Increase of dose associated with decrease in protection against controlled human malaria infection by PfSPZ Vaccine in Tanzanian adults..**Clin Infect Dis. 2020 Dec 31;71(11):2849-2857. doi: 10.1093/cid/ciz1152**

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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.

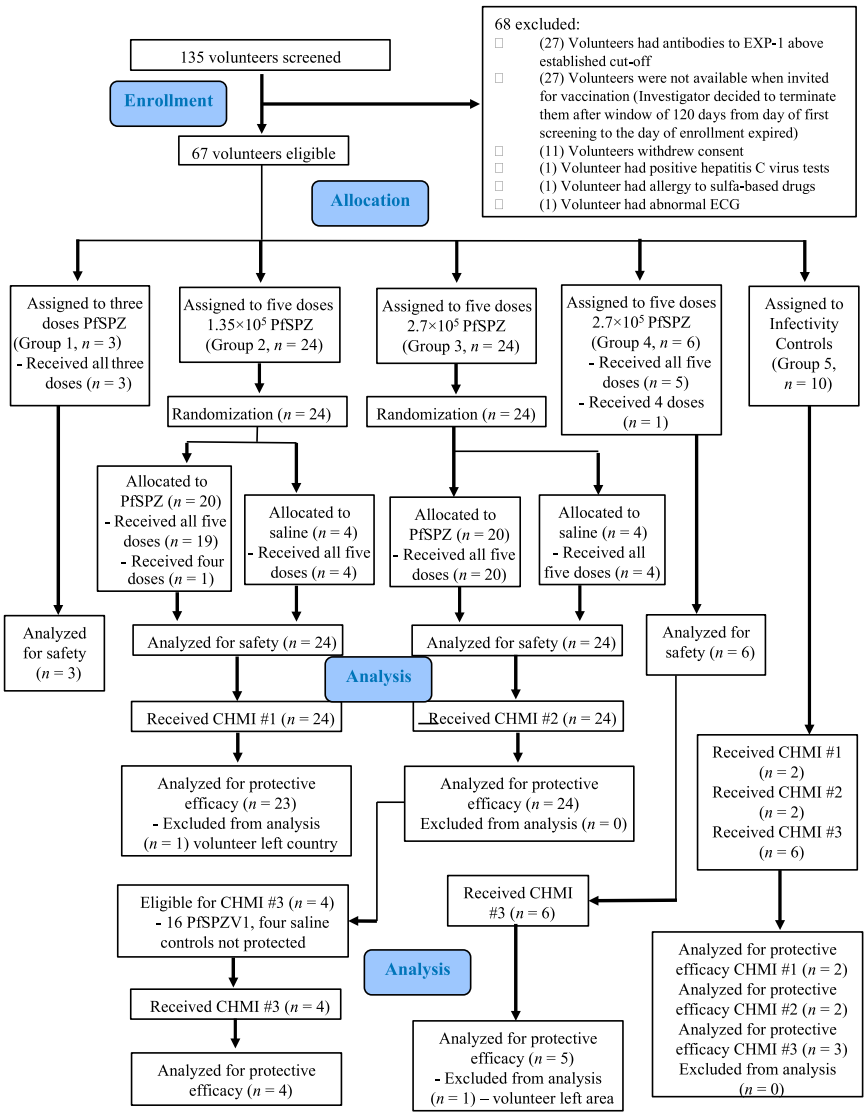


FIGURE 1. Volunteer participation (CONSORT 2010 diagram). This figure appears in color at www.ajtmh.org.

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%.

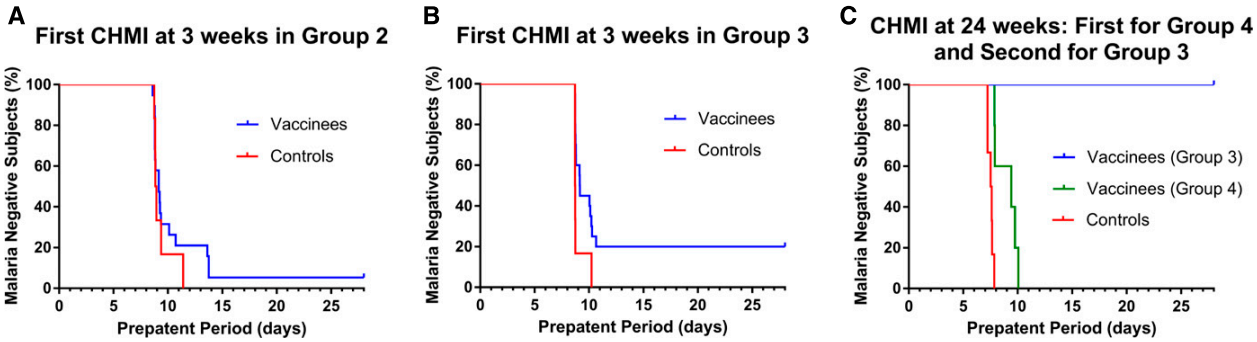
Group 4 (2.7×10^5 PfSPZ). First CHMI at 24 weeks after the last vaccine dose (CHMI #3): 4/5 vaccinees developed parasitemia by TBS and qPCR. The fifth was negative by TBS, but positive by qPCR (see Supplemental Table 10). There was one excluded volunteer (see the previous paragraph). Vaccine efficacy 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

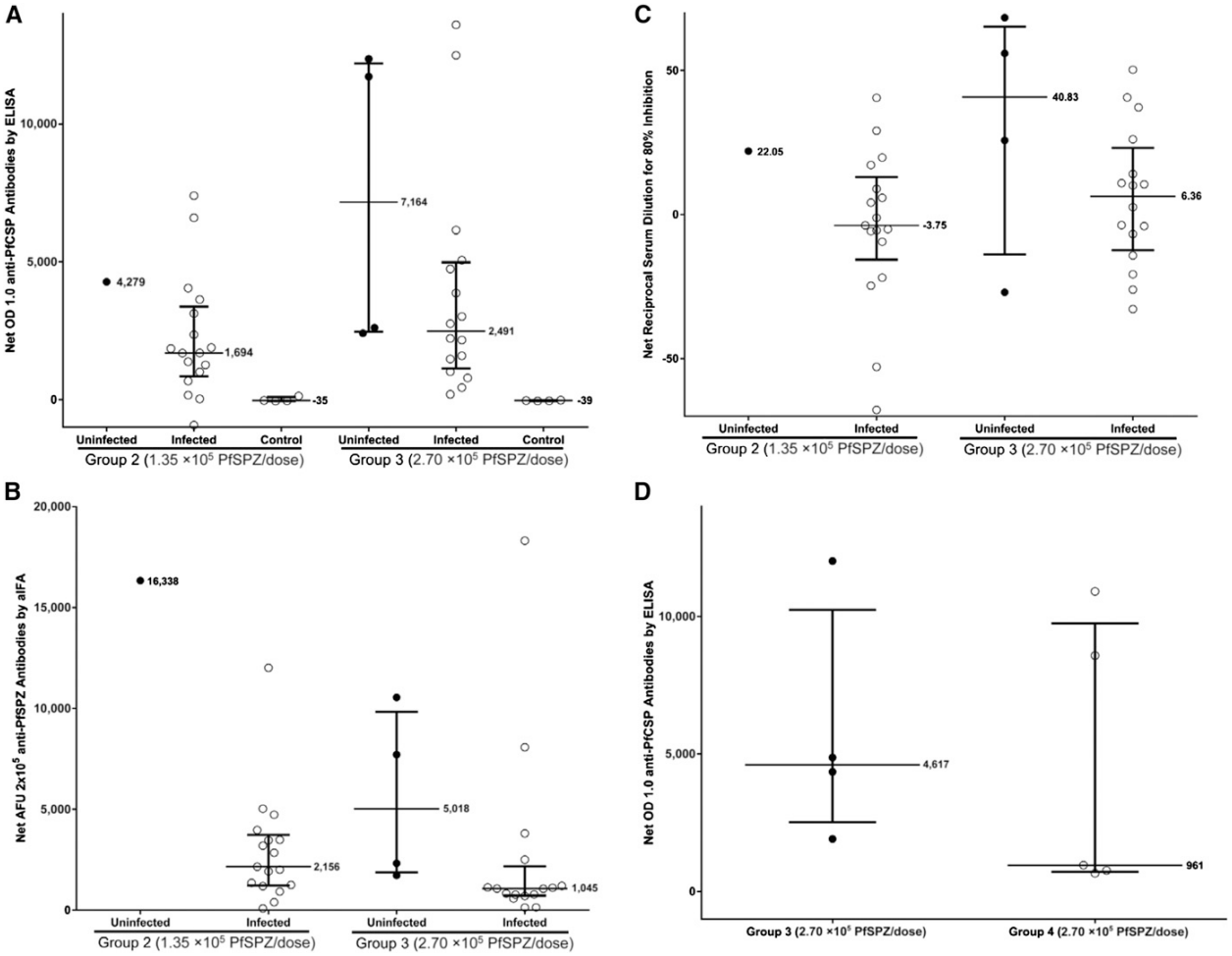

FIGURE 3. Antibody responses to *Plasmodium falciparum* Sporozoites (PfSPZ) and PfCSP before controlled human malaria infection (CHMI). For all assays, uninfected subjects are shown as filled (black) circles and infected subjects are open circles. For each of the defined subject groups, the interquartile 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.

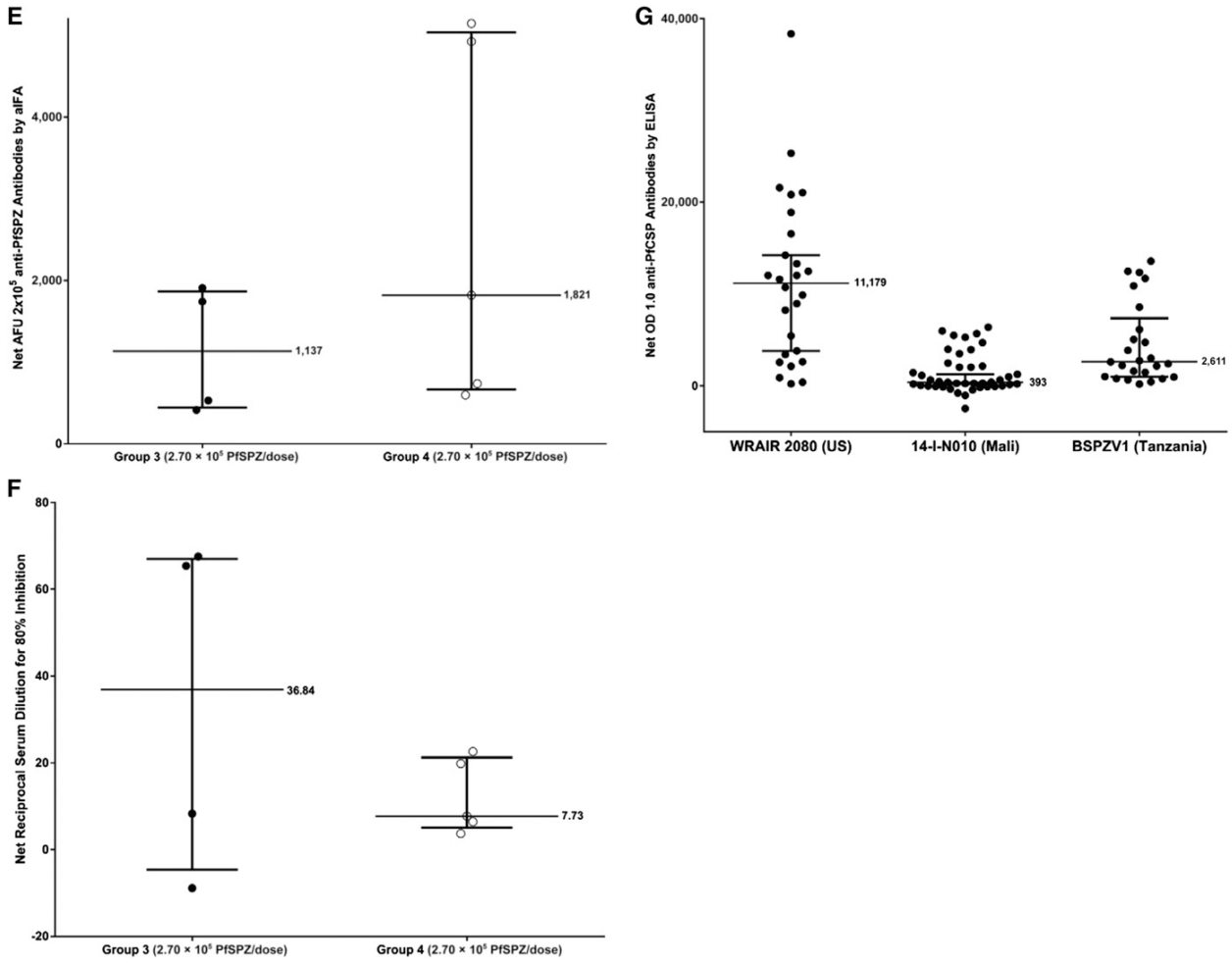

FIGURE 3. (*Continued*)

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

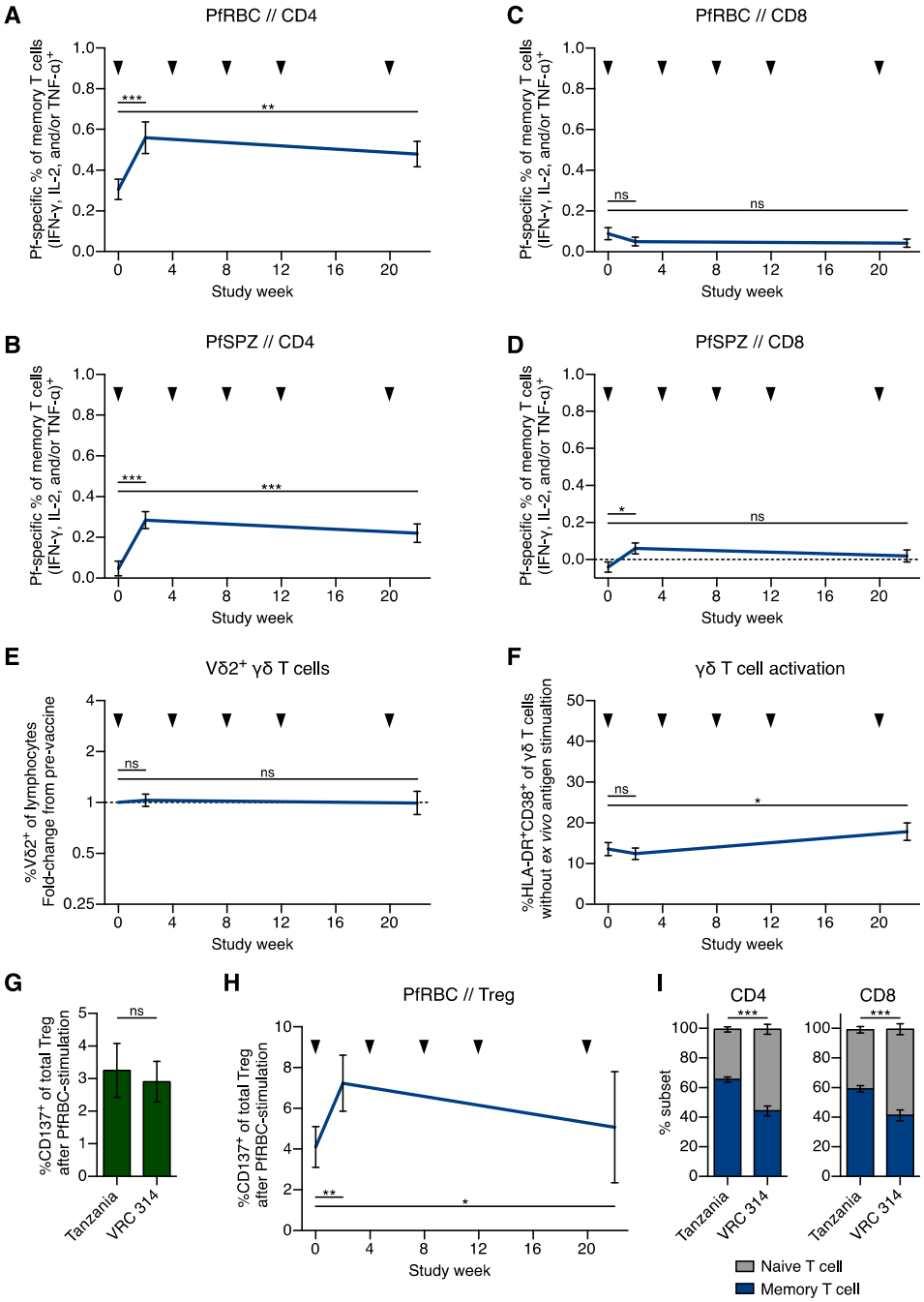

FIGURE 4. *Plasmodium falciparum* Sporozoites (PfSPZ)–specific T-cell responses in vaccine recipients receiving 1.35×10^5 PfSPZ. (**A–D**) PfSPZ-specific T-cell responses. Frequency of cytokine-producing memory CD4 T cells responding to (**A**) PfRBC or (**B**) PfSPZ. Throughout, "naïve T cell" refers 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 Vδ2+ 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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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

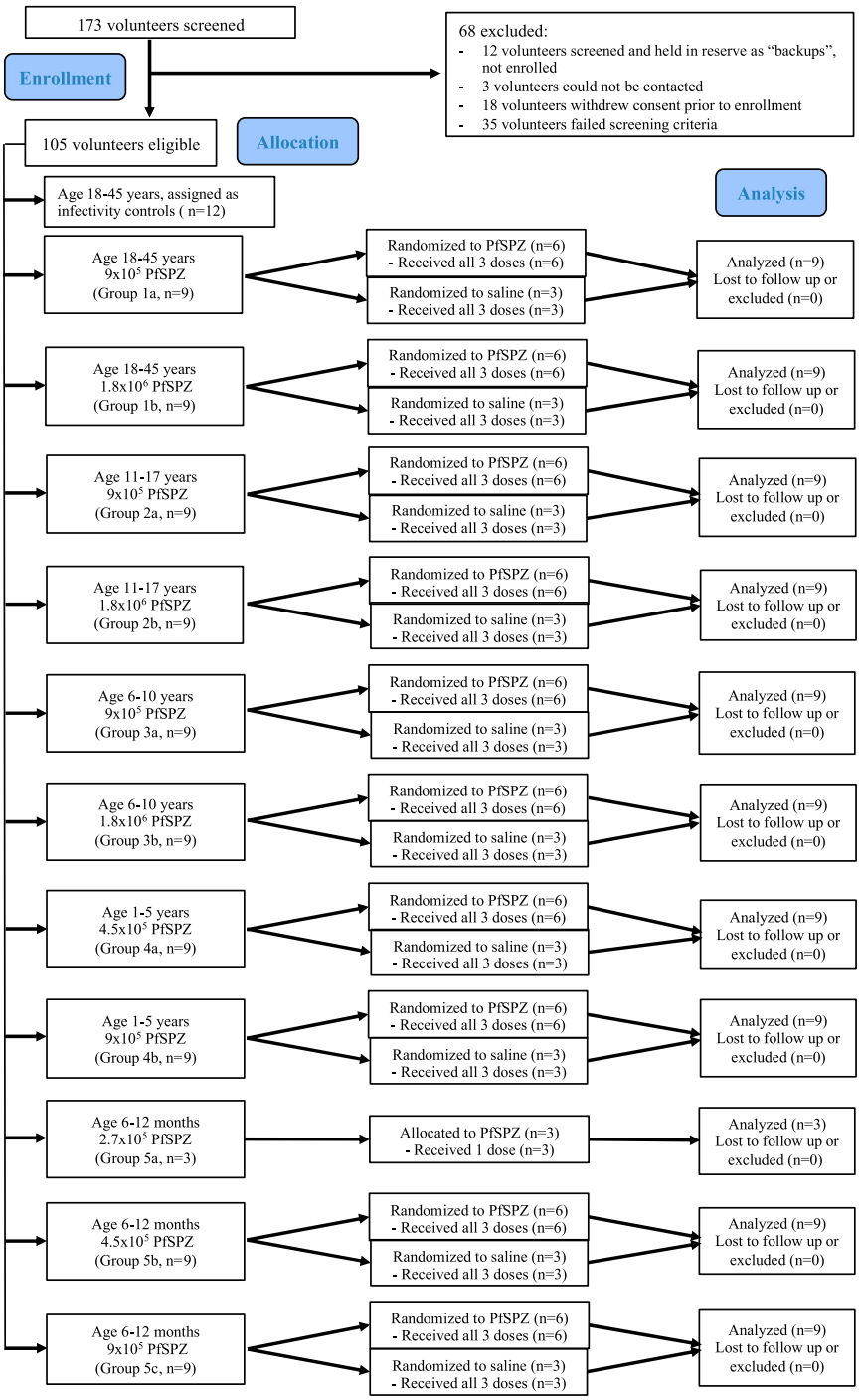

FIGURE 1. Volunteer participation (CONSORT 2010 Diagram). Once allocated, no volunteers were removed, lost to follow-up, or excluded from analysis. This figure appears in color at www.ajtmh.org.

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

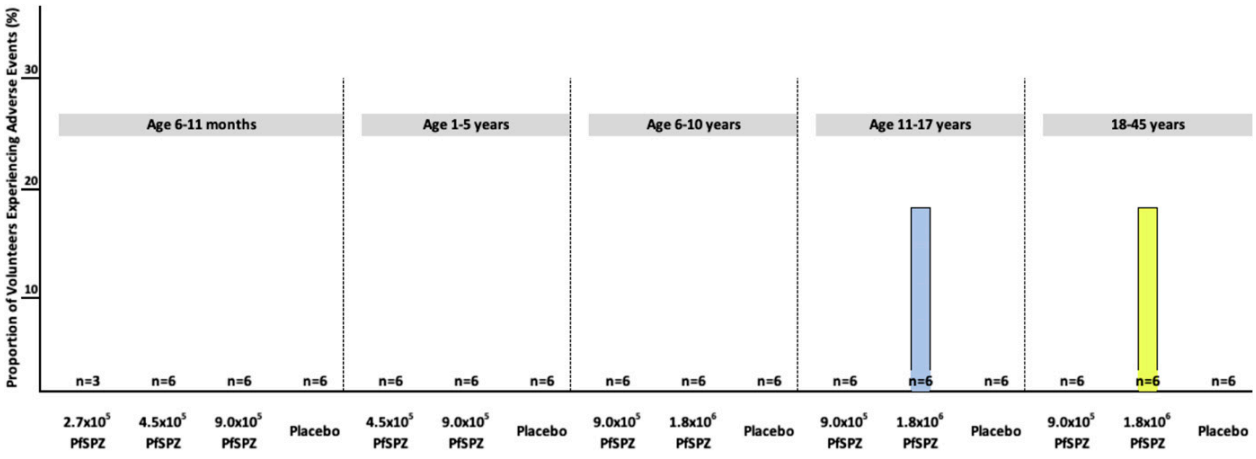

FIGURE 2. Proportion of volunteers experiencing solicited adverse events (AEs) during the 7 days after each immunization. Ninety-one of 93 volunteers injected at least once experienced no solicited AEs during the 7 days after each immunization. One volunteer experienced three Grade 1 local AEs (yellow 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.

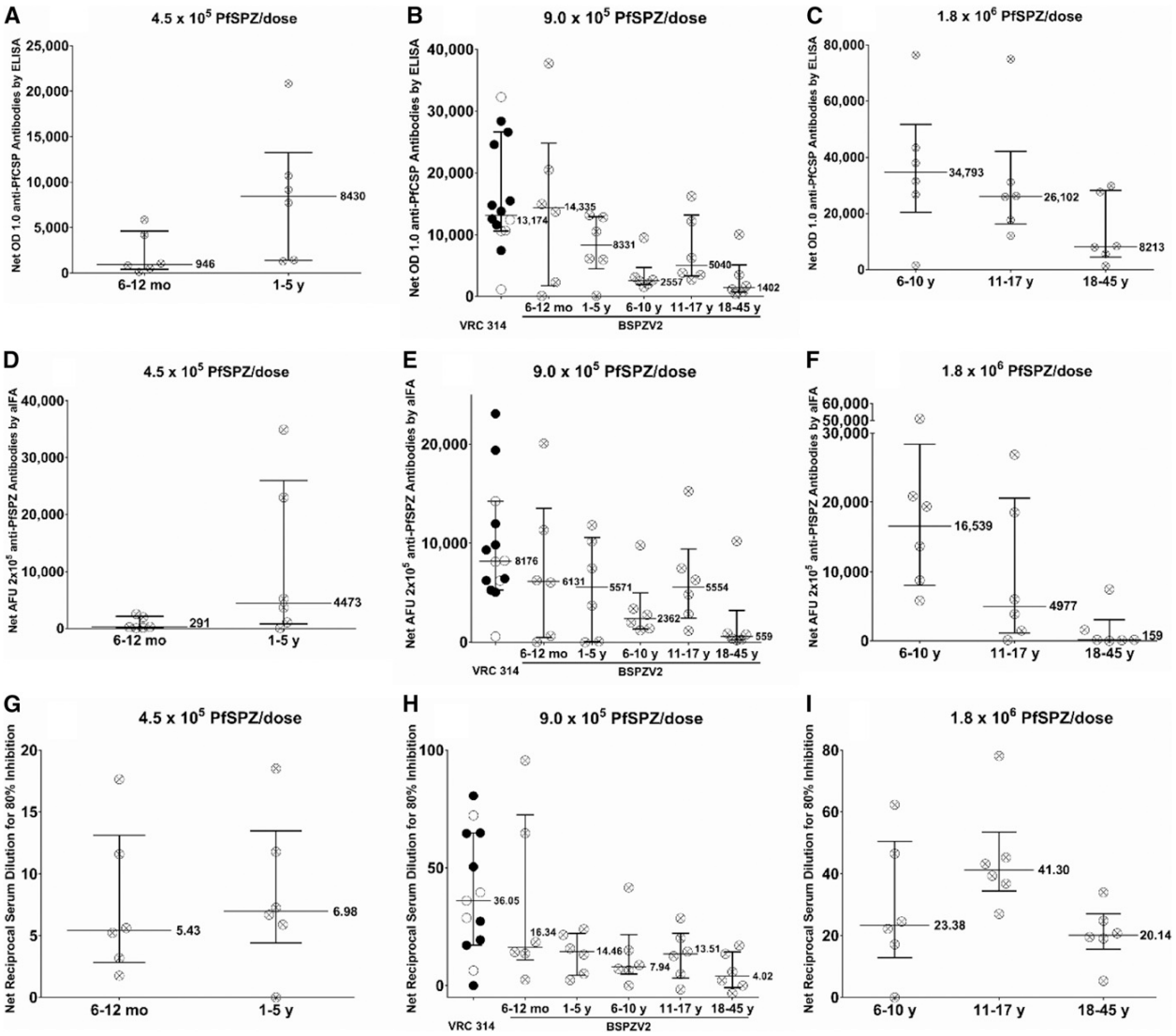

FIGURE 3. Difference between postimmunization and preimmunization antibody results. Antibody assay results by dose in *Plasmodium falciparum* circumsporozoite protein enzyme-linked immunosorbent assay (**A**–**C**), immunofluorescence assay (**D**–**F**), and inhibition of sporozoite invasion (**G**–**I**) 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)

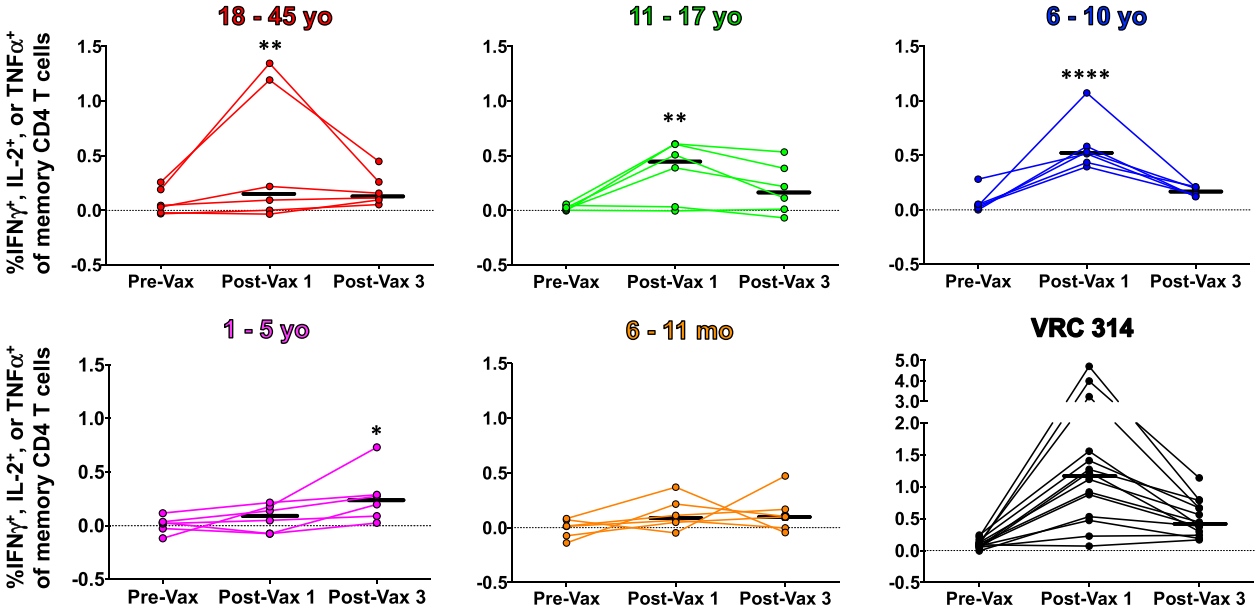

FIGURE 4. *Plasmodium falciparum* sporozoites (PfSPZ)-specific memory CD4 T-cell responses pre- and postvaccination. Percent of memory CD4 T cells in the blood expressing interferon gamma (IFN-γ) interleukin 2 (IL-2) or tumor necrosis factor alpha (TNF-α) at preimmunization or 2 weeks after the first 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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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

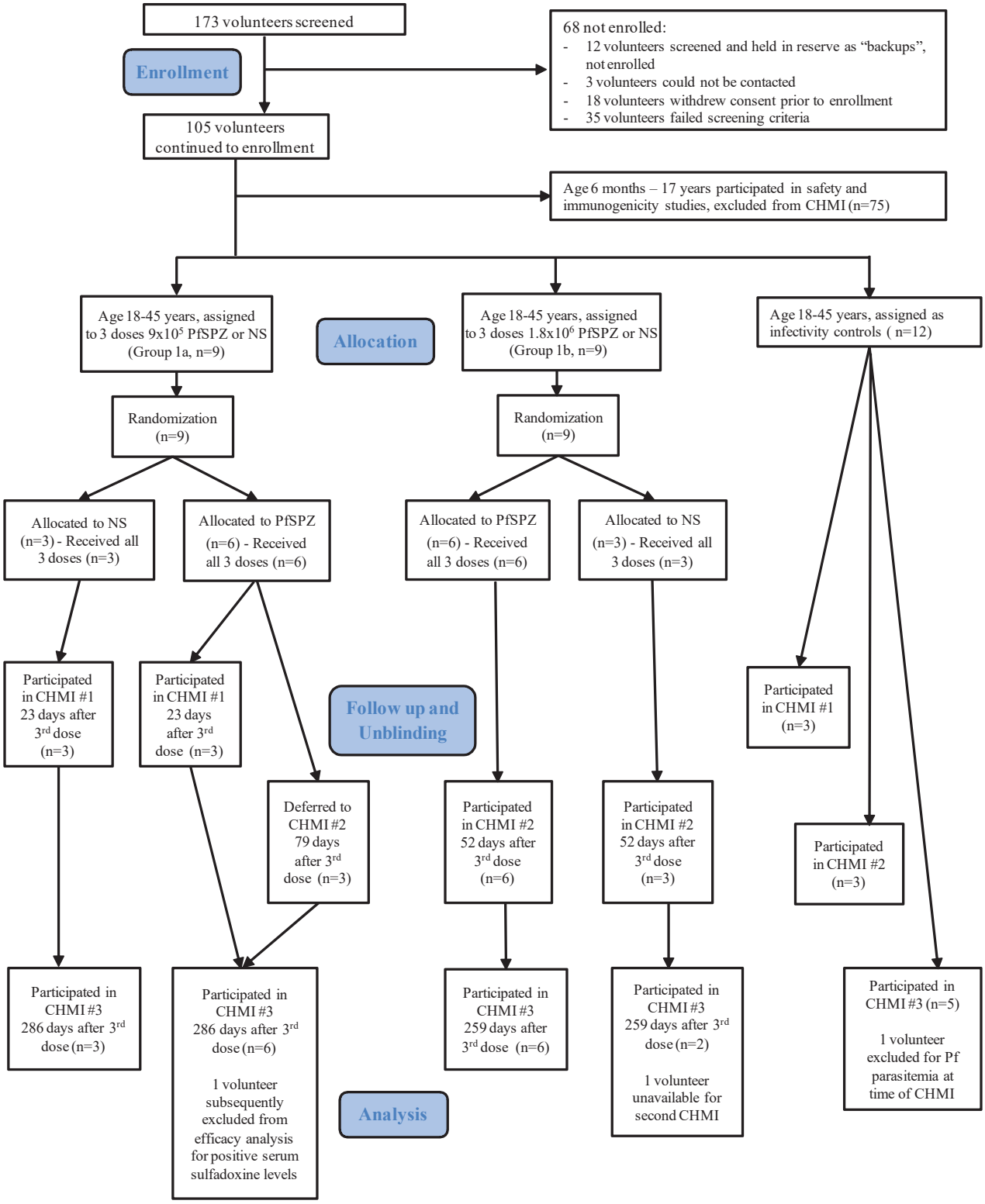

Figure 1. Volunteer participation (CONSORT 2010 Diagram). Abbreviations: CONSORT, Consolidated Standards of Reporting Trials; CHMI, controlled human malaria infection; NS, normal saline; PfSPZ, *Plasmodium falciparum* sporozoite.

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

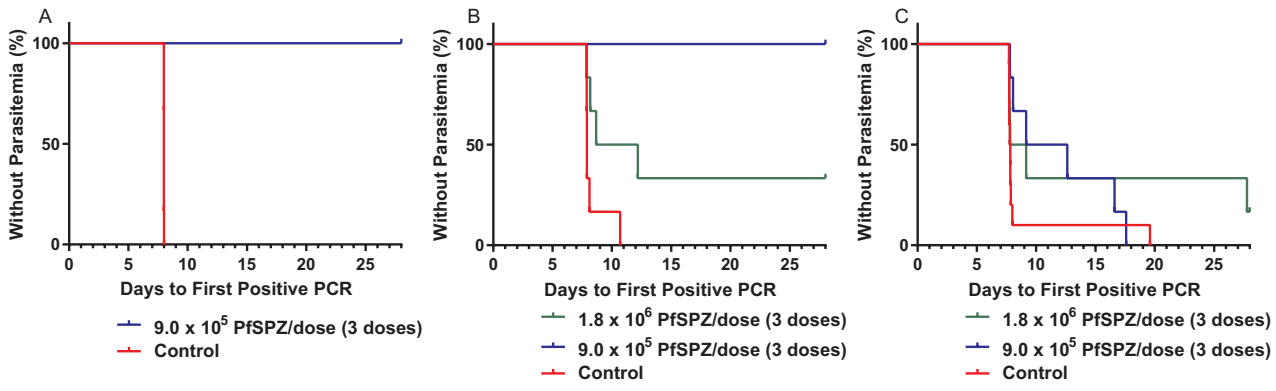

Figure 2. Kaplan-Meier survival curves in immunized volunteers vs controls as assessed by qPCR. Kaplan-Meier curves in volunteers undergoing: *A*, CHMI 23 days after the last of 3 doses with 9.0×10^5 PfSPZ (n = 3) vs NS and infectivity controls (n = 6); *B*, CHMI 79 days after the last of 3 doses with 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

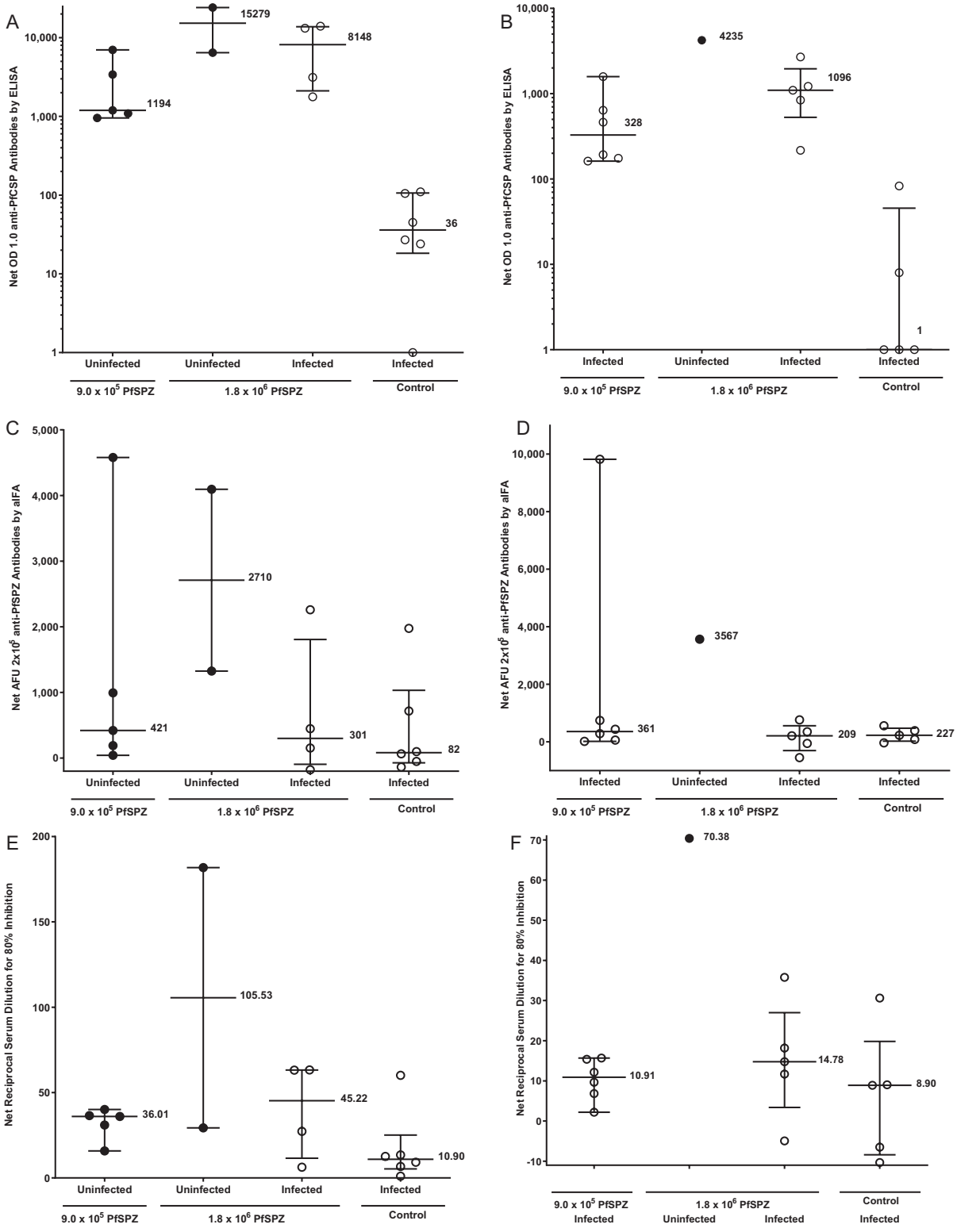

Figure 3. IgG antibodies to PfCSP by ELISA at time of first CHMI (CHMIs no. 1 and no. 2). *A*, IgG antibodies to PfSPZ by aIFA (*C*) and automated inhibition of PfSPZ invasion of hepatoma cells (aISI) (*E*) and at time of second CHMI (CHMI no.3) by ELISA (*B*), aIFA (*D*) and ISI (*F*) in subjects who 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

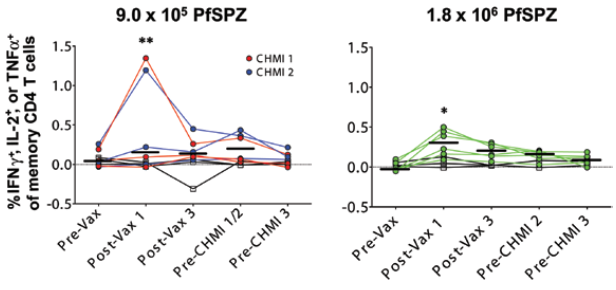

Figure 4. Pf-specific memory CD4 T-cell responses following different PfSPZ Vaccine doses. T-cell responses were assessed by flow cytometry 2 weeks after first and third doses of vaccine, and prior to the first and second CHMIs. We incubated PBMCs from vaccinees and controls with radiation attenuated (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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Chapter 3

Immunogenicity and protective efficacy of radiation-attenuated and chemo-attenuated PfSPZ vaccines in Equatoguinean adults.

This chapter contains the following manuscript

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LRH: JONGO AND OTHERS

RRH: PFSPZ VACCINE AND PFSPZ-CVAC EFFICACY IN EQUATORIAL GUINEA

Immunogenicity and protective efficacy of radiation-attenuated and chemo-attenuated PfSPZ vaccines in Equatoguinean adults

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ABSTRACT

PfSPZ Vaccine (radiation-attenuated, aseptic, purified, cryopreserved *Plasmodium falciparum* (Pf) sporozoites (SPZ)) and PfSPZ-CVac (infectious, aseptic, purified, cryopreserved PfSPZ administered to subjects taking weekly chloroquine chemoprophylaxis) have shown vaccine efficacies (VE) of 100% against homologous controlled human malaria infection (CHMI) in non-immune adults. PfSPZ-CVac has never been assessd against CHMI in African vaccinees. We assessed the safety, immunogenicity and VE against homologous CHMI of 3 doses of $2.7x10^6$ PfSPZ of PfSPZ Vaccine at 8 week intervals and 3 doses of $1.0x10^5$ 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 (AEs) between vaccinees and NS 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 due to Pf parasitemia. Antibody responses were 2.9 times higher in PfSPZ Vaccine recipients as compared to PfSPZ-CVac recipients at time of CHMI. VE 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.

Trial registration. Clinical Trials.gov(NCT02859350).

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 2015-2018[1, 2]. Depending on the estimate[1, 3], there are 16,730 to 28,000 deaths from malaria every 2 weeks. The Bioko Island Malaria Elimination Program (BIMEP) 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 2-14-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 2-14-year-olds has been unchanged for the past 6 years, paralleling the international situation (G. 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* (Pf) sporozoite (SPZ) 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 upon, the optimization of vaccination regimens with PfSPZ vaccines has been empirical. Here we report the safety, immunogenicity and vaccine efficacy (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 (PfSPZ Challenge) administered to subjects taking chloroquine chemoprophylaxis)[9, 13] in healthy 18-35-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 month to 17 year olds and 36-65 year olds (part A) and a safety, immunogenicity and controlled human malaria infection (CHMI) component to assess vaccine efficacy (VE) in 18-35 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 age 18 to 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: Table S1, S2) and successfully completed a test of understanding were 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 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 is comprised of 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 (NS) was the placebo. Chloroquine phosphate (Resochín, Kern Pharma), administered weekly beginning 2 days prior to 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 ($2.7x10^6$ 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 ($1.0x10^5$ 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] and PfSPZ-CVac [9, 13]. The study team was blinded to treatment assignment within each group. PfSPZ Vaccine, PfSPZ Challenge, or normal saline (NS) in 0.5 mL was administered by DVI through a 25-gauge needle. Chloroquine was administered orally under direct observation 2 days prior to first dose of PfSPZ Challenge or NS in the PfSPZ-CVac group and weekly thereafter through 5 days after the final injection of PfSPZ Challenge or NS (Figure 1 and S1); the first dose was 600 mg chloroquine base and subsequent doses were 300 mg chloroquine base.

Vaccine efficacy (VE): VE was assessed by CHMI by DVI of $3.2x10^3$ PfSPZ of PfSPZ Challenge and calculated based on the first positive qPCR result. CHMIs were planned for 10 to 14 weeks after last immunization, although for several subjects the CHMI was delayed (Figure 1 and 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. TBS 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 retrospectvely, unless to confirm a positive TBS, in which case they were run within 24 hours.

Adverse events (AE): Solicited local (Table S3) AEs were collected for 3 days after each immunization. Solicited systemic (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 chloroqine administration (2 days prior to the first immunization) through 12 days after final immunization and 7 days after final chloroquine dose (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 clinic visits. Any subject who reported AEs at home was referred to the clinic. Grading of severity of AEs and relatedness to IP were done according to a pre-specified system (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 pre-specified (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 prior to and 7 days after the TBS was first positive.

Treatment: *Plasmodium* sp. infections diagnosed in subjects prior to CHMI were treated according to national guidelines with artesunate-amodiaquine (AS-AQ) or artemether-lumefantrine (AL). 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 day 22, 24, 26 and 28. TBSs 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 4 parasites/µL blood when ~0.5 µL of blood was assessed.

Parasites were quantified by qPCR using the PlasQ qPCR assay as described[30]. The lower limit of detection for this qPCR assay was 50 copies/mL.

A single positive timepoint was considered positive for infection with Pf. After start of CHMI, the time of first blood sample positivity by qPCR was used to determine infection status

and calculation of prepatent period. During CHMI all samples were analysed by qPCR in real time as they were continuously collected from the subjects . Samples collected during the immunization period were analysed 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[30]. Samples positive for Pf were genotyped by assessing polymorphisms for msp1/msp2[31] and selected microsatellite markers[32]. Additionally, two widespread markers of sulphadoxine-pyrimethamine (SP), dhfr and dhps, were amplified and sequenced[33].

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 day of first administration of IP, which was 2 days after administration of the loading dose of chloroquine (600 mg base) were analyzed in blinded fashion for chloroquine. using high performance liquid chromatography (HPLC). The samples were run in 2 lots, corresponding to the 2 different cohorts enrolled in the PfSPZ-CVac arm of the trial. For the second cohort, plasma samples from the same time point and stored at -80°C were also analyzed by Swiss BioQuant AG, Reinach, Switzerland, using HPLC.

Antibody assays: Blood for immunogenicity testing was drawn prior to the first immunization, 2 weeks after 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) 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 than a 99% Pf infection frequency in controls (α=0.05, 2-tailed), with allowance for loss of up to 2 vaccinees and 1 control. Categorical variables were summarized using absolute (n) and relative (%) frequencies. Continuous variables were summarized using mean and standard deviation, 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 2-sided test was used. Time to event was assessed by Kaplan Meier curves and logrank for trend. Time to event data were analyzed from CHMI injection until positive qPCR result. No corrections were made for multiple comparisons due to the early phase nature of this trial. A p value <0.05 was considered significant.

RESULTS

Vaccine Efficacy (VE):

Normal Saline (NS) Controls: Four of 11 subjects did not undergo CHMI (Figure 1). Two subjects developed malaria prior to 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.

233 *PfSPZ Vaccine*: Three of 20 subjects did not undergo CHMI. One subject became pregnant
234 during immunization, one did not respond to initial treatment for incidental *Loa loa* infection,
235 and one withdrew. Seventeen subjects immunized with $2.7x10^6$ PfSPZ of PfSPZ Vaccine
236 underwent CHMI. One subject was excluded from analysis after he was retrospectively
237 determined to be qPCR-positive for Pf on day of CHMI. Fifteen PfSPZ Vaccine subjects
238 underwent CHMI 14-16 (median 14) weeks after last vaccine dose. Eight were positive by both
239 TBS and qPCR, 2 were negative by TBS but positive by qPCR, and 5 were negative by both
240 TBS and qPCR. The 16^{th} subject immunized with PfSPZ Vaccine underwent first CHMI at 33
241 weeks after last immunization and was negative by both TBS and qPCR (Table 1). VE at a
242 median of 14 weeks after last dose of vaccine was 27% (6 of 16 vaccinees vs. 1 of 7 controls
243 negative by qPCR, p=0.32, Barnard's test, 2-tailed).
244 *PfSPZ-CVac*: Five of 19 subjects did not undergo CHMI. One subject developed *L. loa*
245 infection during immunization and four were positive for Pf by qPCR prior to CHMI. Fourteen
246 subjects immunized with PfSPZ-CVac underwent CHMI. One subject was excluded from
247 analysis because he was retrospectively determined to be qPCR-positive for Pf on day of CHMI.
248 Thirteen PfSPZ-CVac subjects underwent CHMI 14-19 (median, 15) weeks after last vaccine
249 dose. Three were positive by both TBS and qPCR, 2 were negative by TBS but positive by
250 qPCR, and 8 were negative by both TBS and PCR (Table 1). VE at a median of 15 weeks after
251 last vaccine dose was 55% (8 of 13 vaccinees negative vs. 1 of 7 controls negative by qPCR,
252 p=0.051). By time-to-event analysis (Figure 2) there was a significant trend towards improved
253 VE (p=0.033, logrank test for trend) from saline controls to PfSPZ Vaccine to PfSPZ-CVac. The
254 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 as several subjects were positive on the first qPCR measurement taken at day 8 after CHMI and may have been positive earlier. By TBS, the prepatent periods were significantly longer for PfSPZ Vaccine compared with 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 3rd immunization and the day prior to CHMI, which was 98 to 231 days after last immunization. We also assessed pre-CHMI sera for antibodies to the late liver stage/asexual erythrocytic stage protein Pf merozoite surface protein 1 (PfMSP1) by ELISA (Figures 3 and S2; Table S5).

Antibodies to PfCSP:

PfSPZ Vaccine vs. PfSPZ-CVac vs. NS placebo: Antibody responses to PfCSP 2 weeks after the 3rd dose (Fig. 3A and S2A, Table S5) were significantly higher in the PfSPZ Vaccine group (median net OD 1.0 = 2936 and median OD 1.0 ratio = 40.35) than 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, 2 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=.0003). Antibody responses to PfCSP the day prior to CHMI (Fig. 3B and S2B) were significantly higher in the PfSPZ Vaccine group (median net OD 1.0 = 1407 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=.0002 and ratio p<0.0001).

Uninfected vs. infected 2 weeks after 3rd dose: There was no significant difference in antibody levels 14 days after the 3rd dose between subjects who received PfSPZ Vaccine and did not become infected, versus those who became infected (median net OD 1.0 4268 vs 2600, p=0.180 and median OD 1.0 ratio 67.57 vs 40.35, p=0.591) (Fig. 3C and S2C). Likewise, there was no significant difference in antibody levels 14 days after the 3rd dose between subjects who received PfSPZ-CVac who were not infected, versus those who became infected (median net OD 411 vs 258, p=0.833 and median OD 1.0 ratio 3.76 vs 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 vs 411, p=0.0012 and median OD 1.0 ratio 40.35 vs 3.76, p=0.0008).

Uninfected vs. infected prior to CHMI: There was a significant difference in net OD 1.0 antibody levels prior to CHMI between subjects who received PfSPZ Vaccine who were uninfected versus those who were infected (median net OD 1.0 2936 vs 1012, p=0.031) (Fig. 3D and 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 vs 30.97, p=0.219). In subjects who received PfSPZ-CVac who were uninfected or infected the difference the net OD 1.0 and OD 1.0 ratios were higher in the uninfected, but not significantly (median net OD 618 vs 293, p=0.126, and median OD 1.0 ratio 6.04 vs 2.49, p=0.247).

<u>Antibodies to PfMSP1:</u>

Uninfected vs. infected prior to CHMI: In subjects who received PfSPZ Vaccine who were uninfected the PfMSP1 median OD 1.0 measured prior to CHMI was higher than that of infected subjects (median OD 1.0 = 899 vs 55), but not significantly (p=0.515) (Fig. S2E, Table S5). Uninfected subjects who received PfSPZ-CVac also had higher antibodies to PfMSP-1 prior to CHMI than the infected subjects (median OD 1.0 = 3000 vs 423), but the difference was not significant (p=0.178).

Safety:

Solicited adverse events 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 9 of 20 subjects receiving $2.7x10^6$ PfSPZ of PfSPZ Vaccine) were associated with 17 systemic solicited AEs (5 reports of fatigue and 3 each of arthralgias, headache, myalgias and subjective fever) compared with one of 18 injections (1 report of arthralgias) in the 6 subjects receiving NS (p=0.215, Barnard's test 2-tailed). One event (headache in a vaccine recipient) was grade 2, all others were grade 1. All events were considered related to IP (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 6 local solicited AEs, all grade 2 or less; 5 of 14 injections (in 4 of 5 subjects receiving NS with chloroquine) experienced 10 systemic solicited AEs and no local solicited AEs (p=0.822) (Table S6). Seven systemic solicited AEs in 5 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) compared with 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 days 7 to 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 (5 of 14 injections) than the NS controls (1 of 18 injections, p=0.028). Pruritis, a frequently cited adverse effect of chloroquine in African populations, was only noted in 2 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 (Table S7). All laboratory abnormalities were grade 2 or less. Likewise, there were not differences vaccinees and controls in the PfSPZ-CVac arms, except that significantly more subjects receiving PfSPZ-CVac experienced grade 1

or 2 neutropenia compared with controls (p=0.0089) (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 3rd dose, with all prior and subsequent sample results in the normal range.

Unsolicited adverse events following immunization:

Nine unsolicited AEs were reported in 8 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 5 AEs reported in 3 of the 5 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.

Serious adverse events:

Three serious adverse events occurred in 3 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 3rd pregnancy and 9 weeks after her first and only dose of PfSPZ Vaccine. Pregnancy loss before 20 weeks approaches 20% in sub-Saharan Africa [34], however the temporal relationship to immunization led the team to consider the event possibly related to vaccine.

A 29-year-old male in the chloroquine + placebo arm of the PfSPZ-CVac group developed non-Hodgkin's lymphoma. This SAE was considered unrelated to immunization; the details of this case are reported elsewhere (Manock S et al, manuscript in preparation).

Plasmodium parasitemia during PfSPZ-CVac immunizations and prior to CHMI:

Six to 10 days after each immunization, 17/18 subjects who received 3 doses of PfSPZ-CVac developed parasitemia by qPCR after the first dose; 13/18 after the second and 3rd 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).

During the immunization phase 6 subjects had asymptomatic parasitemia outside the 6-10 day window during scheduled PCR sampling - 2 subjects each receiving PfSPZ Vacccine, PfSPZ-CVac and NS control (Table S8). Genotype analysis excluded the PfNF54 strain identified as the cause of parasitemia in 3 subjects and suggested this was not the etiology in 2 additional subjects (Table S9). Four additional subjects in the PfSPZ-CVac arm (3 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. CHMI was well tolerated (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

post administration of PfSPZ Challenge. Nine unsolicited AEs were reported in 8 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 the IC_{50} for the NF54 strain to chloroquine (8.7 ng/mL)[35].

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 (Table S10). Eight of the 9 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; 2 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 3-dose PfSPZ-CVac regimen of $1.0x10^5$ PfSPZ/dose had a VE of 55% (8 of 13, p=0.051), whereas, a 3-dose PfSPZ Vaccine regimen of $2.7x10^6$ PfSPZ/dose had a VE of 27% (5 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 pre-patent 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 pre-patent 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 replicate up to $5x10^4$ times in hepatocytes of the vaccinees and express ~3,000 proteins not expressed by the non-replicating PfSPZ of PfSPZ Vaccine[13]. Three doses of $9x10^5$ PfSPZ of PfSPZ Vaccine protected 72% of vaccinees against homolgous CHMI at 9.5 weeks after last dose in Germany (personal communication, B. Mordmüller) and 64% of vaccinees in the U.S. against homologous CHMI at ~19 weeks after last vaccine dose [12], and three doses of $5x10^4$ PfSPZ of PfSPZ-CVac protected 100% of vaccinees in Germany against homologous CHMI at 10 weeks after last dose[13]. Thus, the better VE with a much lower dose of PfSPZ-CVac in our trial was expected.

Data from mice and non-human primates indicate the VE of radiation-attenuated and chemo-attenuated PfSPZ is dependent on CD8+ T cells; antibodies alone are not adequate[7, 36-38]. 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 time of CHMI uninfected PfSPZ Vaccine vaccinees (2936) had 2.9 times higher levels of antibodies to PfCSP than did infected vaccinees (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 adverse events 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 3rd doses and the intervals between the third dose and CHMI. Naturally acquired, asymptomatic malaria was discovered in some of the subjects prior to 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 7 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 non-immune control subjects in the US and Europe (73/73) [13, 26, 27, 29, 39, 40] and Tanzania (34/34) [17, 18] have been infected with this dose, in other settings in Africa, including Gabon (20/25) [27], the 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 $9.0x10^5$ 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 $1.8x10^6$ PfSPZ administered at 8-week intervals, VE against CHMI conducted 7 weeks after the third

462 immunization was reduced to 33%, suggesting doses can be too high[18]. When the EGSPZV2 trial
463 was designed, it was thought that higher doses would be better – thus the choice of $2.7x10^6$
464 PfSPZ per dose. The resulting VE of 27%, is consistent with the findings in Tanzania, increasing
465 the dose beyond $9.0x10^5$ apparently reduces VE against CHMI. Thus, in our next studies we plan
466 to immunize with $9.0x10^5$ PfSPZ.
467 The dose of PfSPZ-CVac, $1.0x10^5$ PfSPZ, was two times higher than the dose that achieved
468 100% VE in malaria-naïve adults in Tubingen, Germany[13]. A regimen of 3 doses of $2.0x10^5$
469 PfSPZ at one month intervals was tested in Mali for protection against naturally transmitted
470 malaria and gave sub-optimal results (Thera and Laurens, unpublished). The results indicated
471 that higher doses of PfSPZ-CVac are needed in Africa due to naturally acquired immunity and
472 an associated immune hypo-responsiveness to malaria immunogens. Immune hypo-
473 responsiveness could also explain the limited decrease in parasitemia seen after each successive
474 immunization in this study, unlike PfSPZ-CVac trials in malaria-naïve adults, where a
475 consistent decrease in mean parasitemia is seen in all subjects with each successive dose
476 [41]Mordmuller, 2017 #10153}. A subsequent study in Mali assessing the VE of PfSPZ-CVac
477 against naturally transmitted malaria is using 3 doses of $4.0x10^5$ PfSPZ (NCT03952650).
478 For subjects immunized with radiation-attenuated PfSPZ Vaccine there were no significant
479 differences in the number of solicited systemic AEs between PfSPZ Vaccine and NS recipients.
480 Likewise, there were no significant difference between vaccinees and controls in the first 5 days
481 after each dose of PfSPZ-CVac. However, solicited AEs were more frequent in vaccinees
482 receiving PfSPZ-CVac compared with controls during days 7-13 after each immunization
483 ($p=0.073$), which we attribute to symptoms related to parasitemia in the 6 to 12 days after
484 immunization. Additionally , AEs attributed to chloroquine accounted for 1/3 of the AEs

485 reported in vaccinees and controls in the PfSPZ-CVac group and account for the differences in
486 the numbers of adverse events beteen the NS + chlorquine control group compared with the NS
487 control group. Despite parasitemia associated AEs occurring exclusively in vaccinees, there was
488 no significant difference in the number of solicited systemic AEs between those receiving
489 PfSPZ-CVac (PfSPZ Challenge + chloroquine) and those receiving NS and chloroquine. It was
490 noteworthy that only 2 of 19 individuals experienced pruritus during 9 weeks of chloroquine
491 administration; in both cases it was mild, resolved spontaneously, and did not reoccur despite
492 ongoing chloroquine dosing.
493 In summary, as part of an ongoing effort to optimize the dosage regimens for PfSPZ Vaccine
494 and PfSPZ-CVac, we conducted a trial comparing a single dosage regimen of each. PfSPZ-CVac
495 provided higher VE than did PfSPZ Vaccine at a much lower dose. However, neither regimen
496 was optimal. Higher doses of PfSPZ-CVac and lower doses of PfSPZ Vaccine will be assessed
497 next.
498

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Figure legends

Figure 1. CONSORT Diagram – Adult subjects ages 18 to 35 years

Figure 2. Kaplan-Meier survival curves in vaccinees and controls as assessed by qPCR. Kaplan-Meier curves in subjects undergoing CHMI after the last of 3 doses with $2.7x10^6$ PfSPZ (n=16) or $1.0x10^5$ PfSPZ Challenge (n=13) vs. pooled saline (n=5) and oral chloroquine plus saline controls (n=2). The median time from last dose to CHMI was 14 weeks for PfSPZ Vaccine and 15 weeks for PfSPZ-CVac.

Figure 3. Antibodies to PfCSP ELISA. IgG antibodies to Pf circumsporozoite protein PfCSP by ELISA two weeks after the 3rd dose (panel A) and at the time of CHMI (panel B) comparing PfSPZ Vaccine and PfSPZ-CVac. IgG antibodies to Pf circumsporozoite protein PfCSP by ELISA two weeks after the 3rd dose (panel C) and at the time of CHMI (panel D) comparing infected and uninfected subjects in PfSPZ Vaccine and PfSPZ-CVac. Filled circles (●) represent subjects remaining uninfected after CHMI; open circles (○) 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 (41.18%) subjects seroconverted (p=0.00012) when measured 2 weeks after the 3rd dose. When PfCSP antibodies were measured before CHMI 15/16 (93.75%) had positive antibody response in the PfSPZ Vaccine group and 8/13 (61.54%) in the PfSPZ-CVac group (p=0.038) (Table S5).

Antibody responses to PfCSP 2 weeks after the 3rd dose (panel A) were significantly higher in the PfSPZ Vaccine group (median net OD 1.0 = 2936) than in the PfSPZ-CVac group (median

net OD 1.0 = 258) ($p<0.0001$, Wilcoxon signed-rank test, 2 tailed). The PfSPZ-CVac group had higher antibody levels than normal saline controls 2 weeks after 3rd dose (median net OD 1.0 = 1) (net $p<0.0001$, Wilcoxon signed-rank test, 2 tailed). Antibody responses to PfCSP the day prior to CHMI (panel B) were significantly higher in the PfSPZ Vaccine group (median net OD 1.0 = 1407) than in the PfSPZ-CVac group (median net OD 1.0 = 520) (net $p=0.0093$, Mann-Whitney test, 2 tailed). The PfSPZ-CVac group had higher antibody levels than normal saline controls prior to CHMI (panel B, median net OD 1.0 = 26, $p=.0002$, Wilcoxon signed-rank test, 2 tailed).

Median net OD 1.0 of PfCSP antibodies measured 2 weeks after the 3rd dose (panel C) in the PfSPZ Vaccine group were higher in uninfected vs that in infected subjects (median net OD 1.0 4268 vs 2600, $p=0.180$, Wilcoxon signed-rank test, 2 tailed), but the difference was not significant. Likewise, there was no significant difference in antibody levels 2 weeks after the 3rd dose between subjects who received PfSPZ-CVac who were not infected, versus 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 prior to CHMI (panel GD) between subjects who received PfSPZ Vaccine who were uninfected versus those who were infected (median net OD 1.0 2936 vs 1012, $p=0.031$, Wilcoxon signed-rank test, 2 tailed). Net OD 1.0 anti-PfCSP antibody levels prior to 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.

Figure 4. Solicited Systemic Adverse Events as a Percentage of Doses Administered. For subjects in the PfSPZ Vaccine arm, solicited AE are reported for 6 days after each immunization. In the PfSPZ-CVac arm, solicited AE are collected at the time of first chloroquine administration

748 (2 days prior to the first immunization) and continue for 12 days after the 3rd immunization (70
749 days total). These AE are further categorized into predetermined intervals (days 1-6, 7-13, and
750 beyond day 13) following each immunization (#1, #2 or #3) for PfSPZ-CVac. Days 1-6 assessed
751 reactions to vaccination while days 7-13 assessed the impact of Pf parasitemia in PfSPZ-CVac
752 recipients. The period after day 13 was to assess the additional impact of continued chloroquine
753 administration. There was no significant difference in AEs between PfSPZ Vaccine and NS
754 (p=0.215), between PfSPZ-CVac and NS+chloroquine (p=0.822) or between PfSPZ Vaccine and
755 PfSPZ-CVac on days 1-6 (p=0.676) or overall (p=0.128). On days 7-13 more AE occurred in the
756 subjects receiving PfSPZ-CVac than subjects receiving NS + chloroquine (p=0.073, Barnard's
757 test, 2-sided).
758 **Figure 5. Parasitemia by qPCR after first, second and 3rd PfSPZ-CVac immunizations.**
759 Panel A shows the transient parasitemia with each successive dose of PfSPZ Challenge in
760 vaccinees protected after CHMI. Panel B shows the transient parasitemia with each successive
761 dose of PfSPZ Challenge in vaccinees not protected after CHMI. Panel C outlines median Pf
762 parasitemia overall and in subjects protected and not protected during CHMI. Only subjects
763 completing all 3 doses and participating in CHMI (n=13) represented. One subject (#525, solid
764 black line in panel A) was persistently qPCR positive on day 10 after the 2nd and 3rd
765 immunizations and subsequently found to have naturally acquired Pf infection. Two additional
766 subjects positive 10 days after the 3rd (panel A) or 1st (panel B) dose were qPCR negative at the
767 next measurement 18 days later.
768
769
770

Table 1. Vaccine efficacy (VE) 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	Vaccine Efficacy (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	--

* One subject was excluded from analysis in the PfSPZ Vaccine arm and one was excluded in the 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, 2-tailed.

Table 2. Prepatent periods by qPCR and thick blood smear

	PfSPZ Vaccine	PfSPZ-CVac	Controls
CHMI (n), evaluable	16	13	7
qPCR + (n)	10	5	6
Prepatent period, qPCR (days)			
median	11.0	8.0	8.5
min, max	8, 18	8, 16	8, 15
p (vs. control)	p=0.21	p=0.84	--
TBS+ (n)	8	3	4
Prepatent period, TBS (days)			
median	17	14	14.5
min, max	15, 19	14, 26	13, 16
p (vs. control)	p=0.02	p=0.89	--

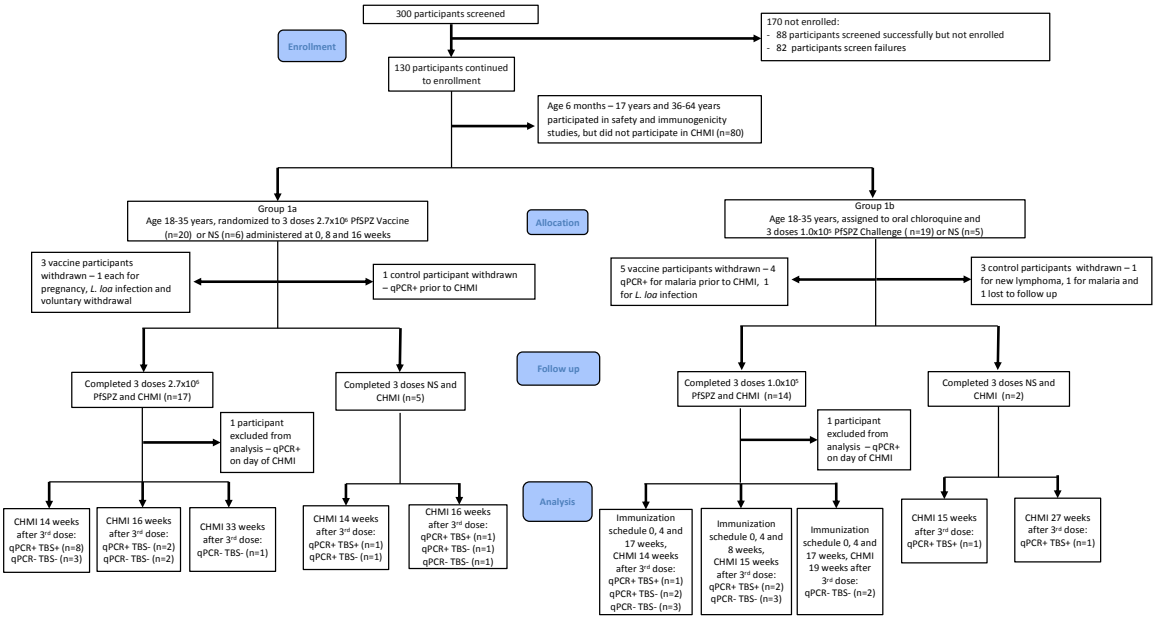

300 participants screened
Enrollment
170 not enrolled:
- 88 participants screened successfully but not enrolled
- 82 participants screen failures
130 participants continued to enrollment
Age 6 months – 17 years and 36-64 years participated in safety and immunogenicity studies, but did not participate in CHMI (n=80)
Group 1a
Age 18-35 years, randomized to 3 doses 2.7x10⁶ PfSPZ Vaccine (n=20) or NS (n=6) administered at 0, 8 and 16 weeks
Allocation
Group 1b
Age 18-35 years, assigned to oral chloroquine and 3 doses 1.0x10⁵ PfSPZ Challenge (n=19) or NS (n=5)
3 vaccine participants withdrawn – 1 each for pregnancy, L. loa infection and voluntary withdrawal
1 control participant withdrawn – qPCR+ prior to CHMI
5 vaccine participants withdrawn – 4 qPCR+ for malaria prior to CHMI, 1 for L. loa infection
3 control participants withdrawn – 1 for new lymphoma, 1 for malaria and 1 lost to follow up
Follow up
Completed 3 doses 2.7x10⁶ PfSPZ and CHMI (n=17)
Completed 3 doses NS and CHMI (n=5)
Completed 3 doses 1.0x10⁵ PfSPZ and CHMI (n=14)
Completed 3 doses NS and CHMI (n=2)
1 participant excluded from analysis – qPCR+ on day of CHMI
1 participant excluded from analysis – qPCR+ on day of CHMI
Analysis
CHMI 14 weeks after 3rd dose: qPCR+ TBS+ (n=8) qPCR- TBS- (n=3)
CHMI 16 weeks after 3rd dose: qPCR+ TBS- (n=2) qPCR- TBS- (n=2)
CHMI 33 weeks after 3rd dose: qPCR- TBS- (n=1)
CHMI 14 weeks after 3rd dose: qPCR+ TBS+ (n=1) qPCR+ TBS- (n=1)
CHMI 16 weeks after 3rd dose: qPCR+ TBS+ (n=1) qPCR+ TBS- (n=1) qPCR- TBS- (n=1)
Immunization schedule 0, 4 and 17 weeks, CHMI 14 weeks after 3rd dose: qPCR+ TBS+ (n=1) qPCR+ TBS- (n=2) qPCR- TBS- (n=3)
Immunization schedule 0, 4 and 8 weeks, CHMI 15 weeks after 3rd dose: qPCR+ TBS+ (n=2) qPCR- TBS- (n=3)
Immunization schedule 0, 4 and 17 weeks, CHMI 19 weeks after 3rd dose: qPCR- TBS- (n=2)
CHMI 15 weeks after 3rd dose: qPCR+ TBS+ (n=1)
CHMI 27 weeks after 3rd dose: qPCR+ TBS+ (n=1)

Figure 1.

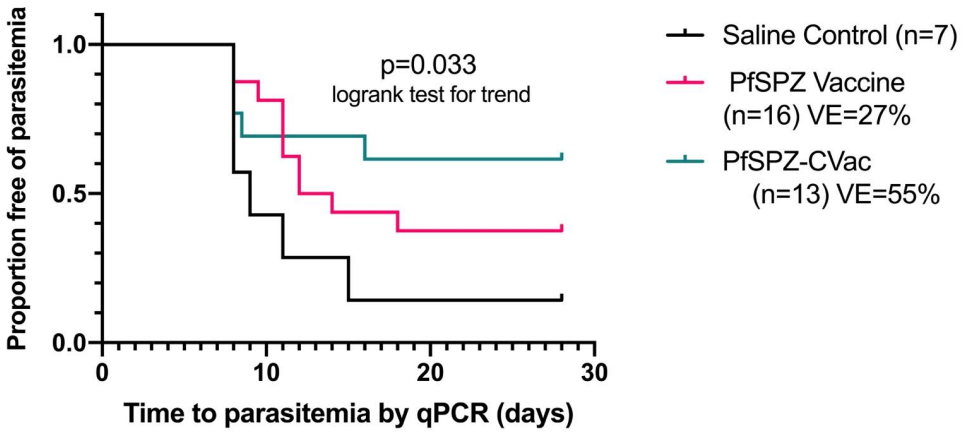
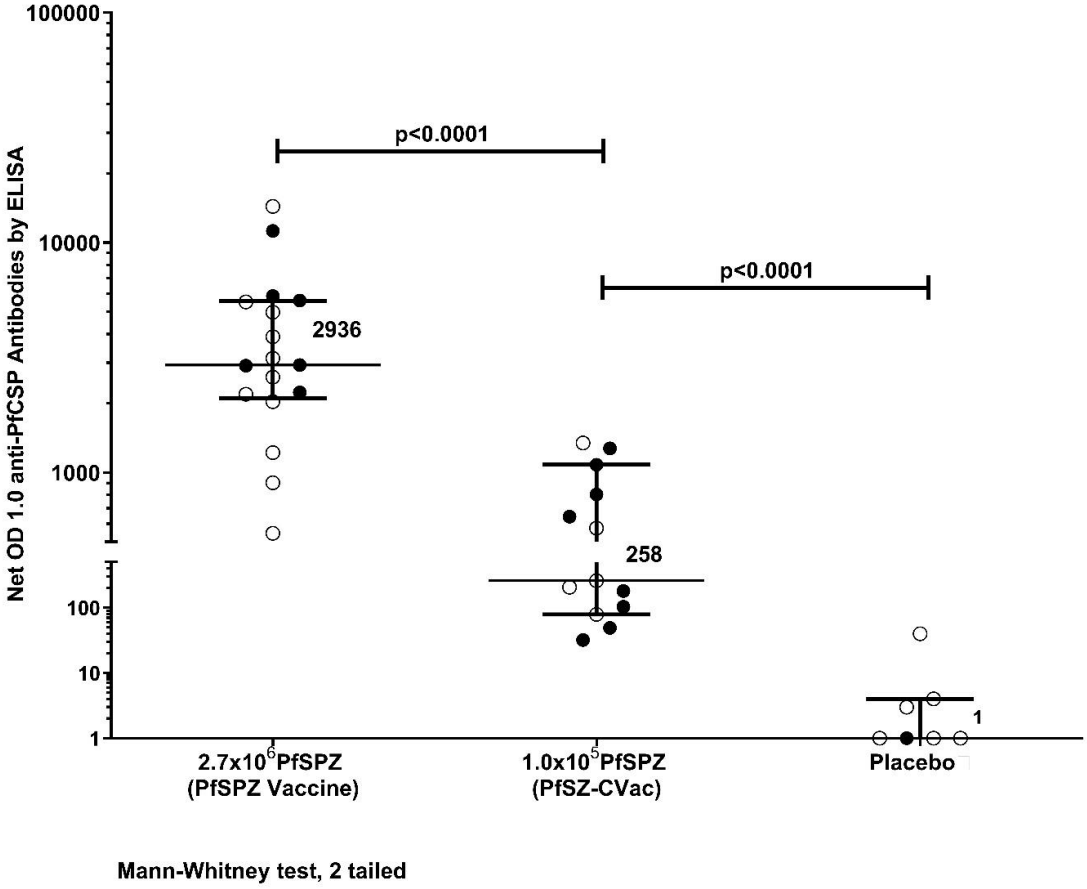

Proportion free of parasitemia
1.0
0.5
0.0
0
10
20
30
Time to parasitemia by qPCR (days)
p=0.033
logrank test for trend
Saline Control (n=7)
PfSPZ Vaccine
(n=16) VE=27%
PfSPZ-CVac
(n=13) VE=55%

Figure 2.

A. 2 weeks after 3rd dose



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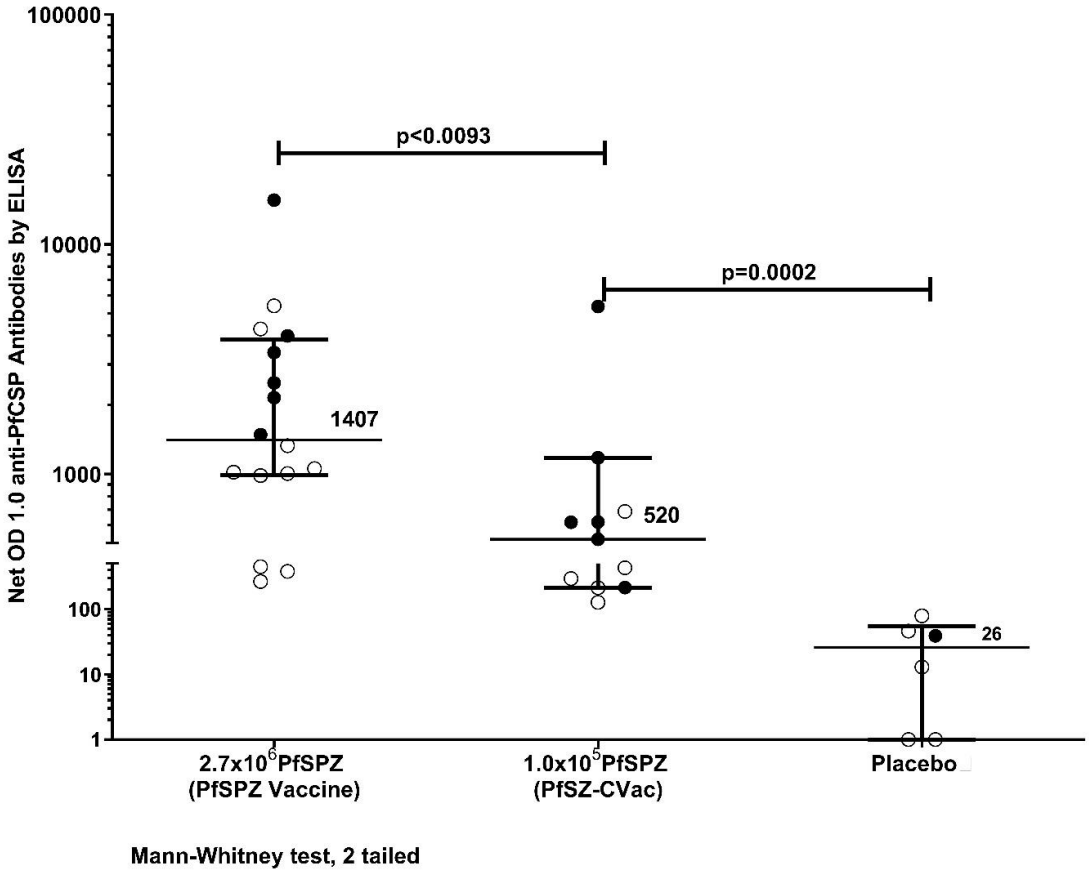
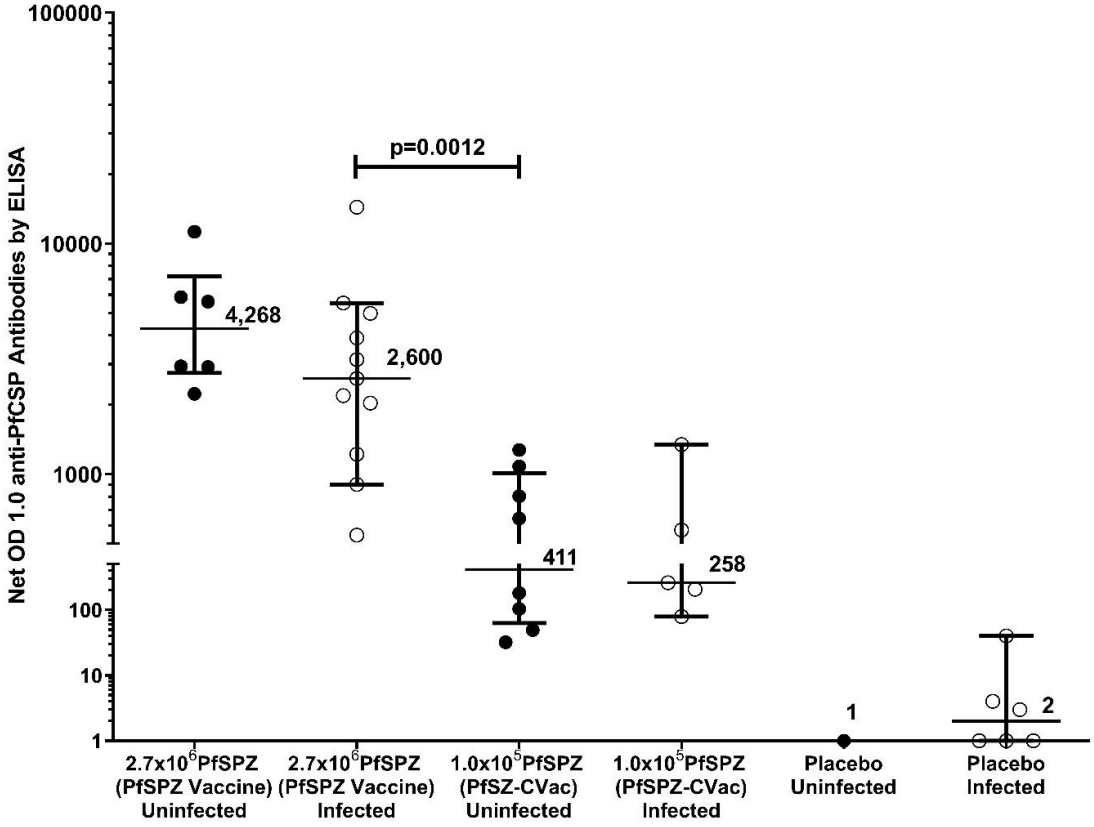
870
871 **B. Pre-CHMI**



872
873

C. 2 weeks after 3rd dose



D. Pre-CHMI

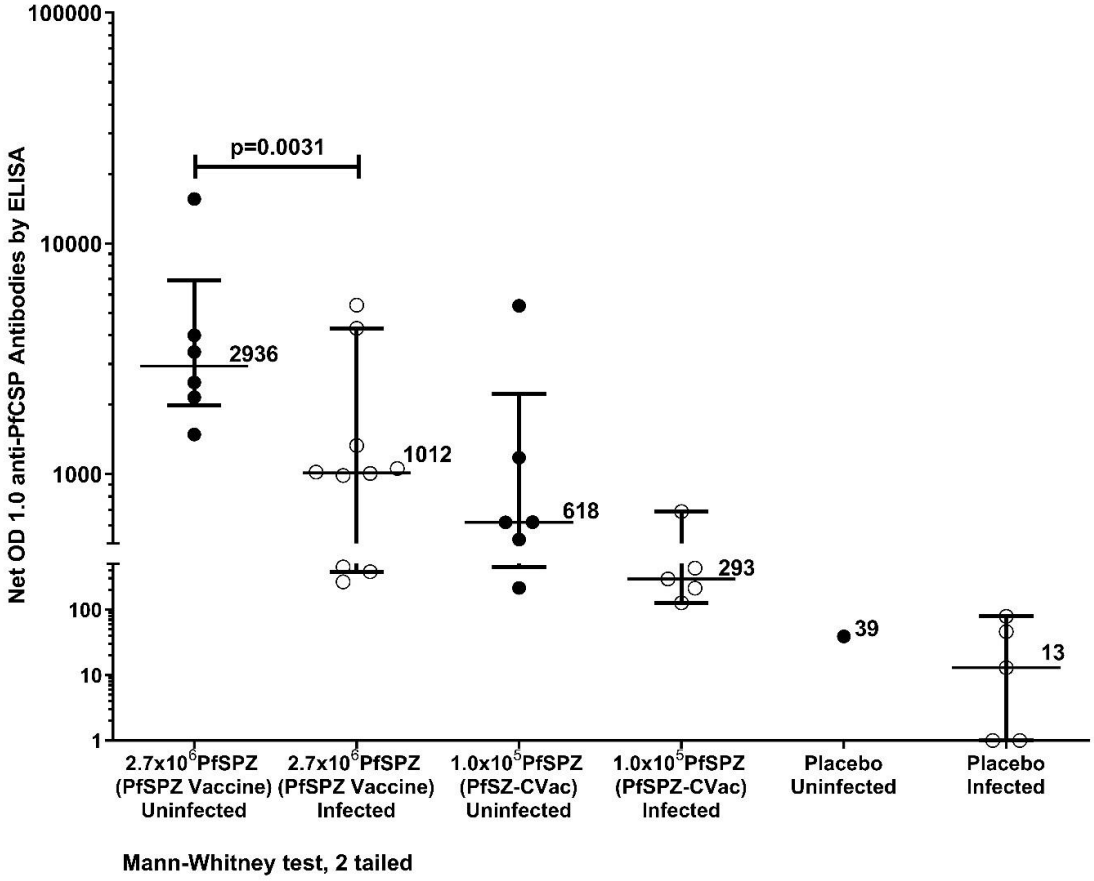


Figure 3.

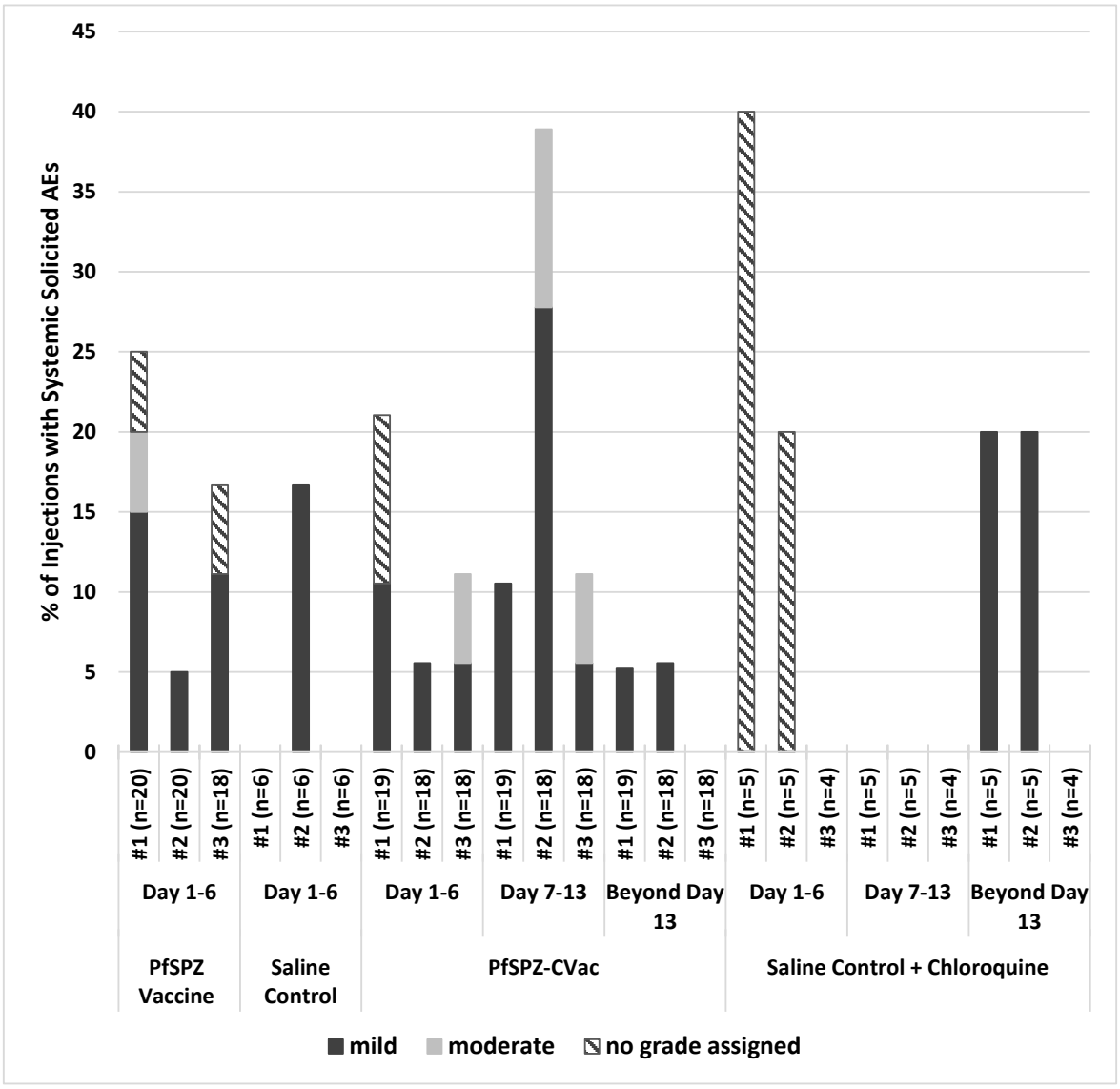
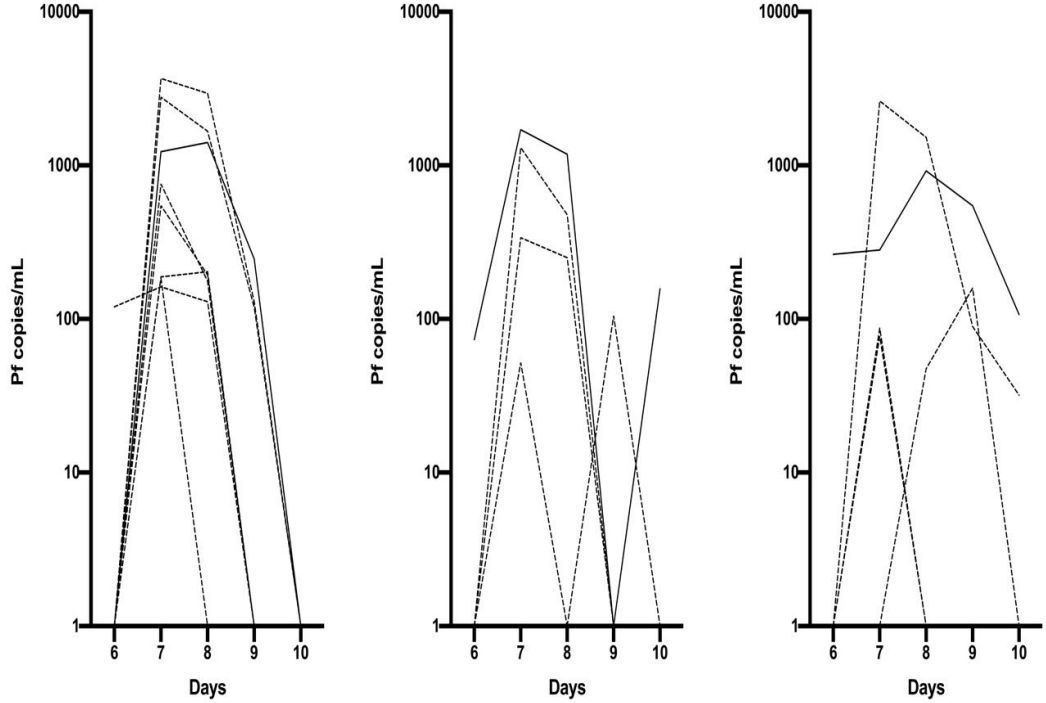
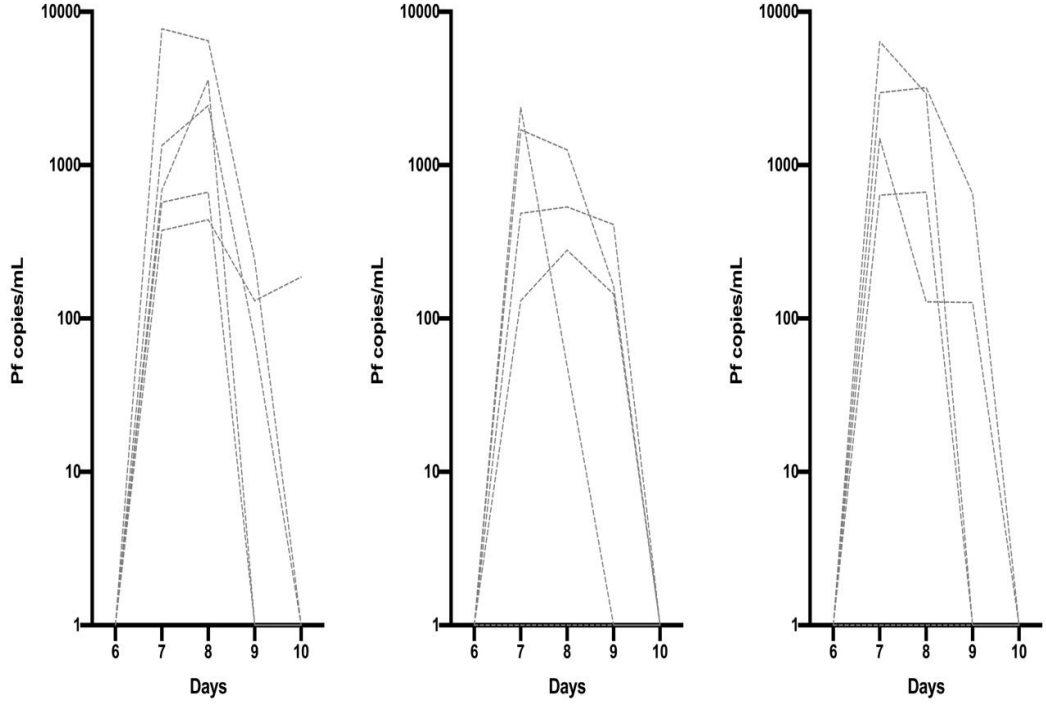

% of Injections with Systemic Solicited AEs
45
40
35
30
25
20
15
10
5
0
#1 (n=20)
#2 (n=20)
#3 (n=18)
#1 (n=6)
#2 (n=6)
#3 (n=6)
#1 (n=19)
#2 (n=18)
#3 (n=18)
#1 (n=19)
#2 (n=18)
#3 (n=18)
#1 (n=19)
#2 (n=18)
#3 (n=18)
#1 (n=5)
#2 (n=5)
#3 (n=4)
#1 (n=5)
#2 (n=5)
#3 (n=4)
#1 (n=5)
#2 (n=5)
#3 (n=4)
Day 1-6
Day 1-6
Day 1-6
Day 7-13
Beyond Day 13
Day 1-6
Day 7-13
Beyond Day 13
PfSPZ Vaccine
Saline Control
PfSPZ-CVac
Saline Control + Chloroquine
mild
moderate
no grade assigned

Figure 4.

A.


10000
1000
100
10
1
Pf copies/mL
6
7
8
9
10
Days

B.


10000
1000
100
10
1
Pf copies/mL
6
7
8
9
10
Days

C.

Group	Immunization #1 (V1)			Immunization #2 (V2)			Immunization #3 (V3		
	No. pos (%)	**Median Pf copies/ mL (range)**	**Median days to peak parasitemia (range)**	**No. pos (%)**	**Median Pf copies/ mL (range)**	**Median days to peak parasitemia (range)**	**No. pos (%)**	**Median Pf copies/ mL (range)**	**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, Mann-Whitney test

Figure 5.

SUPPLEMENTAL DATA

Figure legends

Figure S1. Proposed study schedule and schedule as executed. This trial was originally designed to enroll 26 subjects into the PfSPZ Vaccine arm and 26 subjects into the PfSPZ-CVac arm and randomize within each arm 20 subjects to vaccine and 6 subjects to control. Immunizations in the PfSPZ-Vaccine arm were scheduled for 1, 9 and 17 weeks and PfSPZ-CVac for 9, 13 and 17 weeks so that both groups could undergo CHMI at the same time (10±1 weeks after the 3rd dose). Due to challenges in recruitment the PfSPZ-CVac arm was broken into 2 cohorts, the first of which began immunizations on schedule with the second cohort delayed by 5 weeks. An unanticipated safety hold to evaluate a SAE led to additional delay with the 3rd dose for the second cohort administer 13 weeks after the second dose instead of 4 weeks. For these subjects CHMI was delayed to allow a minimum of 10 weeks between the 3rd dose and CHMI. A few subjects encountered additional dealys due to intercurrent malaria infections from natural exposure.

Figure S2. Antibodies to PfCSP and MSP-1 by ELISA. IgG antibodies to Pf circumsporozoite protein PfCSP by ELISA two weeks after the 3rd dose (panel A) and at the time of CHMI (panel B) comparing PfSPZ Vaccine and PfSPZ-CVac are presented as OD 1.0 ratios and correspond to the net OD 1.0 values presented in Figure 3 panels A and B. IgG antibodies to Pf circumsporozoite protein PfCSP by ELISA two weeks after the 3rd dose (panel C) and at the time of CHMI (panel D) comparing infected and uninfected subjects in PfSPZ Vaccine and PfSPZ-CVac are presented as OD 1.0 ratios and correspond to the net OD 1.0 values presented in Figure 3 panels C and D. IgG antibodies to Pf merozoite surface protein-1 PfPfMSP-1 by ELISA

926 measured at the time of CHMI (panel E) comparing PfSPZ Vaccine and PfSPZ-CVac. Filled
927 circles (●) represent subjects remaining uninfected after CHMI; open circles (○) represent
928 subjects infected after CHMI.
929
930 Antibody responses to PfCSP 2 weeks after the 3rd dose (panel A) were significantly higher in
931 the PfSPZ Vaccine group (median OD 1.0 ratio = 38.70) than in the PfSPZ-CVac group (median
932 OD 1.0 ratio = 2.48) (p=0.0043, Wilcoxon signed-rank test, 2 tailed). The PfSPZ-CVac group
933 had higher antibody levels than normal saline controls 2 weeks after 3rd dose (median OD 1.0
934 ratio = 1.02) (p<0.0001, Wilcoxon signed-rank test, 2 tailed). Antibody responses to PfCSP the
935 day prior to CHMI (panel B) were significantly higher in the PfSPZ Vaccine group (median OD
936 1.0 ratio 43.84) than in the PfSPZ-CVac group (OD 1.0 ratio 4.10) (p<0.0001, Mann-Whitney
937 test, 2 tailed). The PfSPZ-CVac group had higher antibody levels than normal saline controls
938 prior to CHMI (median OD 1.0 ratio = 1.27, p<0.0001, Wilcoxon signed-rank test, 2 tailed).
939
940 Median OD 1.0 ratio of PfCSP antibodies measured 2 weeks after the 3rd dose (panel C) in the
941 PfSPZ Vaccine group were higher in uninfected vs that in infected subjects (median OD 1.0 ratio
942 67.57 vs 40.35, p=0.59, Wilcoxon signed-rank test, 2 tailed), but the difference was not
943 significant. Likewise, there was no significant difference in antibody levels 2 weeks after the 3rd
944 dose between subjects who received PfSPZ-CVac who were not infected, versus those who
945 became infected (median OD 1.0 ratio 3.76 vs 4.90, p=0.93).
946
947 Prior to CHMI (panel D) the uninfected PfSPZ Vaccine group also had a higher median OD 1.0
948 ratio, but this did not reach the level of statistical significance (median OD 1.0 ratio 61.28 vs

949 20.11, p=0.15, Wilcoxon signed-rank test, 2 tailed). In subjects who received PfSPZ-CVac who
950 were uninfected or infected the median OD 1.0 ratios was higher, but not significantly (6.04 vs
951 3.49, p=0.35).
952
953 In subjects who received PfSPZ Vaccine who were uninfected the PfMSP-1 (panel E) median
954 OD 1.0 measured prior to CHMI was higher than that of infected subjects (median OD 1.0 = 889
955 vs 62), but not significantly (p=0.406) (Table S5). Subjects who received PfSPZ-CVac and were
956 uninfected also had higher antibodies to PfMSP-1 prior to CHMI than the infected subjects
957 (median OD 1.0 = 1518 vs 605), but the difference was not significant (p=0.880) (Table S5).
958
959
960

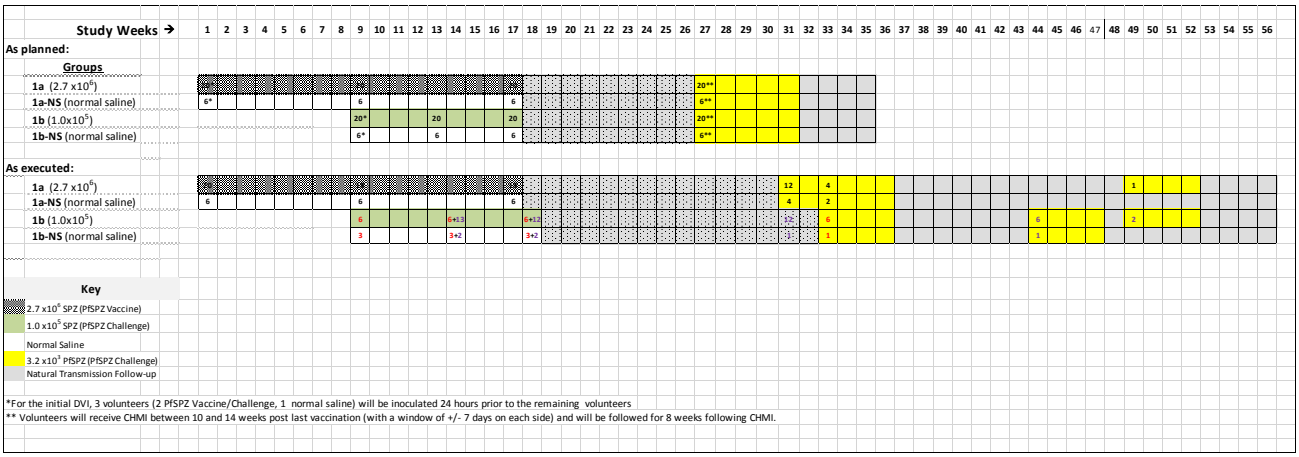
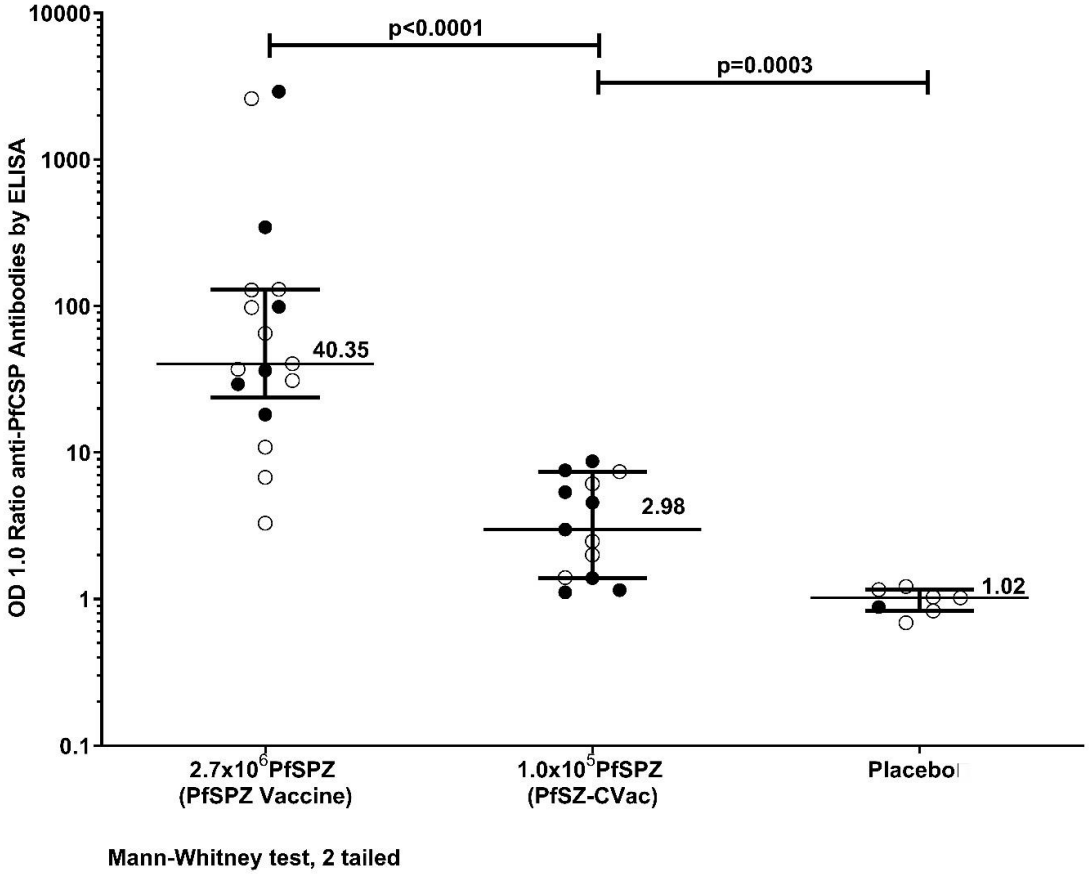

Study Weeks →
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56
As planned:
Groups
1a (2.7 x10^6)
1a-NS (normal saline)
1b (1.0x10^5)
1b-NS (normal saline)
As executed:
1a (2.7 x10^6)
1a-NS (normal saline)
1b (1.0x10^5)
1b-NS (normal saline)
Key
2.7 x10^5 SPZ (PfSPZ Vaccine)
1.0 x10^5 SPZ (PfSPZ Challenge)
Normal Saline
3.2 x10^4 PfSPZ (PfSPZ Challenge)
Natural Transmission Follow-up
*For the initial DVI, 3 volunteers (2 PfSPZ Vaccine/Challenge, 1 normal saline) will be inoculated 24 hours prior to the remaining volunteers
** Volunteers will receive CHMI between 10 and 14 weeks post last vaccination (with a window of +/- 7 days on each side) and will be followed for 8 weeks following CHMI.

Figure S1.

970 **A. 2 weeks after 3rd dose.**



971
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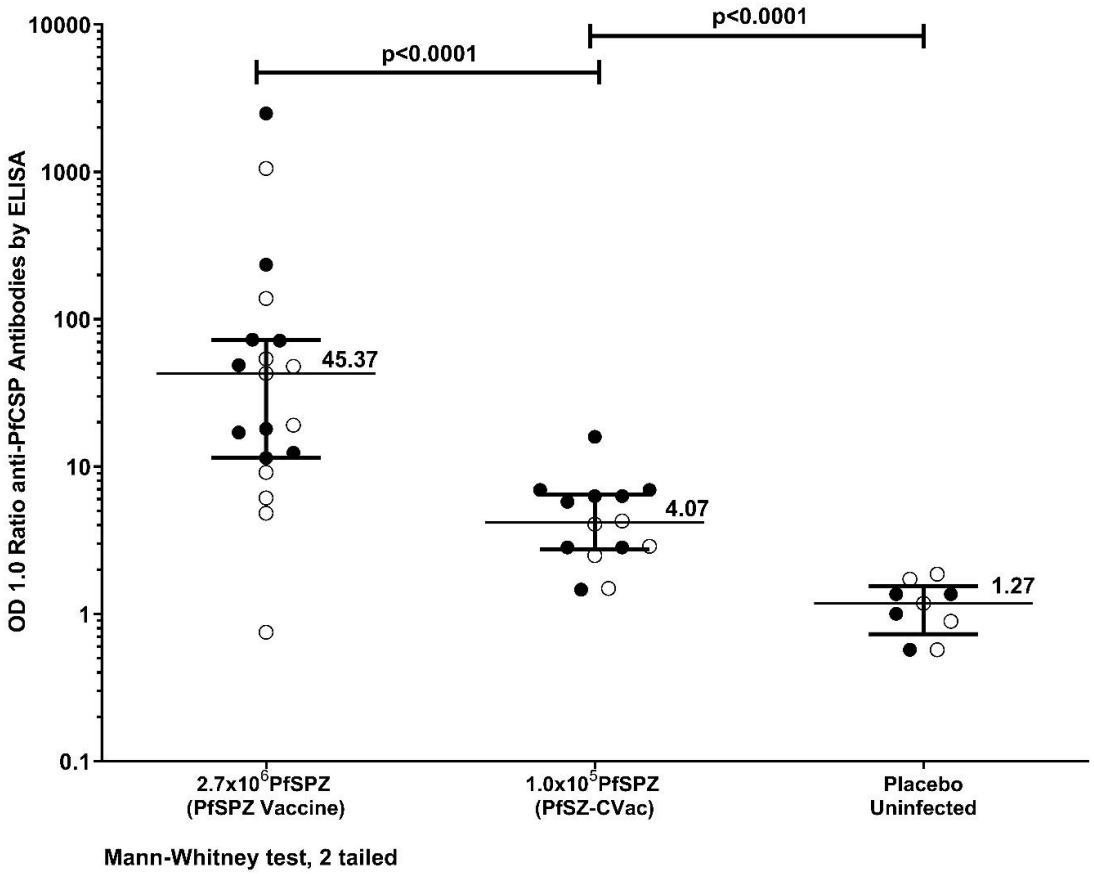
974 **B. Pre-CHMI**



975
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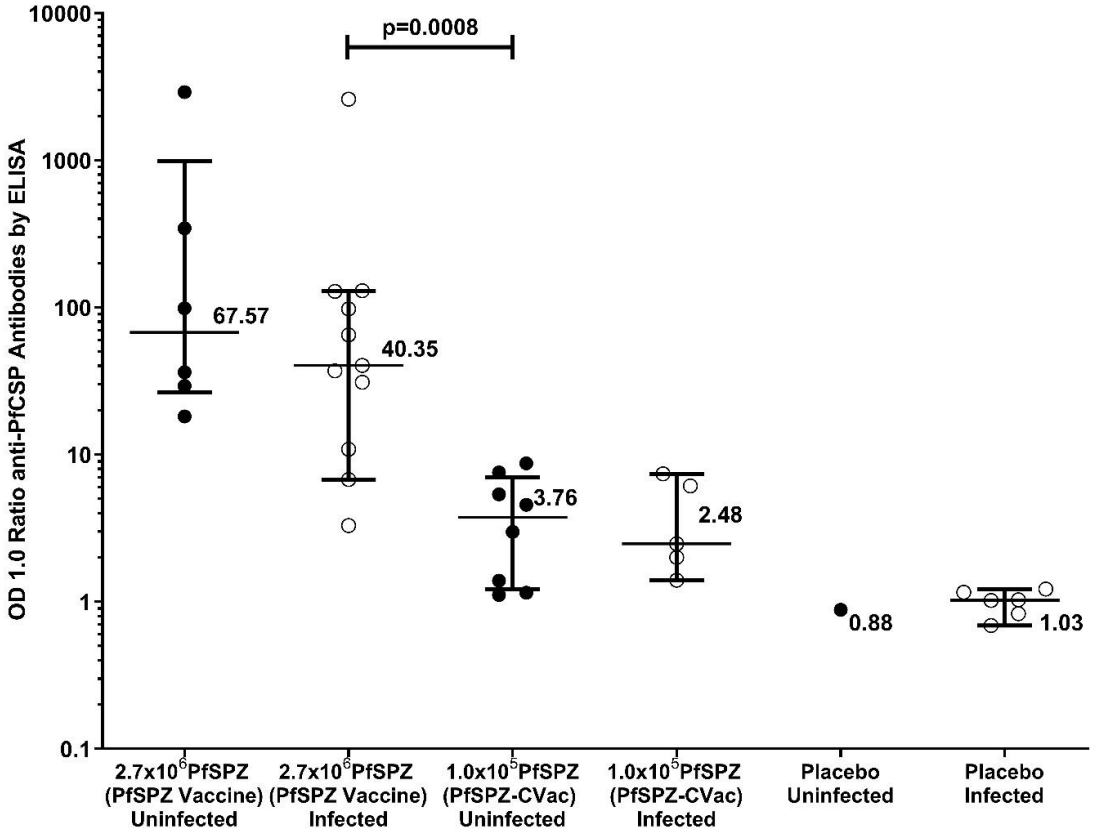
978 **C. 2 weeks after 3rd dose**



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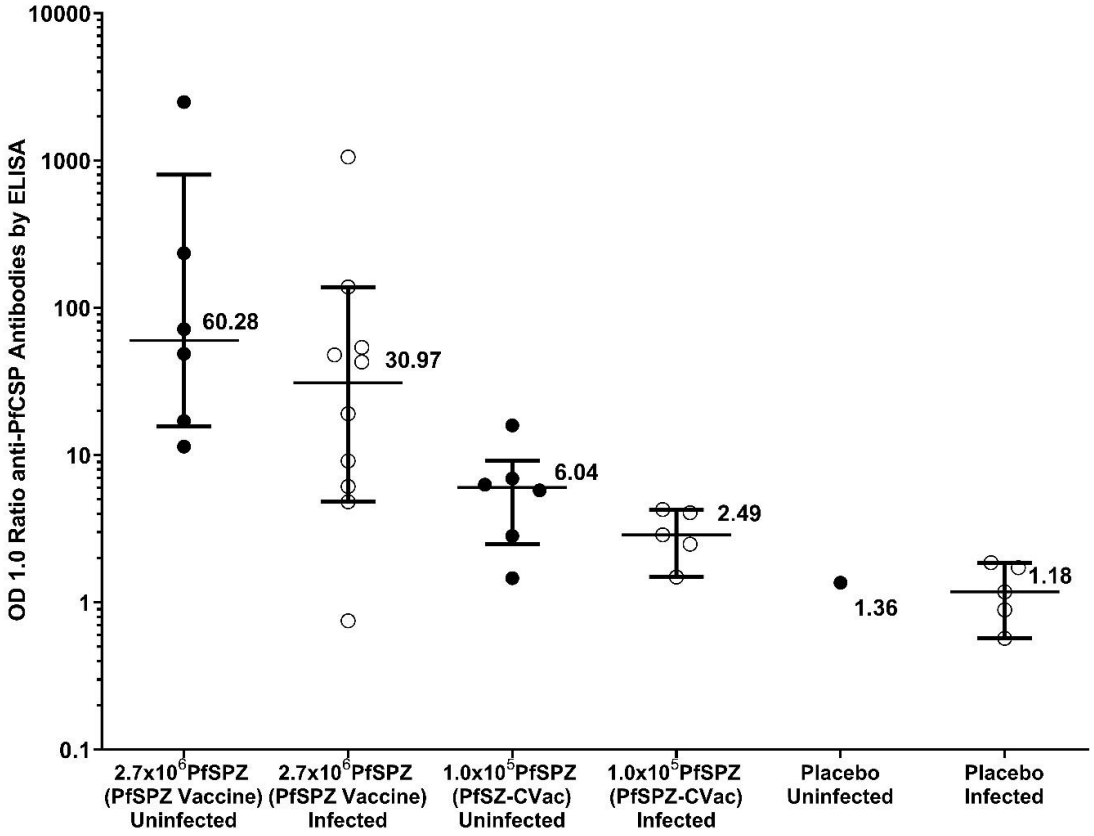
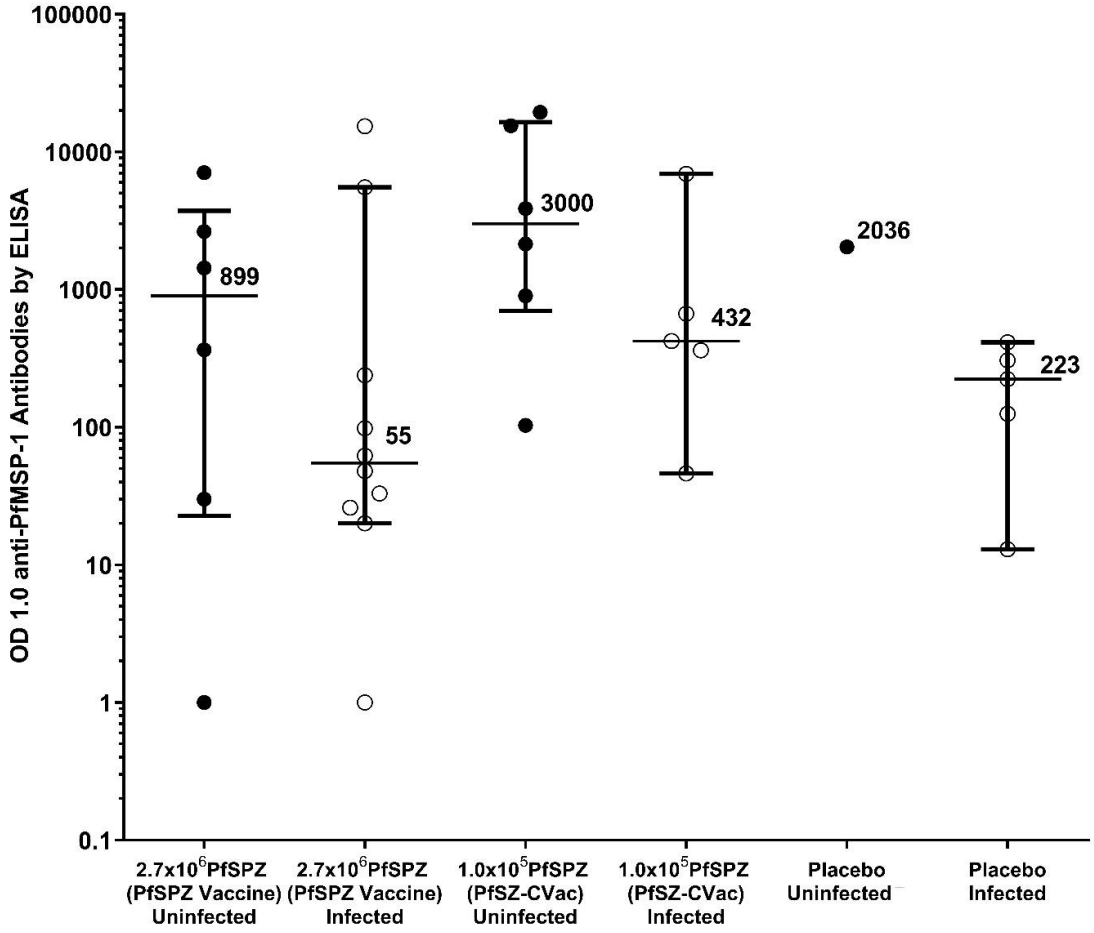
982

983 **D. Pre-CHMI**



984

985 **E. PfMSP-1 pre-CHMI**



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987

988

989 **Figure S2**

Table S1: Inclusion Criteria

1. Healthy males and females, based on clinical and laboratory findings
2. Age 6 months to 65 years
3. Adults with a Body Mass Index (BMI) 18 to 30 Kg/m^2; or adolescents, children and infants with Z-score of the selected indicator ([weight-for-height], [(height and BMI) for age]) category within ±2SD.
4. Long-term (at least one year) or permanent residence in the Baney district or Malabo city
5. Agreement to release medical information and to inform the study doctor concerning contraindications for participation in the study
6. Willingness to be attended to by a study clinician and take all necessary medications prescribed during study period
7. Agreement to provide contact information of a third party household member or close friend to study team
8. Agreement not to participate in another clinical trial during the study period
9. Agreement not to donate blood during the study period
10. Able and willing to complete the study visit schedule over the study follow up period, including the hospitalizations required for protocol compliance
11. Willingness to undergo HIV, hepatitis B (HBV) and hepatitis C (HCV) tests
12. Volunteer (subjects 18 years of age and older) or the parent / guardian signing the informed consent (for subjects <18 years of age) is able to demonstrate their understanding of the study by responding correctly to 10 out of 10 true/false statements (in a maximum of two attempts for those who failed to respond correctly to all true/false statements in the first attempt).
13. Signed written informed consent, in accordance with local practice, provided by adult volunteers, parents or legal representatives and relevant assent for children participants as applicable.
14. Free from malaria parasitemia by blood smear at enrollment and by PCR for group 1
15. Has not been treated with any antimalarial medication for at least two weeks prior to the first immunization.
16. Free from helminth infections (detected by microscopy) at enrollment.
17. Female volunteers aged 9 years and above must be non-pregnant (as demonstrated by a negative urine pregnancy test), and those aged 13 to 49 years provide consent/assent of their willingness to take protocol-defined measures not to become pregnant during the study and safety follow-up period.

Table S2: Exclusion Criteria

1. Previous receipt of an investigational malaria vaccine in the last 5 years
2. Participation in any other clinical study involving investigational medicinal products including investigational malaria drugs within 30 days prior to the onset of the study or during the study period
3. History of arrhythmias or prolonged QT-interval or other cardiac disease, or clinically significant abnormalities in electrocardiogram (ECG) at screening
4. Positive family history in a 1st or 2nd degree relative for cardiac disease at age <50 years old
5. A history of psychiatric disease
6. Suffering from any chronic illness including; diabetes mellitus, cancer or HIV/AIDS
7. Any confirmed or suspected immunosuppressive or immune-deficient condition, including asplenia
8. History of drug or alcohol abuse interfering with normal social function
9. The use of chronic immunosuppressive drugs or other immune modifying drugs within three months of study onset (inhaled and topical corticosteroids are allowed) and during the study period
10. Any clinically significant deviation from the normal range in biochemistry or hematology blood tests or in urine analysis
11. Positive HIV, hepatitis B virus or hepatitis C virus tests
12. Volunteers who are have risk factors for tuberculosis and/or signs and symptoms of tuberculosis (TB), plus a positive tuberculin skin test (TST).
13. Symptoms, physical signs and laboratory values suggestive of systemic disorders 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 volunteers
14. Any medical, social condition, or occupational reason that, in the judgment of the investigator, is a contraindication to protocol participation or impairs the volunteer's ability to give informed consent, increases the risk to the volunteer because of participation in the study, affects the ability of the volunteer to participate in the study or impairs the quality, consistency or interpretation of the study data.
15. History of non-febrile seizures or atypical febrile seizures.

Table S3: List of solicited adverse events with the grading system for severity and grading for relatedness*.

Local Solicited AEs *(at injection site)*	• Pain • Tenderness • Pruritus		1 2 3	Daily activity minimally affected, with or without treatment Daily activity possible but only with treatment Daily activity not possible even with treatment
	• Erythema • Swelling • Induration • Bruising/extravasated blood		1 2 3	2.5 – 5 cm 5.1 – 10 cm >10 cm, necrosis or exfoliative dermatitis
Systemic Solicited (Core List-post Vaccination)	• Fever		1 2 3	38.0°C – 38.4°C 38.5°C – 38.9°C >39.0°C
	<u>Adults, adolescents and older children</u>	• Allergic reaction *(rash, urticaria, pruritis, edema)* • Headache • Subjective Fever** • Fatigue • Malaise • Chills • Myalgia • Arthralgia	1 2	Daily activity minimally affected, with or without treatment Daily activity possible but only with treatment
	<u>Infants and younger children</u>	• Allergic reaction *(rash, urticaria, pruritis, edema)* • Subjective fever* • Drowsiness • Irritability/fussiness • Inability/refusal to eat or drink	3	Daily activity not possible even with treatment
Post CHMI Malaria Signs and Symptoms (In addition to Core List)	• Dizziness • Rigors • Sweats • Cough • Nausea • Vomiting • Abdominal pain • Diarrhea • Chest pain • Palpitations • Shortness of breath		1 2 3	Daily activity minimally affected, with or without treatment Daily activity possible but only with treatment Daily activity not possible even with treatment

*AEs (solicited and unsolicited) were recorded and graded by physicians: mild (easily tolerated), moderate (interfere with normal activity), severe (prevents normal activity) or life threatening (Table S3). Axillary temperature was Grade 1 (38.0-38.4°C), Grade 2 (38.5–38.9°C) or Grade 3 (> 39.0°C). Hematological and biochemical abnormalities were assessed using standard clinical assays. All AEs were assessed for severity and relatedness to IP administration. AEs were classified as definitely related, probably related, possibly related, unlikely to be related, or not related. Definitely, probably, and possibly were classified as related to IP administration; unlikely to be related and not related were classified unrelated.

** *Perceived by the subject and/or subject's guardian*

Table S4: Solcited adverse events for CVac

Chloroquine Only Dosing Period	Post Immunization during CQ Administration	
CQ	**Chloroquine + PfSPZ Challenge (CQ+CH)**	**Chloroquine + Parasitemia (CQ + P)**
CQ solicited AEs will be collected from the Day of the first dose through +7 days after the last dose.	*Six (6) additional signs/symptoms (with CQ solicited AEs) will be solicited from day of PfSPZ Challenge Vaccination through +5 days.*	*Twelve (12) additional signs/symptoms (with CQ solicited AEs) will be solicited from +6 days following PfSPZ Challenge Vaccination through +12 days.*
Days CV-2, CV-1	**Days 1 to 6 (CV_1 to CV_1+5)**	**Days 7 to 13 (CV_1+6 to CV_1+12)**
Days 14 to 28 (CV_1+13 to CV_1+27)	**Days 29 to 34 (CV_2 to CV_2+5)**	**Days 35 to 41 (CV_2+6 to CV_2+12)**
Days 42 to 56 (CV_2+13 to CV_2+27)	**Days 57 to 62 (CV_3 to CV_3+5)**	**Days 63 to 69 (CV_3+6 to CV_3+12)**
1 Nausea	1 Nausea	1 Nausea
2 Vomiting	2 Vomiting	2 Vomiting
3 Diarrhea	3 Diarrhea	3 Diarrhea
4 Abdominal pain	4 Abdominal pain	4 Abdominal pain
5 Dizziness	5 Dizziness	5 Dizziness
6 Tinnitus	6 Tinnitus	6 Tinnitus
7 Blurred vision	7 Blurred vision	7 Blurred vision
8 Photosensitivity	8 Photosensitivity	8 Photosensitivity
9 Insomnia	9 Insomnia	9 Insomnia
10 Pruritus	10 Pruritus	10 Pruritus
11 Headache	11 Headache	11 Headache
12 Fatigue	12 Fatigue	12 Fatigue
13 Myalgia	13 Myalgia	13 Myalgia
14 Anxiety	14 Anxiety	14 Anxiety
15 Confusion	15 Confusion	15 Confusion
	16 Elevated body temperature of >38oC	16 Elevated body temperature of >38oC
	17 Allergic reaction (rash, urticaria, pruritus, edema)	17 Allergic reaction (rash, urticaria, pruritus, edema)
	18 Subjective fever	18 Subjective fever
	19 Malaise	19 Malaise
	20 Chills	20 Chills
	21 Arthralgia	21 Arthralgia
		20 Rigors
		21 Sweats
		22 Cough
		23 Chest pain
		24 Palpitations
CV= PfSPZ Challenge Vaccination		25 Shortness of breath

Table S5. Antibodies to PfCSP and PfMSP1. All out-of-range, negative and zero values are reported as 1.

Group (Age)	PfSPZ/ Dose	Infection	Volunteer ID	ELISA PfCSP OD 1.0							ELISA PfMSP-1 OD 1.0	
				Pre-Immune	2 weeks post-3rd dose	NET (Pre-Post)	Ratio (Post/Pre)	pre-CHMI	NET pre-CHMI	Ratio (Post/Pre)	Pre-Immune	pre-CHMI
1a (18-35 y)	2.7x10^6 PfSPZ Vaccine	Uninfected	E21A317	30	2,966	2,936	98.87	2,182	2,152	71.73	1	1
			E21A371	198	5,797	5,599	29.28	3,575	3,377	17.06	2,283	2,632
			E21A412	130	2,358	2,228	18.14	1,615	1,485	11.42	8,505	7,052
			E21A414	319	11,569	11,250	36.27	15,892	15,573	48.82	355	365
			E21A416	-	2,911	2,911	2,911.00	2,494	2,494	2,494.00	-	1,432
			E21A444	17	5,867	5,850	345.12	4,014	3,997	235.12	1	30
			Median	**130**	**4,382**	**4,268**	**67.57**	**3,035**	**2,936**	**60.28**	**355**	**899**
		Infected	E21A309	39	5,021	4,982	128.74	5,442	5,403	138.54	48	33
			E21A311	108	4,001	3,893	37.05	1,094	986	9.13	121	98
			E21A313	224	14,587	14,363	65.12	4,504	4,280	19.11	67	48
			E21A314	73	2,261	2,188	30.97	520	447	6.12	19	20
			E21A316	21	2,052	2,031	97.71	1,027	1,006	47.90	28	26
			E21A399	1	2,601	2,600	2,601.00	1,057	1,056	1,056.00	24,988	15,331
			E21A402	55	599	544	10.89	320	265	4.82	447	238
			E21A417	1,363	4,499	3,136	3.30	2,381	1,018	0.75	11,376	5,534
			E21A426	958	6,474	5,516	6.76	-	-	-	-	-
			E21A433	31	1,251	1,220	40.35	1,359	1,328	42.84	1	1
			E21A448	7	909	902	129.86	384	377	53.86	83	62
			Median	**55**	**2,601**	**2,600**	**40.35**	**1,076**	**1,012**	**30.97**	**75**	**55**
		Group Median		**64**	**2,966**	**2,936**	**40.35**	**1,899**	**1,407**	**45.37**	**83**	**80**
1b (18-35 y)	1.0x10^5 PfSPZ-CVac	Uninfected	E21B-407	98	740	642	7.55	618	520	6.31	20,612	19,348
			E21B-446	104	906	802	8.71	722	618	6.94	50	103
			E21B-508	337	386	49	1.15	954	617	2.83	4,743	3,864
			E21B-509	359	1,633	1,274	4.55	5,721	5,362	15.94	1,021	2,136
			E21B-518	464	643	179	1.39	677	213	1.46	13,377	15,448
			E21B-525	284	316	32	1.11	-	-	-	-	-
			E21B-526	247	1,327	1,080	5.37	1,425	1,178	5.77	234	899
			E21B-530	52	155	103	2.98	-	-	-	-	-
			Median	**266**	**692**	**411**	**3.76**	**838**	**618**	**6.04**	**2,882**	**3,000**
		Infected	E21B-379	210	1,555	1,345	7.40	898	688	4.28	1	423
			E21B-401	139	345	206	2.48	566	427	4.07	1,377	669
			E21B-458	112	685	573	6.12	323	211	2.88	32	361
			E21B-519	196	275	79	1.40	489	293	2.49	5,919	6,908
			E21B-527	257	515	258	2.00	384	127	1.49	88	46
			Median	**196**	**515**	**258**	**2.48**	**489**	**293**	**2.49**	**88**	**423**
		Group Median		**210**	**643**	**258**	**2.98**	**677**	**520**	**4.07**	**1,021**	**899**

1b (18-35 y)	**Placebo**	**UnInfected**	E21A422	108	95	1	0.88	147	39	1.36	1,525	2,036
		Infected	E21A411	979	814	1	0.83	559	1	0.57	547	223
			E21A303	64	44	1	0.69	57	1	0.89	1	13
			E21A431	254	294	40	1.16	300	46	1.18	191	306
			E21A472	18	22	4	1.22	31	13	1.72	303	414
			E21B-353	92	95	3	1.03	171	79	1.86	97	125
			E21B-459	48	49	1	1.02	-	-	-	-	-
	Group Median			**92**	**95**	**1**	**1.02**	**159**	**26**	**1.27**	**247**	**265**

Table S6: Solicited Adverse Events Post-Vaccination. Adverse events are shown as the number of subjects (% of subjects) experiencing the adverse event by dose and stratified according to the greatest severity reported. Boxes are shaded to highlight the positive responses (blue – no grade assigned; yellow – mild; orange – moderate). Gray shaded boxes represent symptoms not solicited for PfSPZ Vaccine.

		PfSPZ Vaccine						PfSPZ-CVac					
		2.7x10^6			Placebo			1.0x10^5			Placebo		
Solicited Event	**Grade**	**Dose 1 N=20**	**Dose 2 N=18**	**Dose 3 N=18**	**Dose 1 N=6**	**Dose 2 N=6**	**Dose 3 N=6**	**Dose 1 N=19**	**Dose 2 N=18**	**Dose 3 N=18**	**Dose 1 N=5**	**Dose 2 N=5**	**Dose 3 N=4**
Local Adverse Events													
Bruising	Grade 1-Mild	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0
Erythema	Grade 1-Mild	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0
Induration	Grade 1-Mild	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0
Pain	Grade 1-Mild	0	0	0	0	0	0	1 (5.3)	1 (5.6)	1 (5.6)	0	0	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0
Pruritus	Grade 1-Mild	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0
Swelling	Grade 1-Mild	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0
Tenderness	Reported*	1 (5.0)	0	0	0	0	0	0	0	0	0	0	0
	Grade 1-Mild	0	0	0	0	0	0	1 (5.3)	1 (5.6)	1 (5.6)	0	0	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	0	0	0	0

		PfSPZ Vaccine						PfSPZ-CVac					
		2.7x10^6			Placebo			1.0x10^5			Placebo		
Solicited Event	**Grade**	**Dose 1 N=20**	**Dose 2 N=18**	**Dose 3 N=18**	**Dose 1 N=6**	**Dose 2 N=6**	**Dose 3 N=6**	**Dose 1 N=19**	**Dose 2 N=18**	**Dose 3 N=18**	**Dose 1 N=5**	**Dose 2 N=5**	**Dose 3 N=4**
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0
Systemic Adverse Events													
Abdominal Pain	Grade 1-Mild							0	1 (5.6)	1 (5.6)	0	0	0
	Grade 2-Moderate							0	0	1 (5.6)	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Anxiety	Grade 1-Mild							0	0	0	0	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Arthralgia	Reported*	1 (5.0)	0	0	0	0	0	0	0	0	0	0	0
	Grade 1-Mild	0	1 (5.6)	1 (5.6)	0	1 (16.7)	0	0	0	0	0	0	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	1 (5.6)	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0
Blurred Vision	Reported*							0	0	0	0	1 (20.0)	0
	Grade 1-Mild							0	1 (5.6)	0	0	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Chest Pains	Grade 1-Mild							0	0	0	0	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Chills	Reported*	0	0	0	0	0	0	0	0	1 (5.6)	0	0	0
	Grade 1-Mild	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0

		PfSPZ Vaccine						PfSPZ-CVac					
		2.7x10^6			Placebo			1.0x10^5			Placebo		
Solicited Event	**Grade**	**Dose 1 N=20**	**Dose 2 N=18**	**Dose 3 N=18**	**Dose 1 N=6**	**Dose 2 N=6**	**Dose 3 N=6**	**Dose 1 N=19**	**Dose 2 N=18**	**Dose 3 N=18**	**Dose 1 N=5**	**Dose 2 N=5**	**Dose 3 N=4**
Confusion	Grade 1-Mild							0	0	0	0	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Cough	Grade 1-Mild							0	1 (5.6)	0	0	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Diarrhea	Grade 1-Mild							0	1 (5.6)	0	0	0	0
	Grade 2-Moderate							0	0	1 (5.6)	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Dizziness	Reported*							1 (5.3)	0	0	2 (40.0)	0	0
	Grade 1-Mild							0	1 (5.6)	0	1 (20.0)	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Fatigue	Reported*	1 (5.0)	0	1 (5.6)	0	0	0	1 (5.3)	0	0	0	0	0
	Grade 1-Mild	2 (10.0)	0	1 (5.6)	0	0	0	0	0	1 (5.6)	1 (20.0)	0	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0
Fever	Grade 1-Mild	0	0	0	0	0	0	0	1 (5.6)	0	0	0	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0

		PfSPZ Vaccine						PfSPZ-CVac					
		$2.7x10^6$			Placebo			$1.0x10^5$			Placebo		
Solicited Event	**Grade**	**Dose 1 N=20**	**Dose 2 N=18**	**Dose 3 N=18**	**Dose 1 N=6**	**Dose 2 N=6**	**Dose 3 N=6**	**Dose 1 N=19**	**Dose 2 N=18**	**Dose 3 N=18**	**Dose 1 N=5**	**Dose 2 N=5**	**Dose 3 N=4**
Headache	Reported*	1 (5.0)	0	1 (5.6)	0	0	0	1 (5.3)	0	0	0	0	0
	Grade 1-Mild	0	0	0	0	0	0	2 (10.5)	1 (5.6)	0	1 (20.0)	0	0
	Grade 2-Moderate	1 (5.0)	0	0	0	0	0	0	2 (11.1)	0	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0
Insomnia	Grade 1-Mild							0	1 (5.6)	0	0	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Malaise	Grade 1-Mild	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0
Myalgia	Reported*	1 (5.0)	0	0	0	0	0	0	0	0	0	0	0
	Grade 1-Mild	2 (10.0)	0	0	0	0	0	0	0	0	1 (20.0)	1 (20.0)	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0
Nausea	Reported*							0	0	0	0	1 (20.0)	0
	Grade 1-Mild							1 (5.3)	0	0	0	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Palpitations	Grade 1-Mild							0	0	0	0	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0

		PfSPZ Vaccine						PfSPZ-CVac					
		2.7x10^6			Placebo			1.0x10^5			Placebo		
Solicited Event	**Grade**	**Dose 1 N=20**	**Dose 2 N=18**	**Dose 3 N=18**	**Dose 1 N=6**	**Dose 2 N=6**	**Dose 3 N=6**	**Dose 1 N=19**	**Dose 2 N=18**	**Dose 3 N=18**	**Dose 1 N=5**	**Dose 2 N=5**	**Dose 3 N=4**
Photosensitivity	Grade 1-Mild							0	0	0	0	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Pruritus	Reported*							1 (5.3)	0	0	0	0	0
	Grade 1-Mild							1 (5.3)	0	0	0	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Rash, urticaria, pruritus, edema	Reported*	0	0	0	0	0	0	1 (5.3)	0	0	0	0	0
	Grade 1-Mild	0	0	0	0	0	0	1 (5.3)	0	0	0	0	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0
Rigors	Grade 1-Mild							0	0	0	0	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Shortness of Breath	Grade 1-Mild							0	0	0	0	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Subjective Fever	Reported*	0	0	1 (5.6)	0	0	0	0	0	0	0	0	0
	Grade 1-Mild	1 (5.0)	1 (5.6)	0	0	0	0	1 (5.3)	1 (5.6)	0	0	0	0
	Grade 2-Moderate	0	0	0	0	0	0	0	0	0	0	0	0
	Grade 3-Severe	0	0	0	0	0	0	0	0	0	0	0	0

		PfSPZ Vaccine						PfSPZ-CVac					
		$2.7x10^6$			Placebo			$1.0x10^5$			Placebo		
Solicited Event	**Grade**	**Dose 1 N=20**	**Dose 2 N=18**	**Dose 3 N=18**	**Dose 1 N=6**	**Dose 2 N=6**	**Dose 3 N=6**	**Dose 1 N=19**	**Dose 2 N=18**	**Dose 3 N=18**	**Dose 1 N=5**	**Dose 2 N=5**	**Dose 3 N=4**
Sweats	Grade 1-Mild							0	0	0	0	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Tinnitus	Grade 1-Mild							0	0	0	1 (20.0)	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0
Vomiting	Grade 1-Mild							0	0	0	0	0	0
	Grade 2-Moderate							0	0	0	0	0	0
	Grade 3-Severe							0	0	0	0	0	0

Denominators are based on the number of subjects with systemic solicited event records submitted for each vaccine dose at the time of data cutoff

*Symptom was reported but grading was not done.

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Table S7: Abnormal Laboratory Values[a]. Number (and %) of subjects in each group experiencing the listed lab abnormality at least one during the study period.

	Group 1A		Group 1B	
Lab parameter	**$2.7x10^6$ (N=20)**	**Placebo (N=6)**	**$1.0x10^5$ (N=19)**	**Placebo (N=5)**
Red Blood Cells	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Decreased Hemoglobin	2 (10.0)	2 (33.3)	3 (15.8)	0 (0.0)
Decreased Platelets	2 (10.0)	1 (16.7)	2 (10.5)	1 (20.0)
Increased WBC Count	0 (0.0)	0 (0.0)	1 (5.3)	2 (40.0)
Decreased WBC Count	7 (35.0)	3 (50.0)	9 (47.4)	0 (0.0)
Decreased Neutrophils	15 (75.0)	4 (66.7)	18 (94.7)[†]	2 (40.0)
Decreased Lymphocytes	3 (15.0)	2 (33.3)	5 (26.3)	0 (0.0)
Increased Eosinophils	7 (35.0)	3 (50.0)	9 (47.4)	3 (60.0)
Elevated ALT	2 (10.0)	3 (50.0)	4 (21.1)	2 (40.0)
Elevated AST	3 (15.0)	1 (16.7)	5 (26.3)	2 (40.0)
Elevated Total Bilirubin	0 (0.0)	0 (0.0)	1 (5.3)*	0 (0.0)
Elevated Creatinine	4 (20.0)	1 (16.7)	3 (15.8)	0 (0.0)
Hypoglycemia	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

*Includes at least one Grade 3 result.

[†]p=0.0089, Barnard's test, 2-tailed. No other comparison between vaccine and corresponding control was staitistically significant.

Table S8: Asymptomatic parasitemia detected during the study prior to CHMI.

Study number	Group or prospective group	Vaccine	Time point(s)	Species
408	PfSPZ Vaccine	$2.7x10^6$ PfSPZ	Screening	*P. malariae*
			V2	*P. falciparum*
			CHMI-7	*P. falciparum*
			CHMI	*P. falciparum, P. malariae*
415	PfSPZ Vaccine	placebo	V3, V3+28, V3+56	*P. malariae*
416	PfSPZ Vaccine	$2.7x10^6$ PfSPZ	V3	*P. ovale*
			V3+196	*P. falciparum*
431	PfSPZ Vaccine	placebo	Scr3	*P. falciparum, P. malariae*
			V2	*P. malariae*
			V3, V3+28, V3+56	*P. falciparum*
404	PfSPZ-CVac	$1.0x10^5$ PfSPZ	CHMI-7	*P. falciparum*
512	PfSPZ-CVac	$1.0x10^5$ PfSPZ	CHMI-7, CHMI-7 (2)	*P. falciparum**
515	PfSPZ-CVac	placebo	CHMI	*P. falciparum**
519	PfSPZ-CVac	$1.0x10^5$ PfSPZ	Sc3	*P. falciparum*
525	PfSPZ-CVac	$1.0x10^5$ PfSPZ	V3, V3+14, CHMI-7	*P. falciparum**
528	PfSPZ-CVac	$1.0x10^5$ PfSPZ	CHMI-7	*P. falciparum, P. ovale*
530	PfSPZ-CVac	$1.0x10^5$ PfSPZ	CHMI-7	*P. falciparum*

* - genotyping confirmed as wild type or not the PfSPZ Challenge strain (NF54).
For the remaining Pf isolates in the PfSPZ-CVac arm, genotyping was either not performed (2) or inconclusive (2). In the PfSPZ Vaccine arm, all Pf infections were assumed to be naturally acquired field strains.

Table S9: Genotype data:

sample metadata			malaria qPCR data		msp1/msp2 genoptyping					drug resistance marker			microsatellite genotyping						Conclusion
Sid	**grp**	**visit**	**pf/uL**	**Non-Pf**	**msp1_k**	**msp1_m**	**msp1_r**	**msp2_fc**	**msp2_ic**	**k13**	**dhfr**	**dhps**	**Poly-A**	**PFPK2**	**TA-81**	**ARA-2**	**TA-87**	**TA-40**	
culture derived NF54			-	-	250	-	-	-	500	PFNF54	PFNF54	PFNF54	153	172	123	67	100	223	PfNF54
512	G1B	PD	0.35	-	-	200	-	-	-	-	-	-	-	-	-	-	-	-	field strain
525	G1B	PD	10.1	-	200	-	-	-	500; 600	PFNF54	N51I; C59R; S108N	PFNF54	-	-	-	-	-	-	multiple strain infection, PfNF54 unlikley
525	G1B	CH-7	22.1	-	200	-	-	-	500; 600	PFNF54	N51I; C59R; S108N	PFNF54	-	-	-	-	-	-	field strain
528	G1B	CH-7	92.2	Po	250; 400	200	-	350	500	PFNF54	N51I; C59R; S108N	PFNF54	-	-	-	-	-	-	multiple strain infection, PfNF54 can NOT be excluded
529	G1B	CH-7	0.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	inconclusive
530	G1B	CH-7	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	inconclusive
510	G1B	CH-7	0.25	-	-	-	-	-	-	-	-	-	-	-	124	-	-	-	inconclusive
515	G1B	PD	84.9	-	-	200	-	-	700	PFNF54	N51I; C59R; S108N	S436A; G437A	-	164	-	70	109	-	field strain
316	G1A	CH+18 T0	63.2	-	250	-	-	-	500	PFNF54	PFNF54	PFNF54	-	-	122	-	-	-	PfNF54
314	G1A	CH+18 T0	25	-	250	-	-	-	500	PFNF54	PFNF54	-	-	-	122	-	-	-	PfNF54
303	G1A	CH+13 T0	59.3	-	250	-	-	-	500	PFNF54	PFNF54	PFNF54	-	-	-	67	100	-	PfNF54
309	G1A	CH+18 T0	53.5	-	250	-	-	-	500	PFNF54	PFNF54	PFNF54	-	-	122	-	-	-	PfNF54

Table S10. Solicited AE, unsolicited AE and symptoms and signs of malaria. Solicited AE were collected for 5 days after CHMI. Specific symptoms and signs of malaria were solicited at each visit starting at day 7 through to day 29 and were attributed to malaria if they corresponded to Pf parasitemia as described. Unsolicited AE not corresponding to parasitemia and presumed unrelated to malaria were collected from days 1 to 29.

	All (n=36)	TBS+/qPCR+ (n=15)	TBS-/qPCR+ (n=6)	TBS-/qPCR- (n=15)
Number subjects (%) with solicited AEs, CHMI days 1-6	1 (2.8%)			
Number of subjects with symptoms or signs of malaria*	9 (25.0%)	8 (53.3%)	1 (16.7%)	0 (0.0%)
Number subjects with unsolicited AEs, CHMI days 1-29[#]	7 (19.4%)	2 (13.3%)	2 (33.3%)	3 (20.0%)

*Symptoms or signs of malaria were identified using a predefined list of symptoms or signs occurring from 3 days prior to 7 days after the detection of parasitemia by TBS. For the one qPCR+/TBS- subject with symptoms the identified symptoms occurred beginning 5 days after the first positive sample was positive by qPCR.

[#]Unsolicited AE included toothace (3), arthralgias, conjunctivitis, left foot swelling, nipple pain, trauma to the right great toe and upper lip swelling. None were considered related to injection of PfSPZ Challenge.

Chapter 4

HIV-1 positive and HIV-1 negative Tanzanian adults undergoing whole irradiation attenuated *Plasmodium falciparum* sporozoite vaccination mount antibody responses targeting the circumsporozoite protein and merozoite surface protein 5

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HIV-1 positive and HIV-1 negative Tanzanian adults undergoing whole irradiation attenuated *Plasmodium falciparum* sporozoite vaccination mount antibody responses targeting the circumsporozoite protein and merozoite surface protein 5.

HIV-1 positive and HIV-1 negative Tanzanian adults undergoing whole irradiation attenuated *Plasmodium falciparum* sporozoite vaccination mount antibody responses targeting the circumsporozoite protein and merozoite surface protein 5

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Abstract:

Background

Radiation-attenuated, aseptic, purified, cryopreserved *Plasmodium falciparum* (Pf) sporozoites (PfSPZ Vaccine) as pre-erythrocytic stage anti-malaria vaccine has been tested in healthy people of a broad age ranges in several Sub-Saharan African countries. We have conducted recently a clinical trial to evaluate the safety, immunogenicity and efficacy of PfSPZ Vaccine in Tanzanian adult volunteers with or without chronic HIV-1 infection (Acronym: BSPZV3a) (Clinicaltrials.gov: NCT03420053). Volunteers underwent controlled human malaria infection (CHMI) 3 weeks past fifth vaccination to test the protective efficacy in both groups of volunteers. Serum samples from placebos and vaccinated volunteers were collected to analyse PfSPZ Vaccine induced humoral immunity and antibody responses after asexual blood stage parasitemia development after CHMI.

Methods

Serum immunoglobulin G (IgG) and IgM responses were analysed at baseline, 14 days after last PfSPZ Vaccination and 28 days after homologous CHMI using a custom made Pf protein microarray comprising 262 individual features covering 228 pre-selected Pf proteins.

Results

At baseline, HIV-1 positive and HIV-1 negative volunteers did not differ in the breadth of IgG responses and very low IgM responses were measured. Two weeks after the fifth vaccination, antibodies targeting the circumsporozoite protein (CSP) and merozoite surface protein 5 (MSP5) were induced in all vaccinees confirming that MSP5 is immunogenic and expressed either in the sporozoite or during early liver stage of the parasite life cycle. Volunteers showed distinct, personalized antibody titers at baseline and after CHMI.

Conclusion

We identify CSP and MSP5 as immunogenic proteins after PfSPZ Vaccination in HIV-1 positive and HIV-1 negative volunteers. The expression and biological function of MSP5 during the pre-erythrocytic stage of the parasite deserves further studies. Our results confirm a highly personalized IgG and IgM immune response against Pf before and after asexual blood stage parasitemia induced by CHMI.

Keywords
Plasmodium falciparum, PfSPZ Vaccine, protein microarray, controlled human malaria infection, HIV-1, merozoite surface antigen 5, circumsporozoite protein

Background

Malaria is a vector-borne disease with six Plasmodium species known to infect humans including *Plasmodium falciparum*, *P. vivax*, *P. malariae*, *P. ovale curtisi*, *P. ovale wallikeri* and *P. knowlesi* [1]. In 2018, an estimated 228 million cases of malaria infections occurred worldwide (95% confidence interval [CI]: 206–258 million) with an estimated number of 405 000 deaths [2]. *P. falciparum* is the most prevalent malaria parasite in the WHO African Region, accounting for 99.7% of estimated malaria cases [2]. Around US$ 2.7 billion was invested in 2018 for malaria control and elimination efforts globally by governments, industry and private philanthropic organisations [3]. Current malaria control efforts rest on a combination of vector control measures to curb parasite transmission and on rapid detection and treatment of malaria infected or diseased individuals [1]. Malaria elimination, that is interruption of local malaria transmission, would enable stronger economic development of countries located in Sub-Saharan Africa and would reduce the cost burden of conducting malaria control programs [4]. As outlined in the malaria vaccine roadmap of WHO, an effective malaria vaccine targeting the most pathogenic malaria species, *Plasmodium falciparum* (Pf) would be an important, complementary tool to facilitate Pf malaria elimination [4, 5].

A number of malaria vaccine approaches are currently followed targeting the pre-erythrocytic stage of malaria. The most advanced is the subunit vaccine RTS,S, formulated in AS01 adjuvant that is currently in the implementation phase in three different countries [6, 7]. The whole, cryopreserved, attenuated, metabolically active Pf sporozoite vaccine approach (PfSPZ Vaccine) is based on the hypothesis that providing the human immune system with a broad range of antigens that induce both cellular and humoral immunity superior to naturally acquired immunity will provide high levels of sterile protection against re-infection [8]. To evaluate the immunogenicity, safety and protective efficacy PfSPZ Vaccine against CMHI and field challenge, a series of clinical trials have been conducted over the past years in Sub-Saharan in different age groups Africa [9-11]. Malaria and HIV-1 are both common in Sub-Saharan Africa and overlap extensively in their geographic range [12, 13]. HIV-1 infection leads to higher malaria parasitemia and more frequent numbers of clinical malaria episodes particularly as the

CD4 T cell counts diminishes [14]. HIV-1 and malaria co-infection were associated with reduced antibody response to an array of *P. falciparum* antigens [12, 13]. Under field conditions administration of an efficacious vaccine for malaria elimination in Sub-Saharan Africa requires to understand the interaction between HIV-1 infection status and vaccination outcome [15]. The malaria vaccine RTS,S/AS01 has been evaluated in WHO stage 1 and 2 HIV-1 positive children ages 6 weeks to 17 months in Kenya [16]. The safety profile of the RTS,S vaccine was no different from a comparator group receiving the rabies vaccine and there was no association with vaccination and HIV-1 disease progression as determined by clinical course, HIV-1 viral load and CD4 count [17].

Protein microarrays are a versatile tool to probe in an unbiased, hypothesis-free approach humoral immunity against the proteome of pathogens of interest proteins [18]. This technology has been in the malaria field to better understand the natural history of malaria infection in different age groups and naturally acquired protection against clinical malaria [19-22] and stage specific immunity [23, 24]. Microarrays have identified immune-reactive antigens associated with recent malaria exposure for immune-epidemiological studies [25]. Microarray analyses help to understand the impact of malaria vaccination on malaria protection and immunity [26-28] and to examine potential correlates of protection in children and adults from endemic areas [29].

We have conducted recently a randomized, double-blind, placebo-controlled trial to evaluate the safety and immunogenicity of PfSPZ Vaccine administered as five doses of each $9.0x10^5$ PfSPZ at 0, 2, 4, 6 and 28 days to HIV-1 negative and HIV-1 positive volunteers compared to normal saline (NS) controls (ClinicalTrials.gov Identifier: NCT03420053, acronym: BSPZV3a). Protective efficacy of this vaccination regimen was assessed by homologous CHMI conducted 3 – 10 weeks after the fifth vaccination by direct venous inoculation of 3,200 fully infectious sporozoites (PfSPZ Challenge). Serum samples were collected pre-vaccination, 14 days past fifth vaccination and 28 days past CHMI and used to probe a custom-made protein microarray of Pf covering 228 proteins. Our study aimed to compare the baseline response and PfSPZ Vaccine-induced IgG and IgM profiles in HIV-1 positive and HIV-1 negative Tanzanian adults. In addition, we wanted to measure the humoral immune responses after homologous CHMI in non-protected volunteers and placebo controls.

Material and methods

Study design and samples

Study samples originate from a yet unpublished phase 1 clinical trial designed to test safety, immunogenicity and efficacy of PfSPZ Vaccine in HIV positive and negative Tanzanian adults. The study was conducted from February to August 2018 at the Ifakara Health Institute Bagamoyo, Tanzania, and is registered at Clinical Trials.gov under the identifier NCT03420053. The trial was approved by the Institutional Review Board for the Ifakara Health Institute (IHI-IRB) (ref No. IHI/IRB/No.22-2017), Tanzanian Food and Drug Administration (TFDA) (TFDA Auth. No. 0017/CTR/0016/6), Tanzanian National Institute for Medical Research (NIMR) (ref. NIMR/HQ/R.8a/Vol.IX/2642), 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.

Briefly, 22 volunteers aged 18-45 years from the malaria meso-endemic Bagamoyo district (PMID: 30518365) and surrounding coastal areas were enrolled in the trial, including 12 participants with a confirmed HIV infection that classified into the WHO clinical stage 1 of the disease. All HIV-1 positive subjects underwent continuous anti-retroviral treatment and had a CD4+ T cell count above 500 cells/µl at screening visit. The study comprised an unblinded HIV-1 positive pilot group (2a, n = 4), receiving five immunizations of PfSPZ with a dose of 4.5 x 105 sporozoites given each by direct venous inoculation (DVI) at study days 0, +2, +4, +6 and +28. Following safety assessment, the study was extended to the full double blinded cohort of 9 HIV-1 positive (group 2b) and 9 HIV-1 negative volunteers (group 1) that were randomized to placebo and verum treatment at a 1:2 ratio. Both HIV positive (group 2b, n = 6) and negative (group 1, n = 6) verum vaccinees received the full dosing of 9.0 x 105 PfSPZ at each injection, maintaining the immunization schedule above. The placebo control group (n = 6, comprising 3 HIV-1 positive and HIV-1 negative subjects each) was inoculated with a normal saline placebo (NS). Efficacy was assessed via controlled human malaria infection (CHMI) of 3,200 live Pf sporozoites by DVI (PfSPZ Challenge) three to ten weeks after completion of immunization. One HIV-1 negative and one HIV-1 positive verum vaccinee were excluded from the study during immunization and challenge phase, respectively, resulting in a total of 5 participants in both groups 1 and 2b undergoing CHMI. Out of these, 4/5 HIV

negative vaccinees were sterilely protected for at least 20 days following CHMI, while 5/5 HIV positive and 6/6 placebos developed parasitaemia during that period (Figure 1 A).

Serum sample collection and processing

Whole blood samples were collected in 10ml blood collection tubes with clot activators (Becton Dickson) and allowed to stand for 1 hour at room temperature to facilitate serum formation followed by centrifugation at 2000 x g for 10 minutes. Serum was then collected, aliquotted and stored at -80ºC until analysis. Timepoints of serum samples taken from volunteers and analysed in this study included baseline (before first vaccine inoculation), 14 days past fifth vaccination and 28 days past CHMI.

Protein microarray

Microarray slides were obtained from the University of California Irvine, Irvine, California, US [30]. 262 protein fragments comprising 228 defined Pf antigens and ranging in size from 100 to 1800 bp were expressed in an *in vitro E. coli* system and cell lysates were printed onto 16-pad ONCYTE AVID® (PolyAn GmbH, Berlin, Germany) nitrocellulose slides. The represented antigens are a selection of antigens that have been observed as antigenic targets in previous larger scale microarray studies, depicting both naturally acquired immunity [31, 32] and vaccination trials [29, 30, 33].

Serum samples were diluted 1:50 in 0.05 X Super G Blocking Buffer (Grace Bio-Labs, Inc., Oregon, US) supplemented with 10 % *E. coli* lysate (GenScript, Piscataway, New Jersey, US) and incubated for 30 minutes on a shaker at room temperature (RT). Both the positive control, a pool of sera sampled in a malaria-endemic area in Ghana, and the negative control, serum pool of a malaria-naïve European donor, were treated similarly. Microarray slides were rehydrated at RT by addition of 100 µl 0.05 X Super G blocking buffer per pad. After removal of the rehydration buffer, 100 µl/pad of diluted sera samples were added onto the slides and incubated overnight at 4°C on an orbital shaker at 180 rpm. Serum dilutions were removed the following day and slides were washed three times using 1 X TBST buffer (Grace Bio-Labs, Inc.). During each washing step, 200 µl wash buffer/pad was applied and slides were incubated for 5 minutes on the shaker. Secondary anti-IgG (goat anti-human IgG QDot™800, Grace Bio-Labs, #110635) and anti-IgM antibodies (biotin-SP-conjugated goat anti-human IgM, Jackson ImmunoResearch, #109-065-043) were diluted 1:200 and applied at 100 µls onto the slides.

Following an incubation period of two hours at RT on the shaker, slides were washed three times before application of 100 µl/pad of a tertiary Qdot™585 Streptavidin Conjugate (Invitrogen #Q10111MP) at a 1:250 dilution. Slides were incubated for one hour at RT on the shaker, subsequently followed by another three washing steps before rinsing with (ultrapure) water and drying by centrifugation in 50 ml Falcon tubes at 500 g for 10 minutes. Images of arrays were taken in the ArrayCAM® Imaging System (Grace Bio-Labs) with an acquisition time of 4 s and an exposure time of 200 and 5 ms for IgG and IgM images respectively. Median raw signal intensities of the single array spots and surrounding background areas were obtained using the ArrayCAM® 400-S Microarray Imager Software. All pictures were manually checked for correct recognition of spot locations and potential noise signals.

Pre-processing of microarray data

Microarray data analysis was performed in R statistical software package version 3.6.2. Data pre-processing comprised the removal of noise signals, followed by background correction, data transformation and normalization steps. Every spot intensity signal was corrected for local background reactivity by applying the normal-exponential convolution model [34] (available in the LIMMA package [35]) in combination with a saddle-point approximation for initial parameter estimation [36]. The model assumes exponentially distributed true spot signals overlaid by normally distributed background signals and is suggested as preferred correction method to reduce variability in the low-intensity signal range [34]. Resulting antigen signal intensities were log2-transformed to approach a normal distribution. Normalization between arrays and thus adjustment for sample-dependent background reactivity to *E. coli* lysate comprised the subtraction of the median intensity of mock expression spots on that particular array.

Data analysis

Antigens were defined as differentially recognized between the test groups if they yielded a $p < 0.05$ (Welch-corrected Student's t-test) and a fold change > 2 in mean signal intensities. Differential responses were classified as relevant if they furtherly reached a $p < 0.05$ following Benjamini-Hochberg (BH) correction for multiple testing and a high effect size (Hedge´s $g > 0.8$). The effect of group and intervention on the measured signal intensity of single relevant reactive antigens was assessed using a two-way mixed ANOVA model, followed by a post-

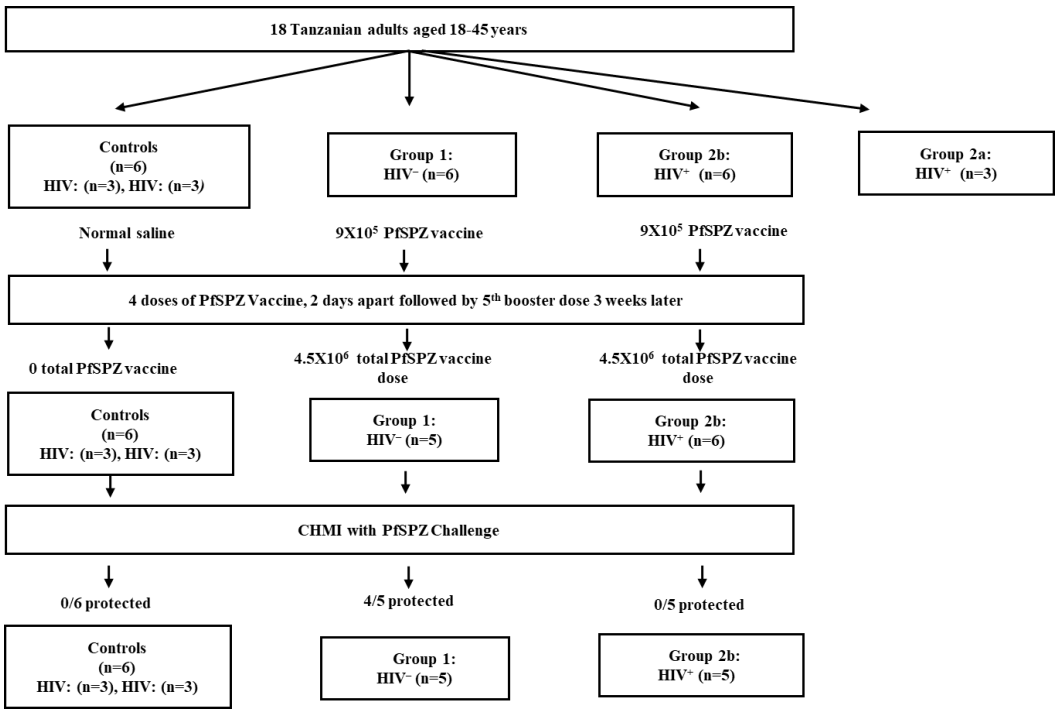
hoc one-way ANOVA with Bonferroni adjusted p-values to specify differences between the study groups at every time point.

An antigen-wise threshold for seropositivity was set at a signal intensity of 3 log levels above the median intensity in the naive controls. The antibody breadth of every sample described the number of seropositive antigens at one particular time point. Baseline differences in antibody breadth between the HIV positive and negative study population were estimated by Wilcoxon-Mann-Whitney tests. The change in antibody breadth over immunization and challenge was assessed in a robust two-way mixed ANOVA on trimmed means, excluding 20 % of highest and lowest values to avoid disturbance of single outliers. Seroconversion within one participant was defined as exceedance of the seropositivity threshold between to time points with a signal increase of at least 2 log levels, sero-reversion as drop below the threshold with a signal decrease of 2 log levels. Signal fluctuations around the threshold with intensity changes smaller than 2 log levels within on sample at different time points were designated as borderline reactivity. Volcano plots were generated in Graph Pad Prism 8, all other plots in R using the ggplot2, gplots, ggbeeswarm, lemon and PAA packages.

Results

Study population and serum sampling

Serum samples for protein microarray analysis were collected at three time points: “baseline” (2 days before first immunization), “immunized” (14 days past the fifth PfSPZ immunization) and “challenged” (28 days past CHMI; Figure 1 B). Baseline study samples were allocated into HIV-1 positive (n = 12, including groups 2a, 2b and placebo recipients) and HIV-1 negative volunteers (n = 6 vaccinees, group1 and n=3 placebo recipients). Analysis of later time points (after immunization and challenge) focused on the three main study groups of placebo controls (n = 6), the higher-dosed HIV-1 positive vaccine group 2b (n = 6 after immunization, n = 5 after challenge) and the HIV-1 negative vaccine group1 (n = 5). All volunteers included in the study had no asexual blood stage parasitemia at the start of the study (measured by malaria thick blood smears and no parasitemia before CHMI (measured by TBS and the more sensitive qPCR) (Jongo et al. manuscript in preparation).



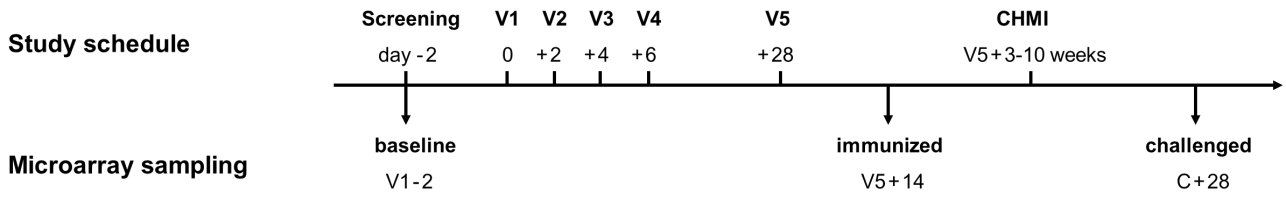


Figure 1. Study design and sampling for microarray analysis. (A) Volunteer group allocation. The trial included an HIV-1 positive pilot group (group 2a) that received 4x105 of PfSPZ Vaccine in each inoculation and did not undergo CHMI. Group 1 and group 2b comprised each six volunteers that were HIV-1 negative and HIV-1 positive, respectively, that received 9x105 of PfSPZ Vaccine in each inoculation. The placebo group had 6 volunteers that were HIV-1 negative (n=3) and HIV-1 positive (n=3). (B) Serum sample collection and study flow chart. Serum samples for microarray analysis were collected at baseline (V1 – 2), 14 days after the fifth injection (V5 + 14), and 28 days after CHMI (C+28).

Baseline immunity of the HIV positive and negative study population

The population-specific immunity was characterized by comparing the baseline microarray reactivity of HIV negative (n = 9) and HIV positive participants (n = 12). The number of antigens recognized by IgG antibodies at baseline was highly variable between individuals. HIV positive samples displayed a lower overall antibody breadth (median, range: 11, 0-82) compared to the positive group (median, range: 23, 2-41) but no significant difference was observed (p = 0.6, Wilcoxon-Mann-Whitney test, Figure 2 A). IgM reactivity was generally very low in both HIV positive (median, range: 0.5, 0-67) and negative subjects (median, range: 0, 0-11; Figure 2 B). Comparing the deviation of median signal intensities of HIV positive and negative samples from the overall weighted median intensity across all microarray antigens, both groups display a similar pattern of the IgG (Figure 2 C) and IgM repertoire (S Figure 1). Notably, IgG antibody levels against the merozoite surface protein 2 (MSP2) and the liver stage antigen 1 (LSA1) are lower in the HIV negative group, while antibody levels against the merozoite surface protein 8 (MSP8) and several intracellular and core proteins (ApiAp2, CryPH, PhLP1, COG4, conserved protein PF3D7_0513200) are clearly elevated. HIV positive samples display an elevated recognition of an exported protein (PHISTc, PF3D7_0801000) but lower reactivity against a variety of proteins, including the rhoptry neck protein 2 (RON2) or a core protein (PHAX domain-containing protein, PF3D7_1021900). Highest overall IgG

baseline responses are observed against some well-described exposure markers such as the liver stage antigen 3 (LSA3) or the merozoite surface protein 2 (MSP2) but also against some uncharacterized proteins (Table S2).

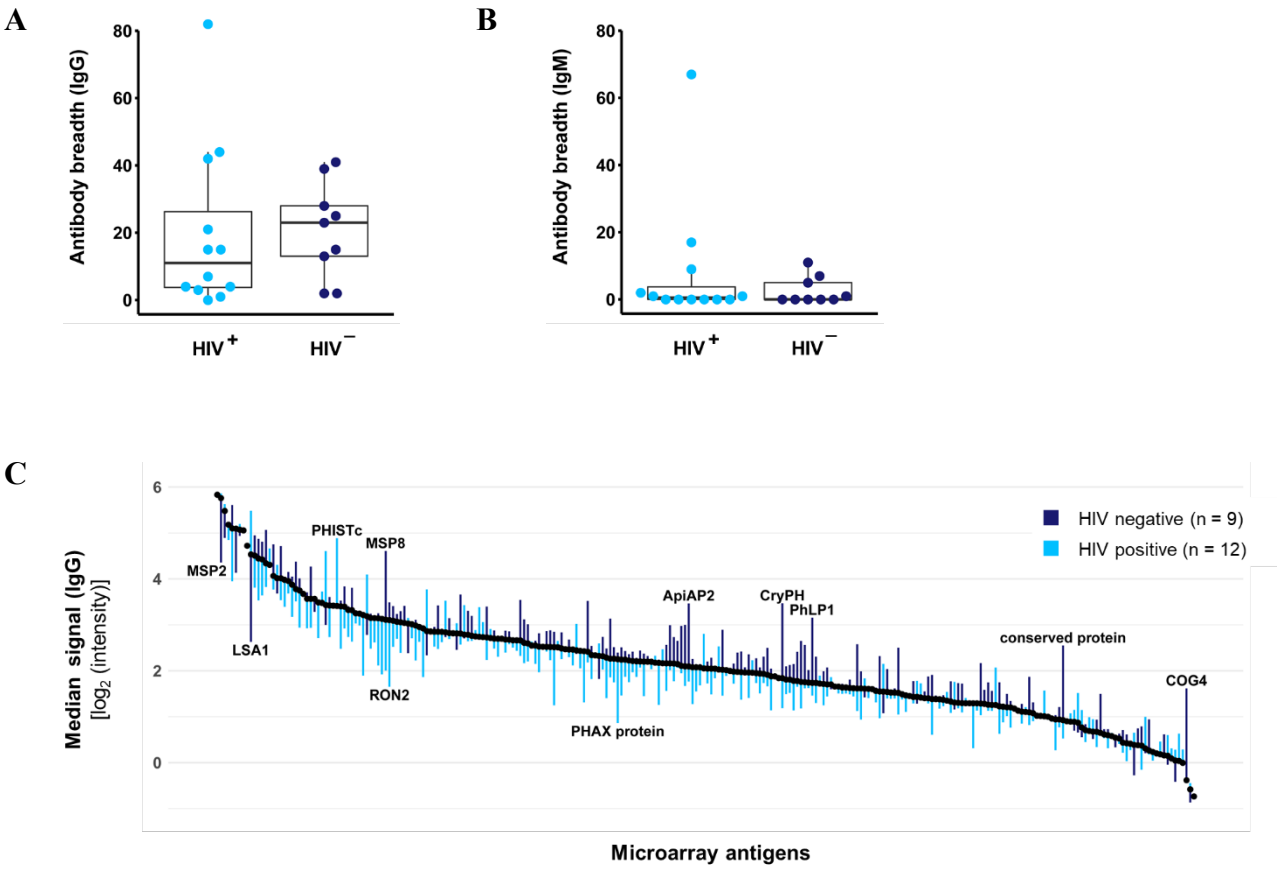


Figure 2. Baseline immunity of the study population stratified by HIV status. Serum samples of HIV negative (n = 9) and HIV positive (n = 12) study participants were collected at screening visit and probed on a protein microarray representing 262 important *P. falciparum* antigens (see Table S1 for array design and antigen abbreviations). Individual breadths of IgG (A) and IgM (B) antibody repertoires were compared between HIV positive and negative participants. Antibody breadth was defined as number of seropositive antibodies exceeding a signal intensity threshold of 3 log levels above a malaria-naïve control. The boxplots give median antibody breadths, interquartile ranges (IQR) and whiskers of length 1.5 × IQR. (C) Antigens are sorted according to their overall median signal intensity, weighted for the different sizes of the HIV positive and negative group. Bars give the deviation of the median signal intensities in the HIV negative (dark blue) and positive group (light blue) from the overall median (see Table S2 for the top 25 antigens). [PHISTc (PF3D7_0801000), conserved protein (PF3D7_0513200)]

Antibody response induced by PfSPZ immunization

Immunization with five doses of 9.0×10^5 PfSPZ Vaccine results in a humoral immune response to a limited number of antigens with little shifting the overall antibody profile. Individual changes in microarray signal intensities between the sampling time points at baseline and after immunization were assessed amongst placebo recipients (n = 6), HIV negative (n = 5, study group 1) and HIV-1 positive vaccinees (n = 6, group 2b). In the placebo group, little changes in the median signal was observed between these two bleeding times, and varying between > 1 and 2 log levels (Figure 3 A). Irrespective of the vaccinees´ HIV-1 status, immunization induced significant IgG reactivity against CSP, MSP5 and a conserved asparagine-rich protein of unknown function (ARP) with a mean fold change > 2 and $p < 0.05$ (paired Welch-corrected student´s t-test) (Figure 3 B). Data stratified by HIV-1 status are provided in S Figure 2 A). A statistical significant effect was only observed for anti-CSP-IgG (Benjamini-Hochberg (BH) adjusted $p = 0.03$, Hedge´s $g = 1.40$).

For IgM, the highest increase in signal intensity was observed for MSP5 in HIV-1 positive and HIV-1 negative vaccinees. Further changes in IgM antibody profile mainly appeared amongst the HIV-1 positive vaccinees against CSP, the serine repeat antigen 4 (SERA4) and the sexual stage-specific protein precursor (Pfs16), the liver stage protein translocon component PTEX150 and a zinc finger protein (Figure 3 C). Further reactive antigens observed amongst vaccinees in general include nucleoporin NUP138 and a SAP domain-containing protein (Figure 3 D). Data stratified by HIV-1 status are given in S Figure 2 B. The strongest, statistically significant PfSPZ vaccination-induced response was detected against MSP5 (BH adjusted $p = 0.003$, Hedge´s $g = 1.9$). PfSPZ vaccine-induced anti-CSP-IgG and anti-MSP5-IgM responses were also observed amongst the three HIV positive vaccinees in study group 2a, receiving 4.5×10^5 PfSPZ per injection (see S Figure 2 for effects of different vaccine doses).

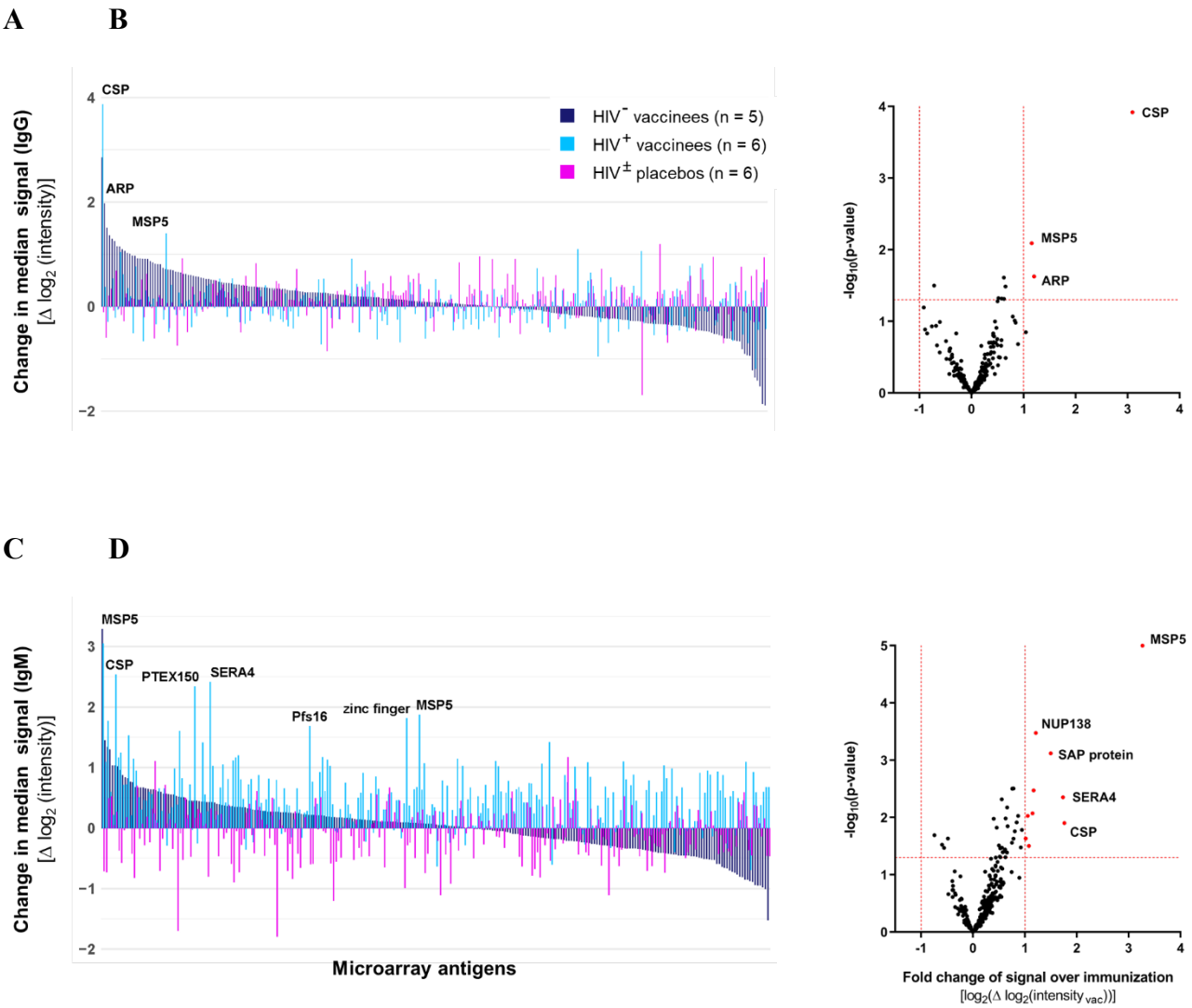


Figure 3. Changes in antibody repertoire induced by immunization. To identify vaccination-induced antibodies, microarray reactivity in samples collected after the completed immunization phase was compared to their individual baseline reactivity. (A, C) Median changes in IgG (A) and IgM (C) signal intensities across 262 microarray antigens over the immunization phase were assessed amongst placebo recipients (n = 6), HIV negative (n = 5) and HIV positive vaccinees (n = 6, study group 2 b only). Antigens are sorted by median intensity changes in the HIV negative vaccinees´ group. (B, D) Fold change in IgG (B) and IgM (D) and p-values (paired Student´s t-test) of average signal intensity in all immunized vaccinees (n = 11) compared to their baseline are given for all microarray antigens. Differentially recognized antigens (p-value < 0.05 and fold change > 2) are depicted in red (see

Table S1 for used antigen abbreviations). [ARP (PF3D7_0108300), zinc finger (PF3D7_1208800)].

Antibody response induced by homologous controlled human malaria infection

Sera of HIV-1 negative (n = 5), HIV-1 positive vaccinees (n = 5) and placebos (n = 6) collected four weeks after CHMI display fundamentally different antibody profiles. No difference was observed between HIV-1 positive or HIV-1 negative participants in either of the study groups (S Figure 4). Comparing the median change of IgG signal intensities over challenge across all microarray antigens, no significant booster effect is seen in vaccinees but rather a decline in most antibody levels, whereas placebo recipients display a very broad and general response to almost all antigens. Sharpest increases in intensity were observed for the early transcribed membrane protein ETRAMP10.2, an exported protein (EXP2), the Pf membrane protein VAR2CSA and the liver stage proteins LSA1 and serptentine receptor SR10 (Figure 4 A). Direct comparison of mean changes in signal intensities over the challenge phase in the three groups reveals a lack of induced reactive antigens in vaccinees compared to a large number of differentially recognized antigens in placebos (mean absolute fold change > 2 and $p < 0.05$ in Welch-corrected student´s t-test), amongst others particularly LSA1, the serine repeat antigen 3 (SERA3) or CSP (Figure 4 B). Observed differences proved not significant following Benjamini-Hochberg (BH) adjustment but a strong effect with Hedge´s $g > 0.8$ was given for almost all identified reactive antigens.

Similar changes appeared in the IgM antibody profile of placebo participants with median signal increases > 2 log levels in a variety of blood stage antigens (including the merozoite surface proteins MSP2 and MSA180, the ring exported protein REX1 and the parasite-infected erythrocyte surface protein PIESP2) alongside with LSA1, the DNA repair antigen RAD14, the sexual stage-specific protein precursor Pfs16 and an uncharacterized conserved protein (PF3D7_0407700; Figure 4 C). In direct comparison of mean signal intensities changes between both groups, no antigens are differentially recognized in the vaccinated group compared to placebo recipients, which in turn display a broad range of reactive antigens with particularly strong differences seen in various LSA1 fragments, CSP, exported proteins (including the PHISTb family member PF3D7_0532300) or a conserved protein (PF3D7_0817300; Figure 4 D).

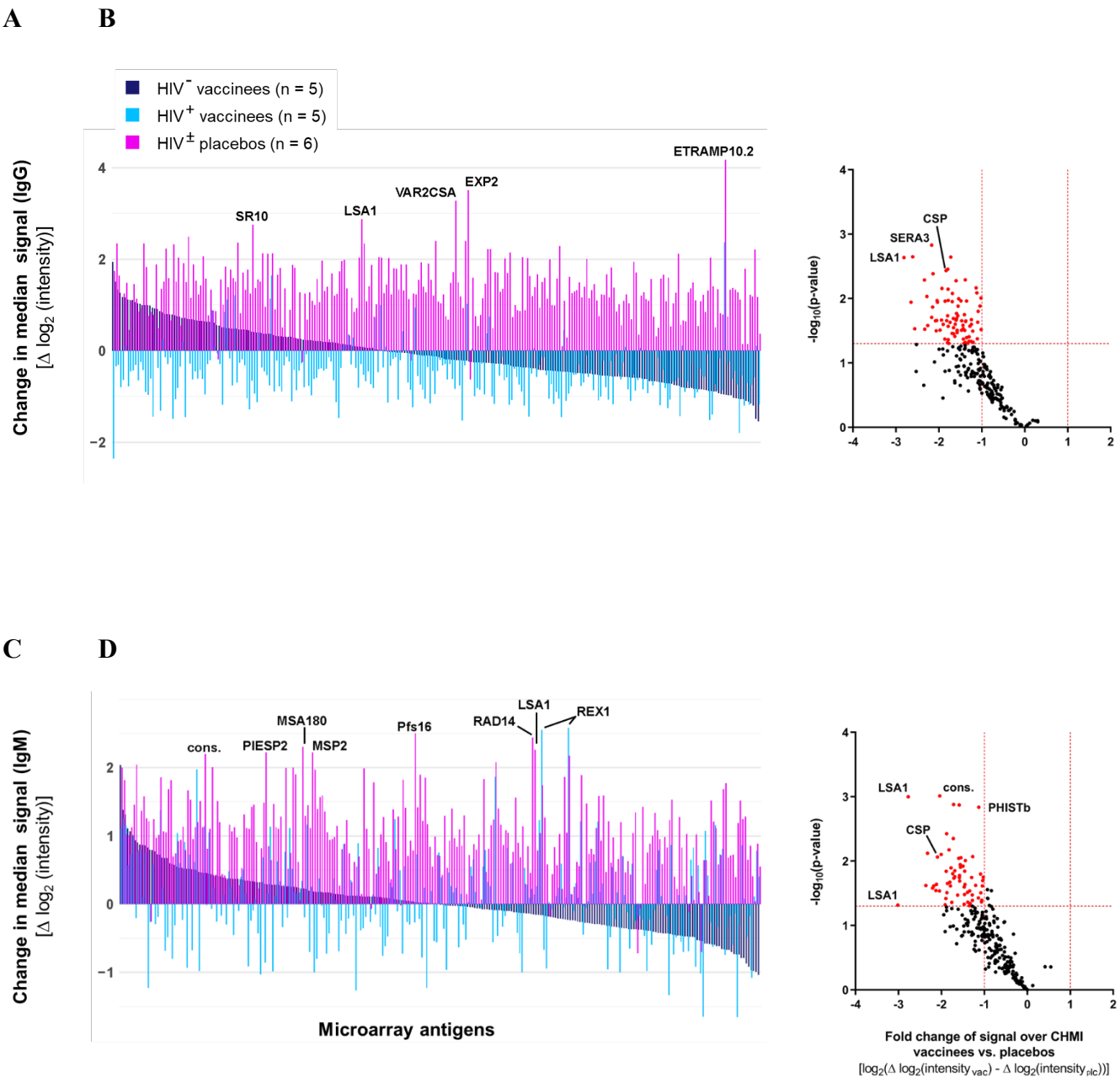


Figure 4. Changes in antibody repertoire induced by homologous challenge. Changes of microarray reactivity due to CHMI were identified by comparing signal intensities in samples collected four weeks after challenge to their individual reactivity levels after immunization. Study participants were allocated into placebo recipients (n = 6) and PfSPZ vaccinees (n = 10) with both groups containing equal numbers of HIV positive and negative subjects. (A, C) Median changes of IgG (A) and IgM (C) signal intensities over challenge are given for 262 microarray antigens. Antigens are sorted by median intensity changes in the vaccinees´ group. (B, D) Mean changes in microarray IgG (B) and IgM (D) signal intensities over CHMI in the vaccines were compared to the placebo group. Fold change and p-values (Welch-corrected

Student´s t-test) are given for all microarray antigens. Differentially recognized reactive antigens (p-value < 0.05 and fold change > 2) are depicted in red (see Table S1 for used antigen abbreviations). [conserved protein (cons., (C): PF3D7_0407700, (D): PF3D7_0817300), PHISTb (PF3D7_0532300)].

Kinetics of anti-CSP-IgG and anti-MSP5-IgM responses

Effects of the assigned study group (placebo, HIV-1 negative and HIV-1 positive vaccinees) and sample collection time point (baseline, immunized and challenged) on the kinetics of the two main vaccination-induced antibodies, anti-CSP-IgG and anti-MSP5-IgM, were evaluated using a two-way mixed ANOVA model (Figure 5 A and B). Study group and time point of sample collection both strongly influenced the measured CSP and MSP5 microarray signal intensity (interaction effects of $p = 0.003$ and $p = 0.001$, respectively). No effect of HIV-1 infection or protection status against CHMI on the measured antibody level was observed. IgG baseline reactivity against CSP was low in all study groups (median microarray signal intensities < 4 log levels). Following immunization, both HIV-1 positive and HIV-1 negative vaccinees similarly displayed elevated signals (median intensities > 6 log levels) compared to the unchanged reactivity in placebos (Bonferroni adjusted $p < 0.001$, post-hoc one-way ANOVA). Challenge induced no further increase in signal intensities amongst vaccinees but elevated antibody levels in placebo recipients, resulting in reduced group differences ($p = 0.003$). Antibody levels of anti-MSP5-IgM followed a very similar kinetic. Almost no baseline reactivity was detected in any of the study groups (median microarray signal intensities close to zero). Placebo recipients maintained a low reactivity throughout the immunization phase, whereas both HIV-1 positive and HIV-1 negative vaccinees strongly responded to the vaccine (Bonferroni adjusted $p = 0.003$, post-hoc one-way ANOVA). Notably, antibody levels of placebo recipients approached those of vaccinees after challenge (median signal intensities around 2 log levels).

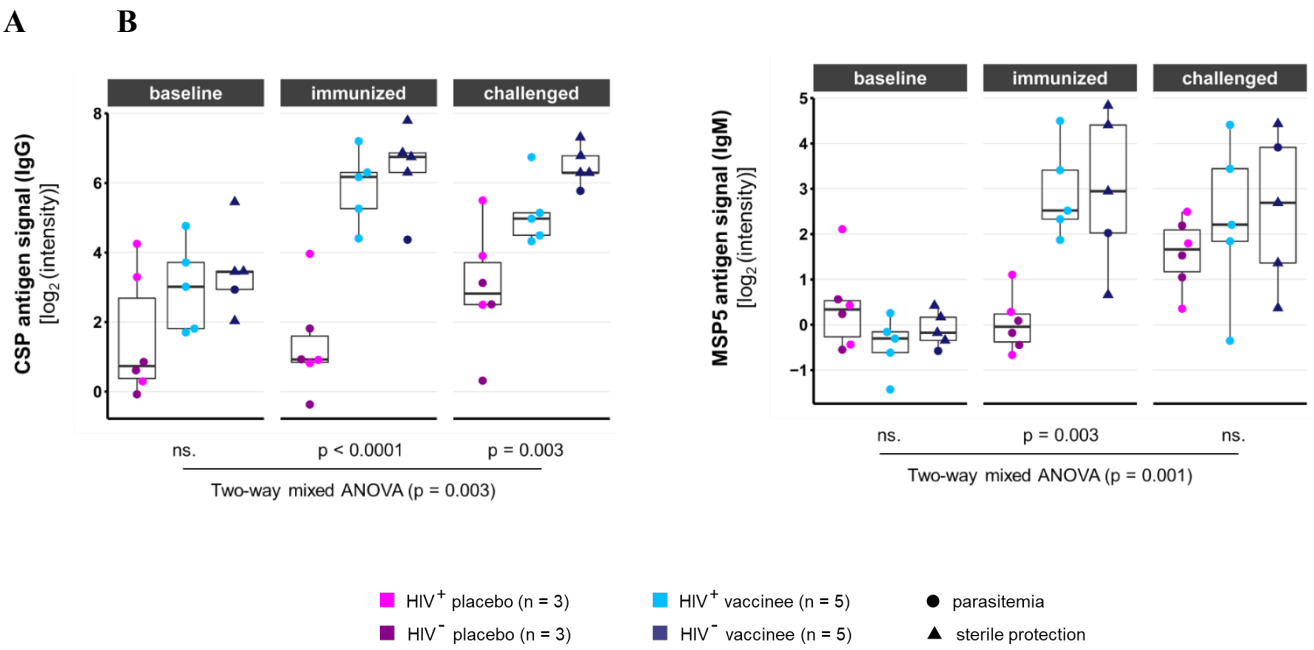


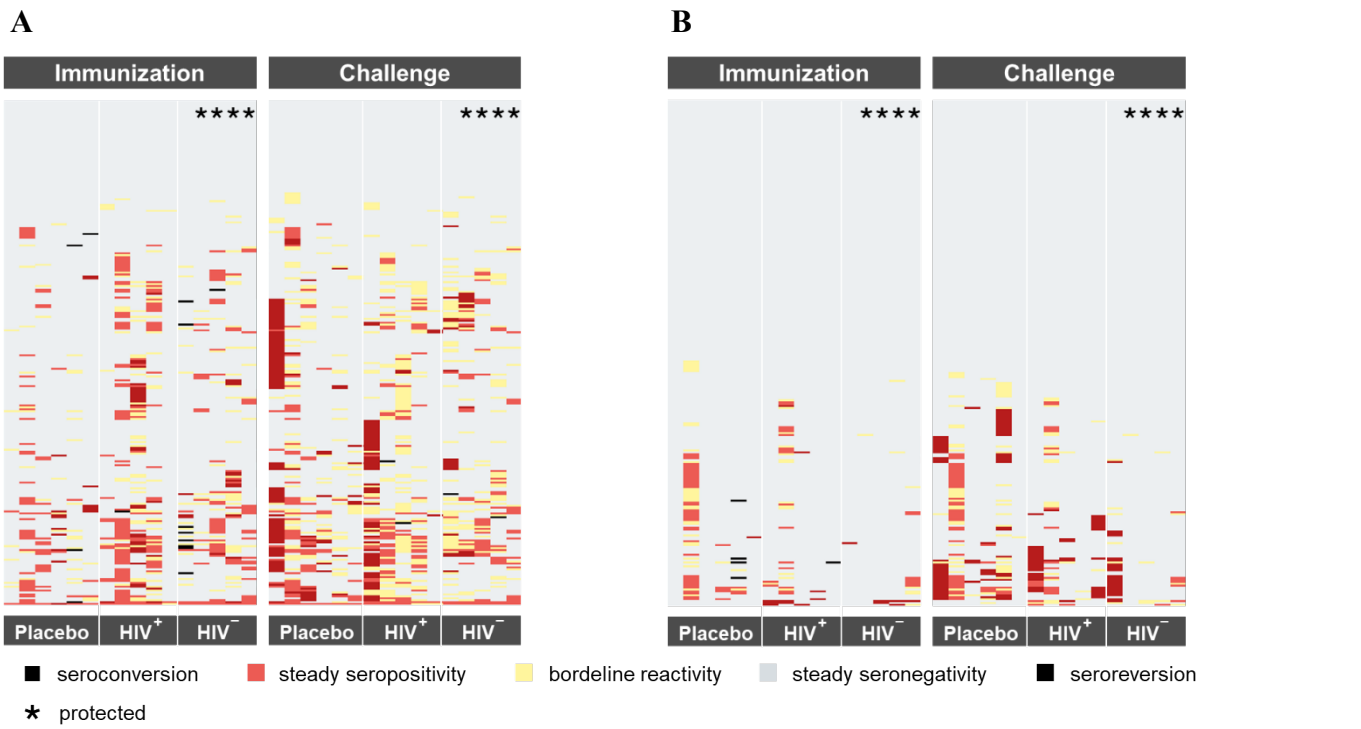
Figure 5. Antibody kinetics of vaccine-induced antibodies. Individual anti-CSP-IgG (A) and anti-MSP5-IgM (B) microarray intensities are compared at baseline, 14 days after immunization and 28 days after challenge. Samples were allocated to placebo recipients (n = 6), HIV positive (n = 5) and HIV negative vaccinees (n = 5). Subjects sterilely protected from challenge are marked with a triangular symbol. The influence of study group and sampling time point on the measured microarray antigen signal intensity was evaluated using a two-way mixed ANOVA model. Differences between the groups at single time points were assessed in a post-hoc one-way ANOVA, reporting Bonferroni-adjusted p-values. The boxplots give median antibody breadths, interquartile ranges (IQR) and whiskers of length 1.5 × IQR.

Individual acquisition of novel antibodies over immunization and challenge phase

Acquisition of novel antibody specificities following immunization and CHMI are presented for each volunteer individually (Figure 6 A and B). Seroconversion, defined as the acquisition of a novel antibody signal, was based on a signal exceeding the seropositivity threshold together with a signal increase of > 2 log levels between the two time points compared. Seroreversion, the disappearance of an antibody signal, was defined as drop of signal intensity of < 2 log levels between the two time points compared. Borderline reactivity is defined as signal fluctuations around the seropositivity threshold with intensity changes < 2 log levels between the time points compared. The majority of antigens were not recognized at all-time

points tested by IgG and IgM (Figure 6 A and B). Some antigens were recognized but remained unchanged upon PfSPZ Vaccination and CHMI while few antigens showed seroreversion. As already seen in Figure 3, few novel IgG or IgM antibodies binding the microarray presented antigens to were acquired during the immunization phase in vaccinees. Next, we compared serum reactivity before and after CHMI and as expected, the acquisition of novel antibody responses was highest in the volunteers who developed asexual blood stage parasitemia after CHMI (non-protected). In summary, a highly personalized response was observed for both IgG and IgM at baseline that was essentially maintained after PfSPZ vaccination and changed exclusively in volunteers that developed asexual blood stage parasitemia (non-protected).

Antibody breadth is defined as number of seropositive antigens recognized exceeding a predefined threshold of 3 log levels above the malaria-naïve control. Using a simplified model, considering only the individual changes in antibody breadth regardless of signal fold changes, confirmed that neither immunization nor challenge had a general impact on the IgG or IgM antibody repertoire within the study groups (Figures 6 C and D). No interaction effect for group and study phase on the change in antibody breath was detected ($p = 0.4$ for both IgG and IgM, robust two-way mixed ANOVA on trimmed means).


A
B
Immunization
Challenge
****
Placebo
HIV+
HIV−
seroconversion
steady seropositivity
bordeline reactivity
steady seronegativity
seroreversion
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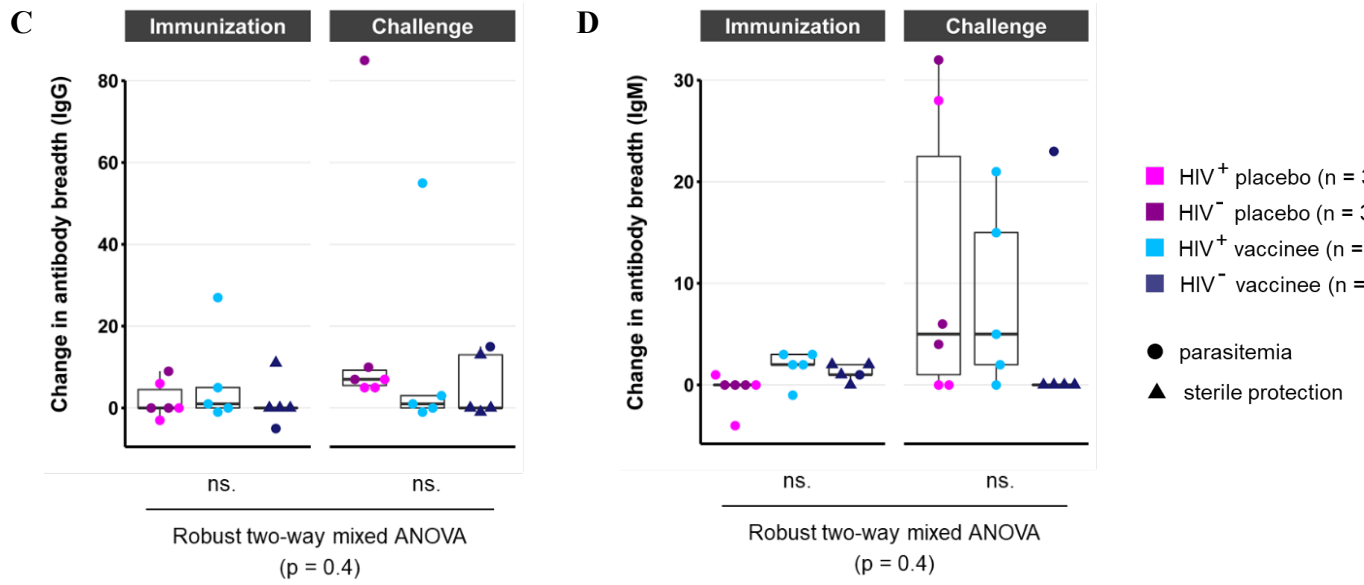
C
D
Immunization
Challenge
Change in antibody breadth (IgG)
Change in antibody breadth (IgM)
ns.
Robust two-way mixed ANOVA
(p = 0.4)
HIV+ placebo (n = 3)
HIV− placebo (n = 3)
HIV+ vaccinee (n = 5)
HIV− vaccinee (n = 5)
parasitemia
sterile protection

Figure 6. Antibody acquisition and change in antibody breadth. Changes in antibody repertoire over the immunization and challenge phase were compared between placebo recipients (n = 6), HIV positive (n = 5) and HIV negative vaccinees (n = 5). The individual antibody breadth gives the number of seropositive antigens at a certain time point (signal intensity of > 3 log levels above a malaria-naïve control). Seroconversion, the acquisition of a novel antigen, was defined as exceedance of the seropositivity threshold accompanied by a signal increase of > 2 log levels between the time points. Serorevesion was defined as drop below the threshold with a signal decrease of > 2 log levels. Smaller signal fluctuations around the threshold were designated as borderline reactivity. (A-B) Changes in antigen recognition after immunization and challenge are shown as heatmap for IgG (A) and IgM (B) with antigens depicted row-wise and samples column-wise. Subjects sterilely protected from challenge are marked with an asterisk. (C-D) Changes in the IgG (C) and IgM (D) antibody breadth over immunization and challenge are compared between the three study groups of placebos, HIV positive and negative vaccinees. The HIV status of placebo recipients is shown by different symbol colours. Protected participants are marked with a triangular symbol. The influence of study group and phase on the change in antibody breadth was evaluated using a robust two-way mixed ANOVA model. Differences between the groups at single time points were assessed in a post-hoc one-way ANOVA. The boxplots give median antibody breadths, interquartile ranges (IQR) and whiskers of length 1.5 × IQR.

Discussion

We report first time to our knowledge a comparative proteomic analysis of serum samples collected from HIV-1 positive and HIV-1 negative volunteers in a malaria-endemic area, undergoing either PfSPZ vaccination or placebo immunization followed by homologous CHMI. IgG and IgM antibody profiles of both PfSPZ and placebo vaccinees were assessed at baseline, two weeks after immunization and after challenge on a protein microarray comprising 262 selected Pf antigen fragments. Interestingly, humoral immune responses to immunization and CHMI did not substantially differ between HIV-1 positive and HIV-1 negative vaccinees despite the obvious lack of protective vaccine efficacy in the HIV-1 infected population that was undergoing ART (0 % vs. 80 % protection in HIV positive compared to HIV negative vaccinees). At baseline, the breadth of Pf-specific IgG repertoires was similar in the HIV-1 positive and HIV-1 negative study population. No gaps were detected in the antibody repertoire of the HIV-1 infected population but higher IgG titers against several liver and blood stage antigens including LSA1, MSP2, MSP4, MSP11, MSA180 and PHISTc while both groups showed a similar baseline reactivity against CSP and MSP5. IgG directed against MSP2 has been described as a marker of recent malaria exposure [25] pointing towards a higher pre-exposure due to increased malaria susceptibility in the HIV-1 positive study population [37]. In accordance to previously published results, our data confirm that despite the broad range of potential antigenic targets presented on whole sporozoites, PfSPZ vaccination hardly affects the overall antibody profile but induces only a very limited number of specific antibodies, most apparently anti-CSP-IgG and anti-MSP5-IgM.

CSP is already known as one of the most immunodominant antigens with regards to the PfSPZ Vaccine [38]. CSP is one of the best studied proteins at the molecular, functional and structural level reported **[39, 40]**. CSP is a protein of 40 – 60 kDa, contains an N terminus that aids attachment to host cells and is proteolytically processed during host cell invasion [41, 42]. The centrally localized NANP-repeat region is highly immunogenic and conserved across clinical *P. falciparum* isolates [43]. The C terminus contains several well-characterized CD4 and CD8 T cell epitopes [44, 45]. CSP undergoes conformational shifts throughout the life cycle of the parasite and unveils functionally essential important epitopes prior to hepatocyte invasion **[46, 47]**. An elegant study of Kumar et al., has shown that although hundreds of other Plasmodium genes are expressed in sporozoites, CSP is an immunodominant and also protective antigen [48]. Peptide microarray analysis of serum samples taken before and after PfSPZ Vaccination in adult Tanzanian volunteers inoculated five times with a total dose of 1.3 x 10^6 sporozoites

(BSPZV1 study) demonstrated that IgG antibodies targeting CSP is one of the immunodominant antigen recognized [38]. Purified IgM antibodies specific for CSP obtained from serum samples of the same cohort activated complement and blocked in vitro invasion of purified sporozoites into hepatocytes supporting the view that PfPSZ vaccine induced IgM targeting CSP contribute functionally to vaccine induced protection [49]. Using a second, newly designed microarray panel and printed antigens, these findings are confirmed and extended here. CSP specific IgG and IgM are induced upon PfSPZ Vaccination in malaria pre-exposed volunteers, irrespective of their HIV-1 infection status.

In addition to CSP, all vaccinees developed IgG and especially IgM against MSP5 past vaccination. The *msp5* gene codes for a 272-residue protein with a C-terminal EGF-like domain and a GPI attachment motif [50]. MSP5 is located on the surface of merozoites and seems to be highly conserved **[51]**. The biological function of MSP5 is apparently not critical for blood stage parasite survival since viable knock-out mutants can be isolated with no apparent growth defect under *in vitro* culture conditions [52]. However, both proteomic and transcriptomic evidence also suggests the expression of MSP5 during the salivary gland sporozoite stage of *P. falciparum* but its subcellular localization as well as its potential functionality in this pre-erythrocytic stage remains unknown [53-55]. Antibodies against MSP5 have indeed been shown to be associated with protection from malaria in a mouse model [56, 57] and from clinical malaria in field studies in Guinea [58, 59], thus further studying seems worthwhile.

CHMI of placebo recipients induced a very broad IgG and IgM response to most antigens represented on the array, with sharpest increase in antibodies against some typical infection markers such as ETRAMP10.2 [60, 61] reflecting the observed blood stage parasitaemia. In contrast, both HIV-1 positive and HIV-1 negative vaccinees hardly reacted to any of the antigens with antibody titers rather declining over the challenge phase. In particular, no boosting effect of the novel vaccination-acquired IgG and IgM antibodies directed against CSP and MSP5 was observed. The only exception is a clear IgG response to ETRAMP10.2 and IgM response to REX1 in HIV positive vaccinees, again marking the clinical infection.

The lack of immunogenicity of CHMI in HIV-1 negative vaccinees is sufficiently explained by the fact that most of these volunteers were sterilely protected in the challenge, indicating little exposure to blood stage infection since parasites do not egress from the liver or are

immediately cleared. A previous protein microarray study already found protection from *P. vivax* infection to be associated with an attenuated response to CHMI [62]

However, none of the HIV-1 positive vaccinees was protected from CHMI, thus an equally broad humoral immune response as observed amongst the placebos might have been expected. A reduced B-cell functionality due to HIV infection seems unlikely since all infected participants are under ART and the CD4+T cell counts did not substantially differ from the HIV-1 negative population at baseline. Also, the antibody profiles observed at baseline did not differ between these two groups of vaccinees.

The humoral immune response to CHMI in this study is neither determined by HIV-1 infection nor protection status but solely depends on whether the PfSPZ vaccine was administered. Even in CHMI unprotected vaccinees, is it conceivable that the immunization induces some effect in reducing the parasite burden egressing from the liver, as it is also reflected in a longer pre-patent time to treatment in unprotected vaccinees compared to placebo recipients. Findings again support former *P. falciparum* microarray studies suggesting a strong correlation between the cumulative parasite exposure and measured increase in antibody intensities over CHMI [60]

In the absence of effective antiretroviral treatment, HIV-1 infection results in a steady and progressive decline of CD4+ lymphocytes, decreased ability to mount effective immune responses to novel stimuli, loss of already existing immunity and increased complications from infections [63]. Similar to other immunosuppressive conditions, HIV-1 infection can impair the effectiveness of vaccines and increase the risk of serious adverse events, particularly from live vaccines like BCG and yellow fever [63]. The magnitude of vaccine induced antibody response is often inversely correlated with the CD4+ lymphocyte count and fewer HIV-1 infected children and adults develop protective antibody levels after vaccination compared to HIV-1 uninfected persons. Vaccine induced antibody responses decline at a faster rate in HIV-1 infected versus HIV-1 non-infected people. Generally, routine vaccines given to HIV-1 positive adults seem to result in lower antibody titers when compared to age matched HIV-1 negative groups [64]. Here, the cohort of HIV-1 positive volunteers enrolled into the BSPZV3a study had stable CD4+ lymphocyte counts > 500 cells/µl and were classified as WHO stage 1 of HIV1 infection. All volunteers, irrespective of their HIV infection status developed antibodies against CSP and MSP5. While all HIV-1 positive volunteers were non-protected against homologous CHMI, one out of five HIV-1 negative volunteers (80 %) were non-protected. This stark difference in protective efficacy in the absence of clear differences of

malaria specific antibody-breath and pattern at baseline and after PfSPZ Vaccination strongly indicates that cellular immune effector mechanisms either interacting with vaccine induced antibodies or on their own are essential for PfSPZ Vaccine induced protection.

Authors' contributions

Planning of study: CD, RF, BM,

Conduct of microarray experiment: JD, AT, FRL, RF,

Analysis of data: FRL, RF, CD, BM

Supervision of study: CD, RF

Writing of manuscript: AT, FRL, CD, RF,

Clinical study design: SA, SJ, SHL

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Competing interests

The authors declare that they have no competing interests. SLH is salaried, full-time CSO and CEO of Sanaria Inc., the developer and sponsor of Sanaria PfSPZ Vaccine.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article as supplementary tables.

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Chapter 5

Role of Pegivirus infections in whole *P. falciparum* sporozoite vaccine induced humoral immunity and controlled human malaria infections in African volunteers

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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. Overall, HPgV-1 viremia levels were not significantly altered after CHMI.

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, whole sporozoite vaccination, human pegivirus, HPgV-1, CHMI, 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) (Supplementary 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 Supplementary 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) (Supplementary 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 (Supplementary Fig. 1 B–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,

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 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: 55ºC for 10 min, 95ºC for

1 min, 45 cycles at 95ºC for 15 sec and 55ºC 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 50ºC and 10 min at 94ºC. 3 ul of cDNA generated by reverse transcription were used for the first round of PCR amplification with forward primer 5'-AAAGGTGGTGGATGGGTGATG-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 95ºC, followed by 28 cycles of 95ºC for 30 sec, 56ºC for 30 sec and 72 ºC for 30 sec with a final extension step at 72ºC 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 out-group. 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

297 calculated from the time of PfSPZ challenge to first positivity detected by qPCR and TBS [9,
298 10, 29, 30]. Parasite multiplication rate (PMR) was assessed using a linear model fitted to log10-
299 transformed qPCR data as published [44]. PMR was calculated for all volunteers that developed
300 asexual blood-stage parasitemia which lasted for at least two 48-hour cycles [44].
301
302 **Statistical analysis**
303 Figures and statistical analyses were generated in R version 3.5.1 and GraphPad Software
304 (Prism V5). Wilcoxon rank sum test or Mann-Whitney test were used to compare continuous
305 variables. Chi-square test was used to compare categorical variables. Absolute values for
306 antibody titers and concentrations of cytokines and chemokines were plotted. Data were log
307 transformed only when investigating the anti-PfCSP antibody titres and viremia levels.
308 Spearman correlation was used to investigate the potential effect of HPgV-1 infection status
309 and viremia with antibody titres and cytokine levels. Data for cytokines, chemokines and
310 growth factors were not analysed for multiple correction as we considered this question as
311 exploratory. P-value $\leq$ 0.05 was considered significant. Differences in viral diversity,
312 abundance and prevalence were assessed using Linear discriminant analysis effect size [45] and
313 GraphPad Software (Prism V5), respectively.
314
315 **RESULTS**
316
317 **Unbiased search for RNA molecules encoding human viruses**
318 We aimed to identify viruses present in peripheral blood of our volunteers participating in
319 PfSPZ Vaccine studies. These analyses included samples from 28 participants of the BSPZV1
320 study collected at multiple time points including baseline, 2 days after first vaccination, 7 days
321 after the first and second vaccination and before CHMI, 2 and 9 days after CHMI. Sequences
322 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 (Supplementary 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. 2 B-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 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 (Fig. 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 EGSPZV2. 23 cytokines, chemokines and growth factors were detected above their pre-defined lower limits of detection (Supplementary Table 1). Although there was a trend of overall higher cytokine levels in HPgV-1 infected individuals (Supplementary 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

399 IL-2 and IL-17A might contribute to differentiation of naïve B cells into plasma cells and
400 support the survival of activated B cells [46, 47]. We examined the potential of HPgV-1
401 infection to impact on PfSPZ vaccine-induced humoral immunity. PfCSP is the most immuno-
402 dominant protein recognized after PfSPZ vaccination. Anti-PfCSP titres were measured at
403 baseline in all volunteers (n=70) and 14 days past last vaccination in all vaccinated volunteers
404 (n=54) participating in BSPZV2, BSPZV3a and EGSPZV2 (Fig. 7 A-B). Similar results were
405 observed when PfSPZ vaccine-induced antibody responses were analysed as net increase (titres
406 at 14 days post last vaccination minus baseline titres) (Fig. 7 C) and as fold change (titres 14
407 days past last vaccination divided by baseline titres) (Fig. 7 D). There was no significant
408 correlation between HPgV-1 infection status and anti-PfCSP antibody titre at baseline and after
409 vaccination.
410
411 **Effect of HPgV-1 infection on PfSPZ vaccine efficacy**
412 The high prevalence of HPgV-1 positive volunteers in our cohort allowed us to investigate a
413 potential impact of ongoing viral infection during PfSPZ vaccination on vaccine-induced
414 protection. Protective efficacy of the vaccine was evaluated by presence or absence of asexual
415 blood-stage parasitemia following homologous PfSPZ challenge (CHMI) (Supplementary Fig.
416 3). While none of the placebo-receiving participants was protected (0/20), the overall protection
417 in the vaccinated group was 55% (26/47). The HPgV-1 prevalence was comparable in these
418 two groups (placebos and vaccines) with 35% (7/20) versus 34% (16/47), respectively,
419 suggesting that HPgV-1 infection does not facilitate protection against CHMI. We further
420 compared the CHMI protection levels in HPgV-1 positive and negative participants in the
421 vaccinated group only (Fig. 8A). HPgV-1 positive vaccinees showed slightly higher protection
422 levels after CHMI (62.5%; 10/16) compared to HPgV-1 negative individuals (51.6%; 16/31).
423 We also assessed anti-PfCSP antibodies titres at the peak response which is 14 days past last
424 vaccination. Slightly higher anti-PfCSP levels were seen in protected compared to non-

425 protected individuals (Fig. 8B), without reaching statistical significance. PfCSP antibody levels
426 tended to be lower in the HPgV-1 positive individuals (Fig. 8B).
427
428 **Interaction of HPgV-1 and controlled human malaria infection induced asexual blood-**
429 **stage parasites**
430 HPgV-1 co-infection has been associated with favourable clinical outcomes in HIV-1 and Ebola
431 co-infected individuals [21]–[23]. So far, the HPgV-1 impact on *P. falciparum* infection and
432 immunity is unknown. We evaluated parasite multiplication rates and pre-patent periods in the
433 placebo volunteers only, that have not been vaccinated (n=20) undergoing CHMI. Comparable
434 asexual blood-stage multiplication rates and pre-patent periods were observed between HPgV-
435 1 positive and negative individuals (Fig. 9 A-B).
436
437 **Effect of CHMI on HPgV-1 viremia levels**
438 *P. falciparum* infection is known to impact viremia levels of some common viruses like HIV-1
439 and EBV [48, 49]. We therefore evaluated the effect of an acute *P. falciparum* infection on
440 HPgV-1 viremia by comparing the viral load before and 28 days post CHMI in 21 eligible trail
441 participants. Detectable levels of HPgV-1 at both time points were quantified in 9 individuals;
442 5 of those showed an increased HPgV-1 viral load and the other 4 had decreased viremia post
443 CHMI (Fig. 9C). In addition, 12 trial participants were HPgV-1 positive only for one of the two
444 tested time points; 6 were positive before CHMI and 6 were positive at 28 days post CHMI
445 (Fig. 9C).
446
447
448 **Discussion**
449 The role of chronic asymptomatic viral infections in modulating immune responses in health
450 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.

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].

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

503 recruited from East and West-central Africa, we had expected to find higher HPgV-1 genetic
504 diversity. Studies in neighbouring countries including Cameroon, the Democratic Republic of
505 Congo and Gabon revealed a high prevalence of genotype 1 [61]–[65]. Genotypes 2 and 5 were
506 also seen when phylogenetic studies included molecular markers other than 5' UTR region like
507 envelope protein 1 (E1), non-structural protein 3 (NS3) and non-structural protein 5A [62], [66].
508 The limitations of amplification of the 5' UTR, a highly conserved region, to discriminate
509 closely related isolates is known [67]. Due to its high variability, E2 provides better genotyping
510 resolution compared to 5' UTR. We amplified and sequenced the E2 region from subjects with
511 high viremia levels in serum (n=9). Based on the E2 sequences, these 9 isolates clustered with
512 strains described elsewhere in Africa. It is possible that the failure to amplify E2 from all
513 volunteers positive by 5' UTR detection is either due to the low sensitivity of the assay used or
514 the high genetic diversity of the E2 region [42]. While it is known that the detection of HPgV-
515 1 based on amplification of the E2 region is highly specific, it requires higher amounts of RNA
516 input [42] and individuals with low HPgV-1 viremia are likely missed. Alternatively, it is
517 possible that E2 genetic variants could not be amplified with the primers used due to nucleotide
518 sequence mismatch. The E2 region is highly variable and this diversity contributes to structural,
519 functional and immunogenic properties of the virus [68]. The inconsistent genotyping results
520 of isolates 6EG and 14EG based on 5' UTR and E2 amplification might be resolved by whole
521 genome sequencing of the virus. Vitrenko et al., reported similar findings in samples from
522 Ukrainian females donating fetal tissues [67].
523
524 Cytokines, chemokines and growth factors are important for inter-cellular communication and
525 regulation of immune processes [69]. Any changes in levels of these immune mediators can act
526 as markers of inflammation, immunity or vaccine uptake [26, 70, 71]. We therefore investigated
527 if altered levels of cytokines and chemokines unique to ongoing HPgV-1 infection could be
528 identified. We analysed serum samples taken at baseline for 45 cytokines in a Luminex

529 platform. Volunteers with chemokine and cytokine levels above the lower limit of detection
530 were stratified according to the HPgV-1 infection status. Of all 23 differentially detected
531 cytokines and chemokines, IL-2 and IL-17A were significantly higher in HPgV-1 positive
532 compared to HPgV-1 negative individuals.
533
534 IL- 2 is an essential survival factor for T and B lymphocytes [47, 72] and induces the
535 development and survival of regulatory CD4 T cells critical for the maintenance of immune
536 tolerance [73]. Fama et al., showed increased levels of circulating soluble IL-2 receptor (sIL-
537 2R) in HPgV-1 positive volunteers but the authors did not quantify IL-2 levels [74]. The
538 increased concentrations of IL-2 seen amongst the HPgV-1 positive individuals could be linked
539 to either on-going antiviral immunity [75] or serves as a survival mechanism used by the virus
540 to establish persistence in immune cells. A similar mechanism has been described in the
541 apicomplexan pathogen *Theileria parva* that infects T and B lymphocytes in cattle [76].
542 Contrary to our observations are results from HPgV-1/HIV-1 coinfection studies which have
543 shown reduced T-cell activation and IL-2 release in coinfected individuals [77, 78]. The HPgV-
544 1 envelope protein 2 (HPgV1-E2) has been implicated in these outcomes, due to its ability to
545 inhibit T cell-receptor mediated signalling and IL-2 signalling pathways [77, 78].
546
547 IL-17A induction has been associated with bacterial, fungal, autoimmune and inflammatory
548 diseases [79]. IL-17A stimulates production of chemokines such as monocyte chemoattractant
549 protein-1 which mediates tissue infiltration of monocytes. The role of IL-17A in the context of
550 HPgV-1 infection is unknown. However, in other viral infections like HIV-1 and Hepatitis C,
551 IL-17A has been shown to promote T-cell mediated anti-viral responses through activation and
552 recruitment of dendritic cells, monocytes and neutrophils [80, 81]. Other cytokines and
553 chemokines which could be detected, albeit not significantly different in HPgV-1 positive
554 individuals included SCF (lower) and IL-1beta, IL-12p70, MCP-1, LIF, VEGF.A, HGFand

555 TNF-α (higher). BDNF, EGF, Eotaxin, GRO-alpha, IFN-ɣ, IL-7, IP-10, MIP1-a, Mip-1b,
556 PDGF.BB, PIGF.1, RANTES, SDF-1a, and VEGF.D were comparable between the two groups.
557 The levels of these measured cytokines and chemokines are within ranges previously reported
558 [26, 27]. While most of the previous HPgV-1 studies had focused on at risk populations,
559 particularly on HIV-1 positive persons, our investigations are in healthy individuals [26, 74],
560 therefor some of the observed differences could be due to health status.
561
562 Here, we observed lower, albeit not statistically significant, median anti-PfCSP titres in the
563 HPgV-1 positive versus the negative group at baseline and 14 days past last vaccination. These
564 observations mirror findings by Avelino-Silva et al., who showed no association between
565 HPgV-1 infection status/viremia with yellow fever specific neutralizing antibody titres in HIV-
566 1 positive individuals immunized with yellow fever vaccine [82]. While studies have
567 extensively tried to understand potential inhibition mechanisms induced by HPgV-1 (and other
568 Flaviviruses) on T cell activation [77, 83], activation pathways that might be affected in B cells
569 are less explored. It is also possible that the effect of HPgV-1 viruses on immune responses
570 against vaccines is negligible when studied singly, but this impact is significantly synergized in
571 the presence of other, co-infecting viruses like EBV, CMV and HSV [85, 86]. Hence, the
572 potential role played by the combined human virome in shaping vaccine-induced responses in
573 different populations needs to be further explored in larger cohorts.
574
575 Clinically silent, chronic viral infections are known to modulate host immunity [16] and in turn,
576 acute co-infections are known to drive the re-activation of asymptomatic viral infections [49].
577 Several viruses, like HIV-1, Ebola and HCV have been implicated in the pathogenesis and
578 clinical outcome of ongoing malaria infections through a range of different mechanisms [84]–
579 [86]. It has been suggested that HIV-1 infections worsen *P. falciparum* presentations by
580 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.

List of 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

DECLARATIONS

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

Availability of data and materials

Data are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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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

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Figures, tables and additional files:

Figure 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 0-3; green indicating low number and red high number of reads.

Figure 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 18-35 years of age. Chi square with Yates correction for group comparisons (*, $P<0.05$).

Figure 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.

Figure 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.

Figure 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.

Figure 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.

Figure 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.

Figure 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 hours 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.

Figure 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

Supplementary Materials

Sup. Table 1: Sensitivity and standard curve range for the 45 cytokines, chemokines and growth factors

The tables shows the 45 cytokines, chemokines and growth factors their sensitivities and standard curve ranges.

Sup. Figure 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

Sup. Figure 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 (PIGF.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.

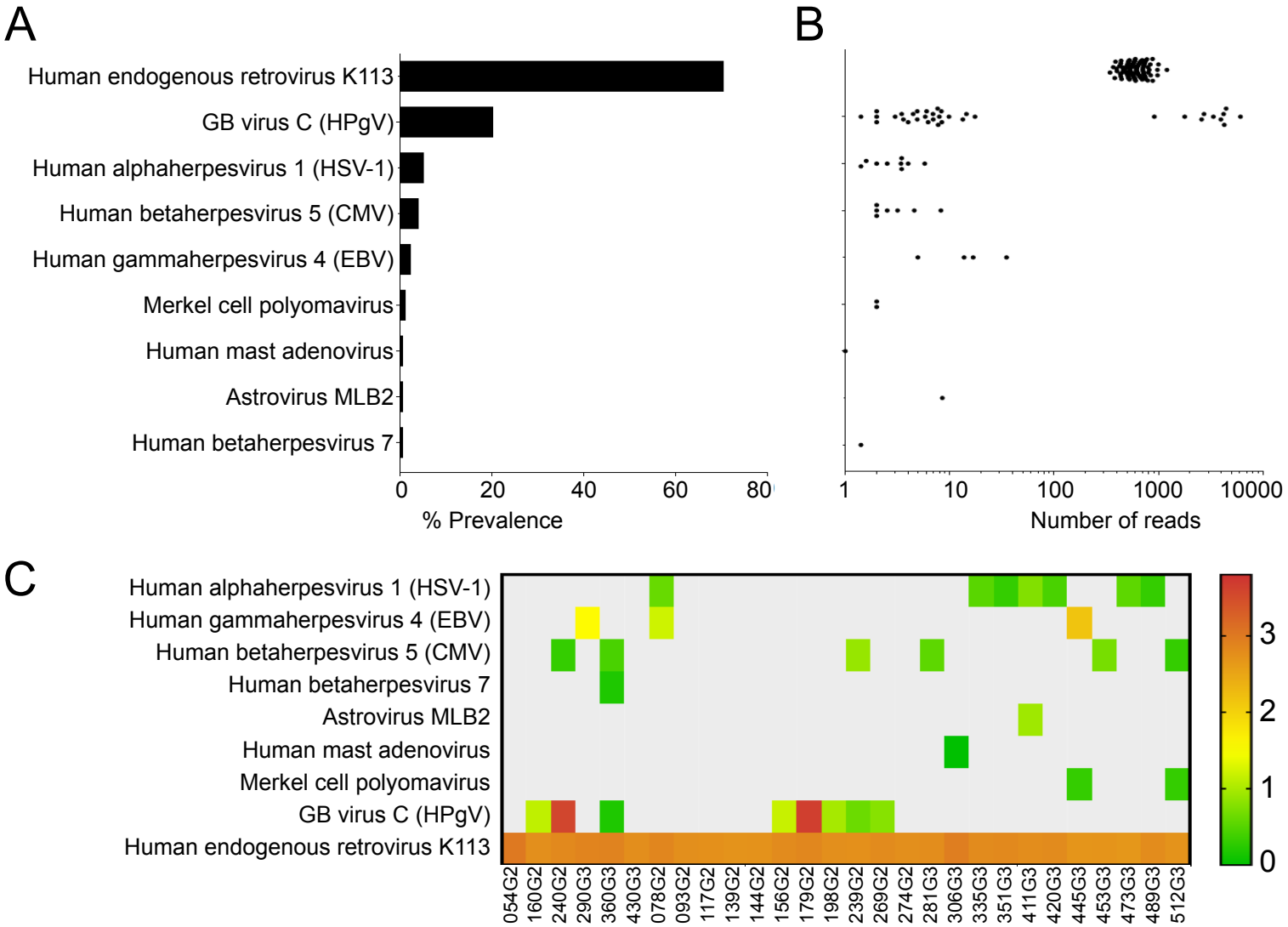
Sup. Figure 3: Vaccine trial design and procedures.

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.

Sup. Figure 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.


A
Human endogenous retrovirus K113
GB virus C (HPgV)
Human alphaherpesvirus 1 (HSV-1)
Human betaherpesvirus 5 (CMV)
Human gammaherpesvirus 4 (EBV)
Merkel cell polyomavirus
Human mast adenovirus
Astrovirus MLB2
Human betaherpesvirus 7
0
20
40
60
80
% Prevalence
B
1
10
100
1000
10000
Number of reads
C
Human alphaherpesvirus 1 (HSV-1)
Human gammaherpesvirus 4 (EBV)
Human betaherpesvirus 5 (CMV)
Human betaherpesvirus 7
Astrovirus MLB2
Human mast adenovirus
Merkel cell polyomavirus
GB virus C (HPgV)
Human endogenous retrovirus K113
054G2
160G2
240G2
290G3
360G3
430G3
078G2
093G2
117G2
139G2
144G2
156G2
179G2
198G2
239G2
269G2
274G2
281G3
306G3
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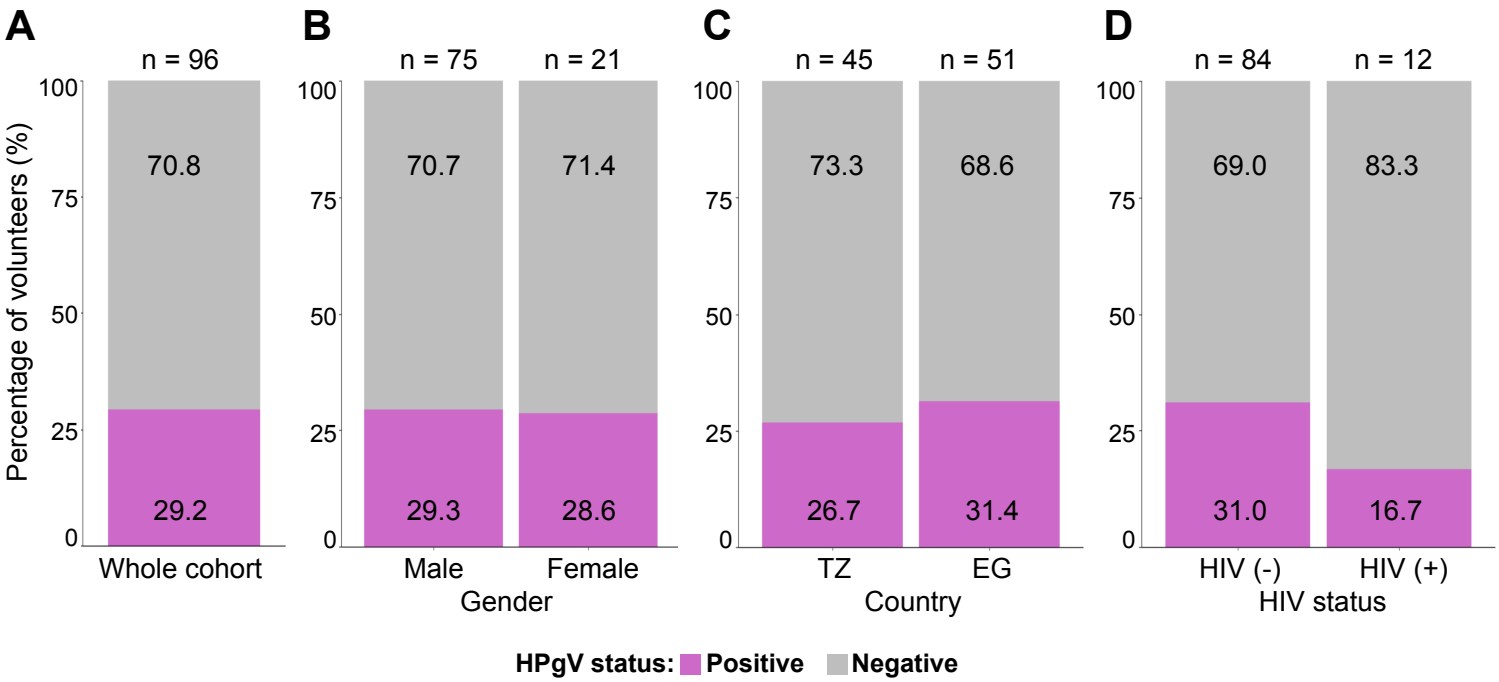
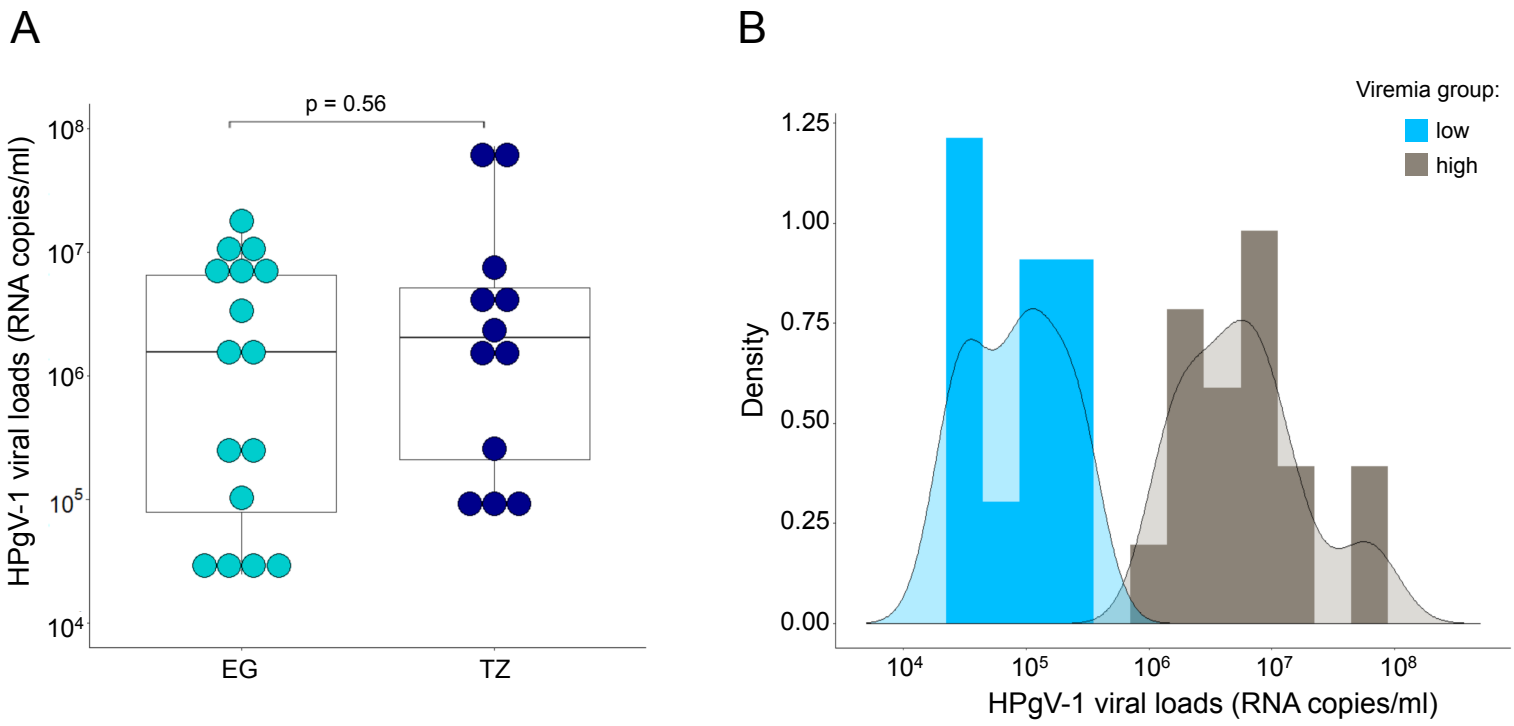
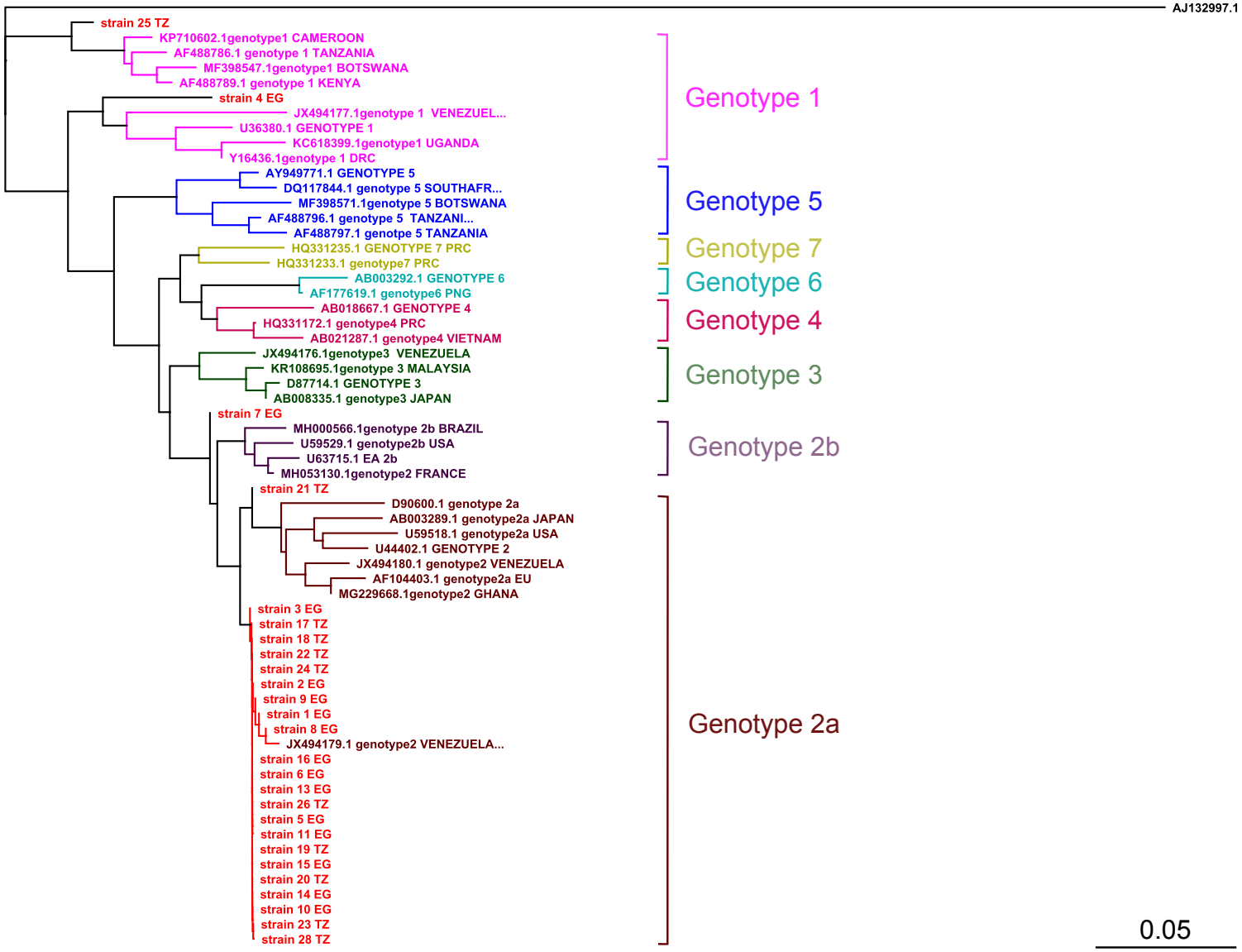
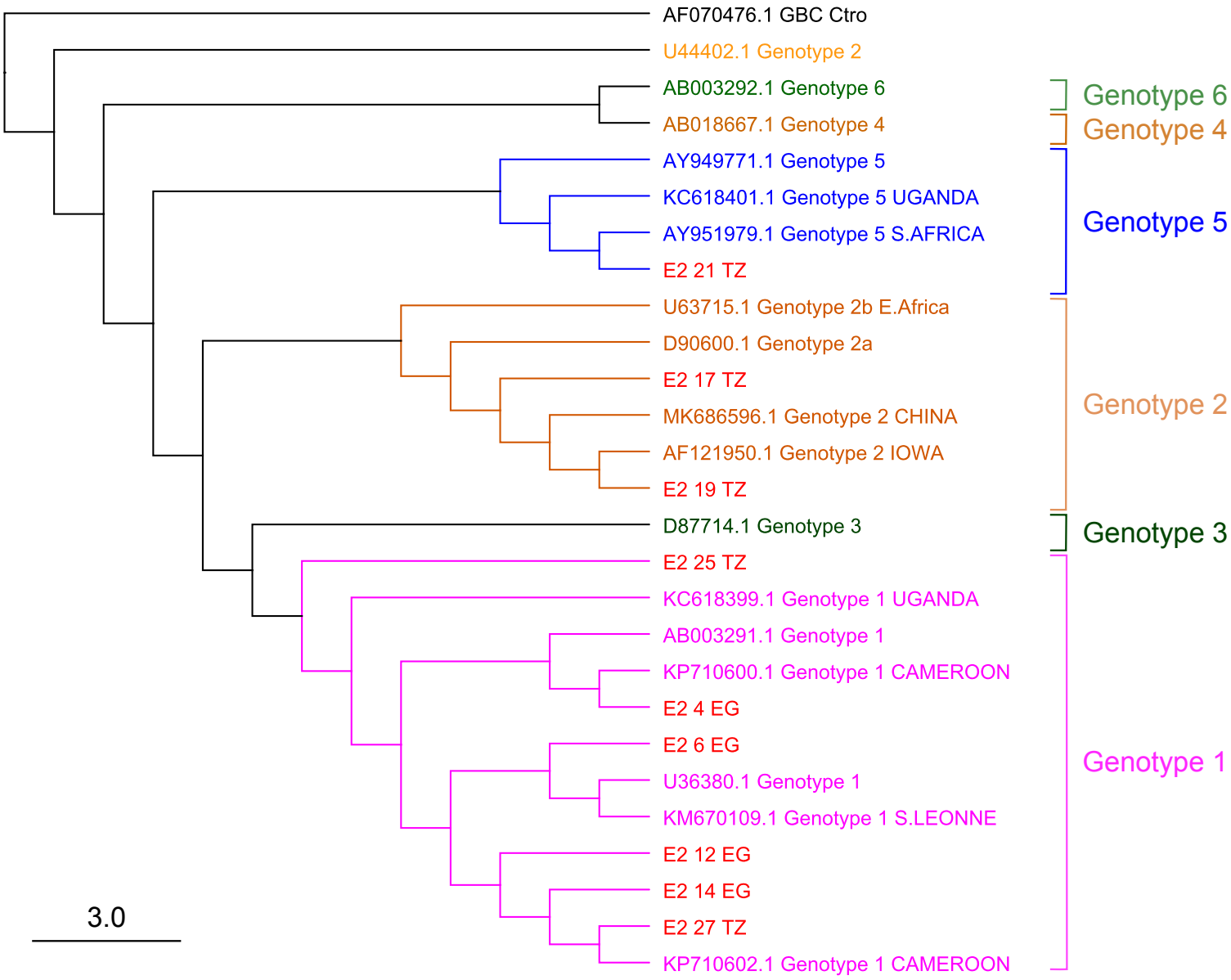
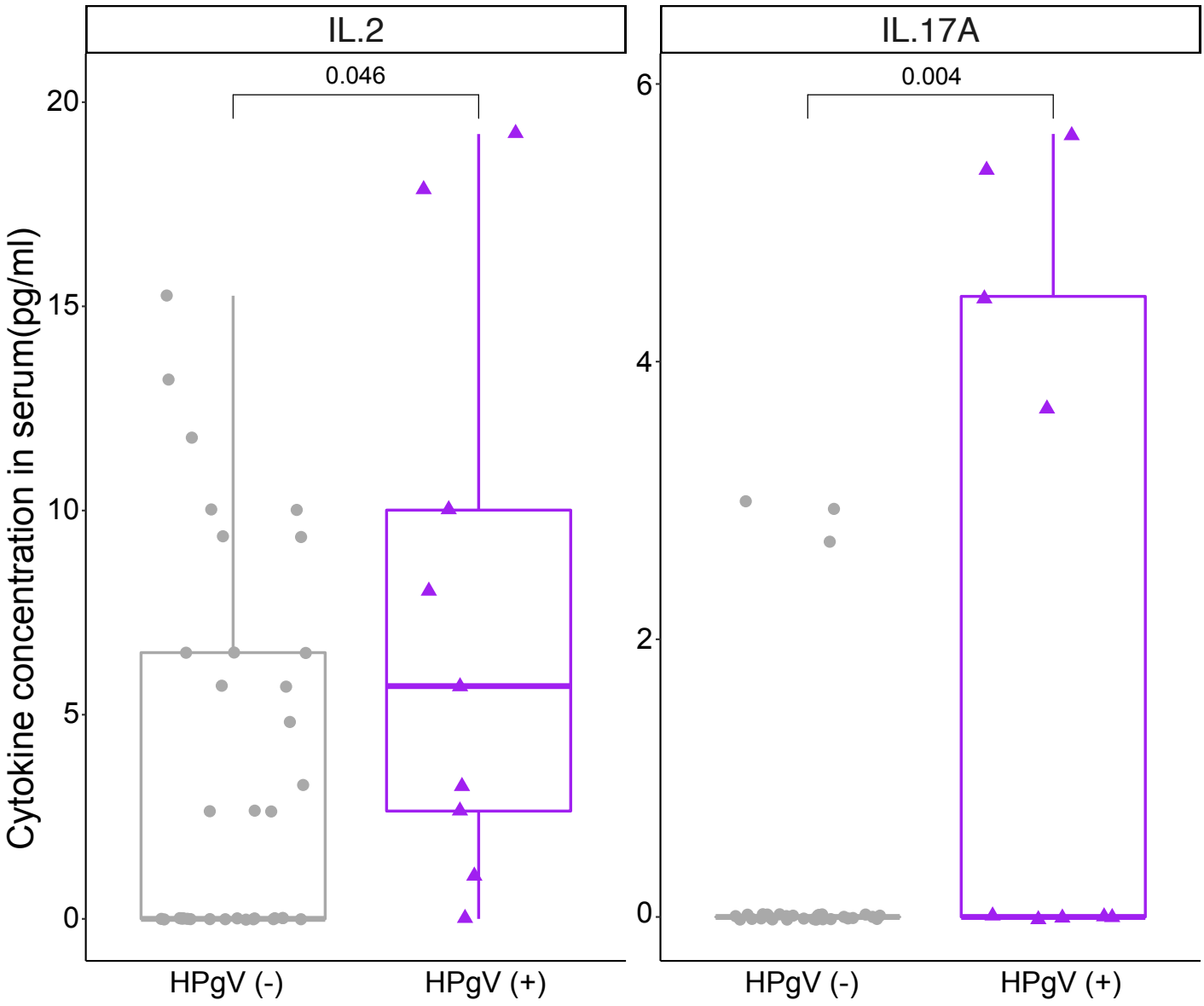
420G3
445G3
453G3
473G3
489G3
512G3
3
2
1
0

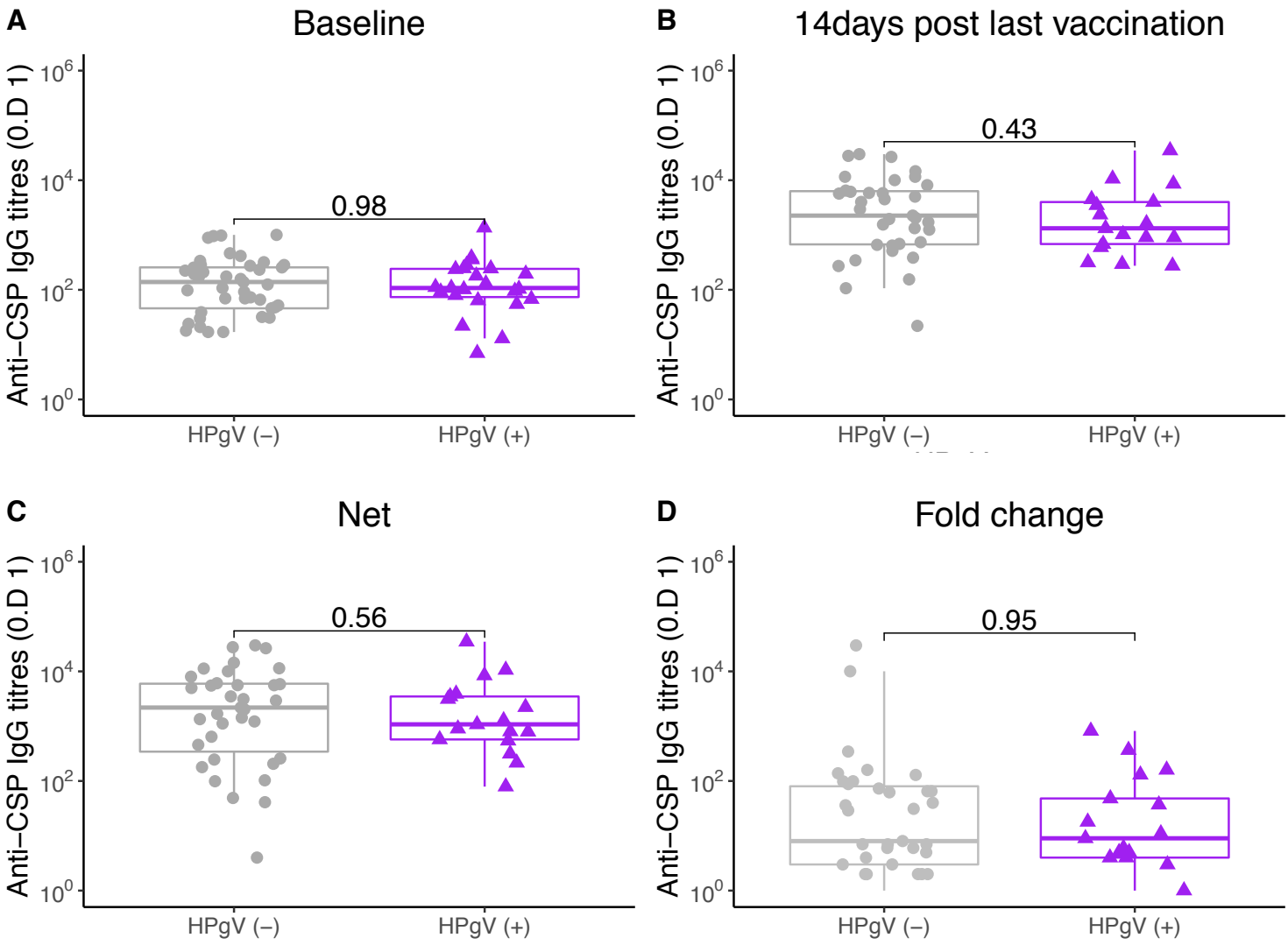

A
B
C
D
n = 96
n = 75
n = 21
n = 45
n = 51
n = 84
n = 12
Percentage of volunteers (%)
100
75
50
25
0
70.8
29.2
70.7
29.3
71.4
28.6
73.3
26.7
68.6
31.4
69.0
31.0
83.3
16.7
Whole cohort
Male
Female
Gender
TZ
EG
Country
HIV (-)
HIV (+)
HIV status
HPgV status: Positive Negative

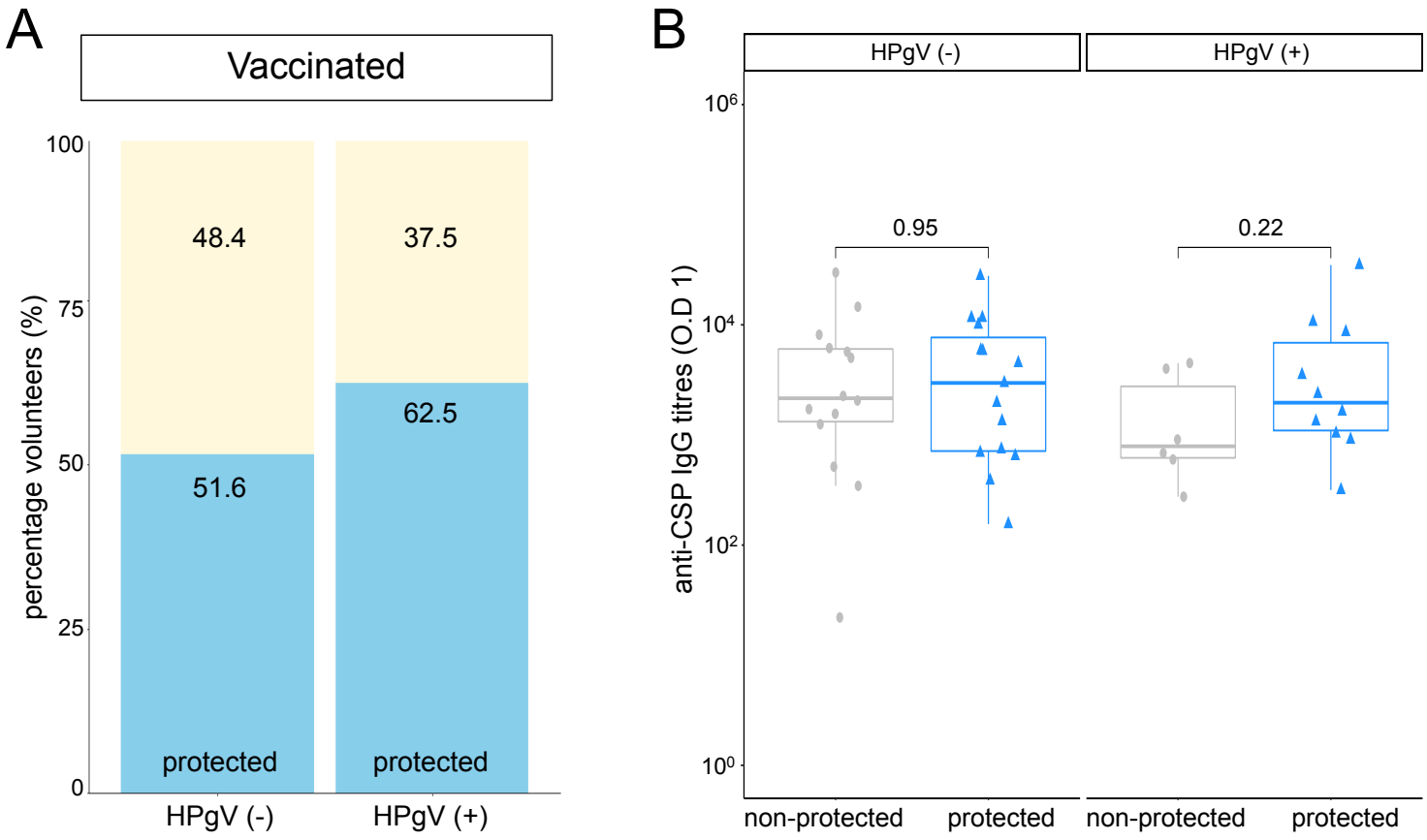

A
p = 0.56
HPgV-1 viral loads (RNA copies/ml)
10^8
10^7
10^6
10^5
10^4
EG
TZ
B
Viremia group:
low
high
Density
1.25
1.00
0.75
0.50
0.25
0.00
10^4
10^5
10^6
10^7
10^8
HPgV-1 viral loads (RNA copies/ml)

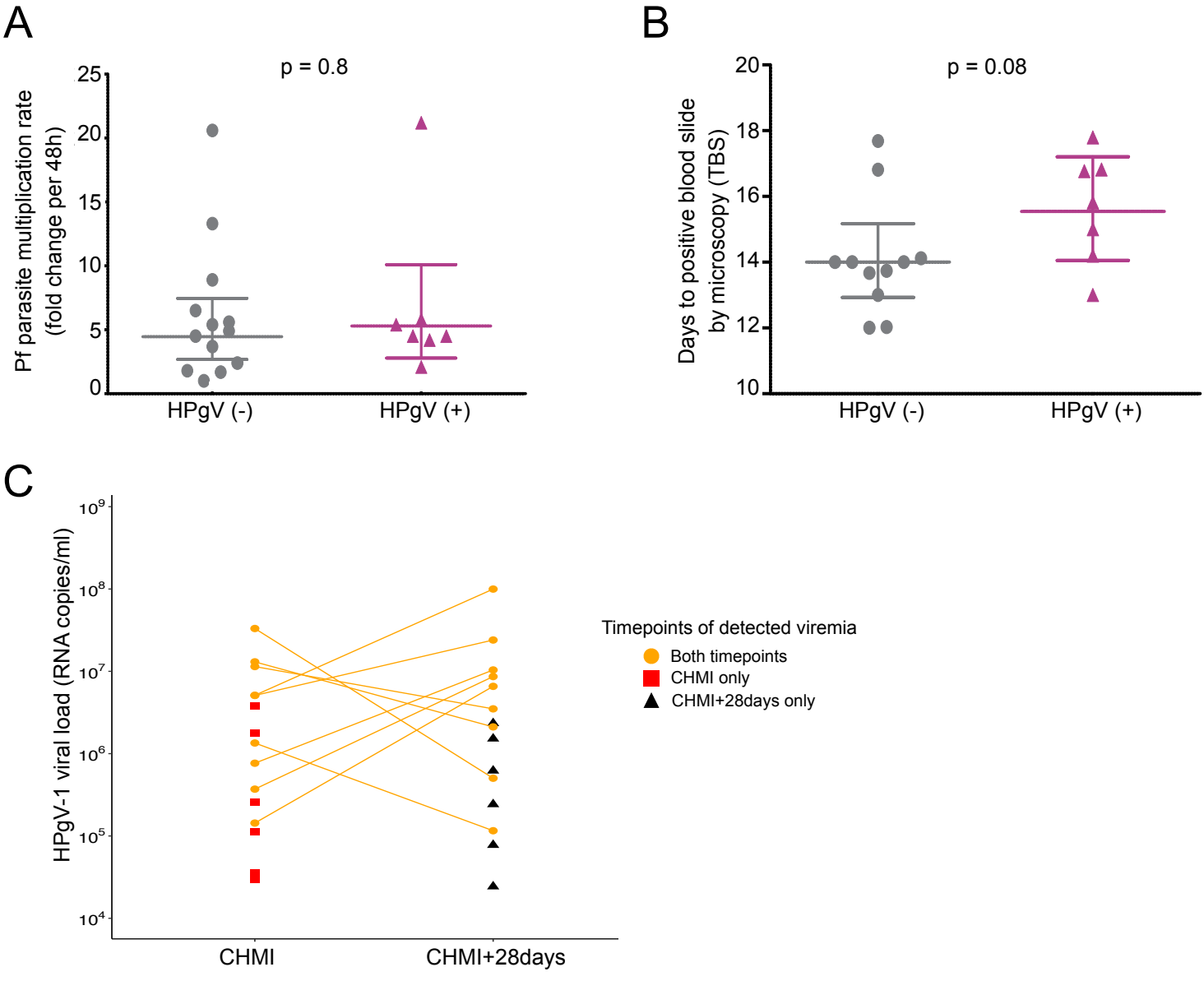

AJ132997.1
strain 25 TZ
KP710602.1genotype1 CAMEROON
AF488786.1 genotype 1 TANZANIA
MF398547.1genotype1 BOTSWANA
AF488789.1 genotype 1 KENYA
strain 4 EG
JX494177.1genotype 1 VENEZUEL...
U36380.1 GENOTYPE 1
KC618399.1genotype1 UGANDA
Y16436.1genotype 1 DRC
AY949771.1 GENOTYPE 5
DQ117844.1 genotype 5 SOUTHAFR...
MF398571.1genotype 5 BOTSWANA
AF488796.1 genotype 5 TANZANI...
AF488797.1 genotpe 5 TANZANIA
HQ331235.1 GENOTYPE 7 PRC
HQ331233.1 genotype7 PRC
AB003292.1 GENOTYPE 6
AF177619.1 genotype6 PNG
AB018667.1 GENOTYPE 4
HQ331172.1 genotype4 PRC
AB021287.1 genotype4 VIETNAM
JX494176.1genotype3 VENEZUELA
KR108695.1genotype 3 MALAYSIA
D87714.1 GENOTYPE 3
AB008335.1 genotype3 JAPAN
strain 7 EG
MH000566.1genotype 2b BRAZIL
U59529.1 genotype2b USA
U63715.1 EA 2b
MH053130.1genotype2 FRANCE
strain 21 TZ
D90600.1 genotype 2a
AB003289.1 genotype2a JAPAN
U59518.1 genotype2a USA
U44402.1 GENOTYPE 2
JX494180.1 genotype2 VENEZUELA
AF104403.1 genotype2a EU
MG229668.1genotype2 GHANA
strain 3 EG
strain 17 TZ
strain 18 TZ
strain 22 TZ
strain 24 TZ
strain 2 EG
strain 9 EG
strain 1 EG
strain 8 EG
JX494179.1 genotype2 VENEZUELA...
strain 16 EG
strain 6 EG
strain 13 EG
strain 26 TZ
strain 5 EG
strain 11 EG
strain 19 TZ
strain 15 EG
strain 20 TZ
strain 14 EG
strain 10 EG
strain 23 TZ
strain 28 TZ
Genotype 1
Genotype 5
Genotype 7
Genotype 6
Genotype 4
Genotype 3
Genotype 2b
Genotype 2a
0.05


AF070476.1 GBC Ctro
U44402.1 Genotype 2
AB003292.1 Genotype 6
AB018667.1 Genotype 4
AY949771.1 Genotype 5
KC618401.1 Genotype 5 UGANDA
AY951979.1 Genotype 5 S.AFRICA
E2 21 TZ
U63715.1 Genotype 2b E.Africa
D90600.1 Genotype 2a
E2 17 TZ
MK686596.1 Genotype 2 CHINA
AF121950.1 Genotype 2 IOWA
E2 19 TZ
D87714.1 Genotype 3
E2 25 TZ
KC618399.1 Genotype 1 UGANDA
AB003291.1 Genotype 1
KP710600.1 Genotype 1 CAMEROON
E2 4 EG
E2 6 EG
U36380.1 Genotype 1
KM670109.1 Genotype 1 S.LEONNE
E2 12 EG
E2 14 EG
E2 27 TZ
KP710602.1 Genotype 1 CAMEROON
Genotype 6
Genotype 4
Genotype 5
Genotype 2
Genotype 3
Genotype 1
3.0

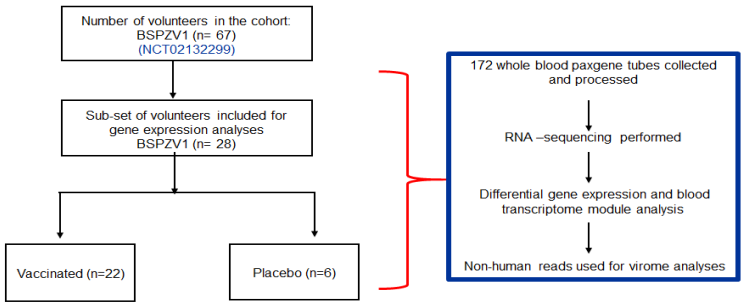

IL.2
0.046
IL.17A
0.004
Cytokine concentration in serum(pg/ml)
0
5
10
15
20
0
2
4
6
HPgV (-)
HPgV (+)
HPgV (-)
HPgV (+)


A
Baseline
Anti–CSP IgG titres (0.D 1)
10^6
10^4
10^2
10^0
0.98
HPgV (–)
HPgV (+)
B
14days post last vaccination
Anti–CSP IgG titres (0.D 1)
0.43
HPgV (–)
HPgV (+)
C
Net
Anti–CSP IgG titres (0.D 1)
0.56
HPgV (–)
HPgV (+)
D
Fold change
Anti–CSP IgG titres (0.D 1)
0.95
HPgV (–)
HPgV (+)

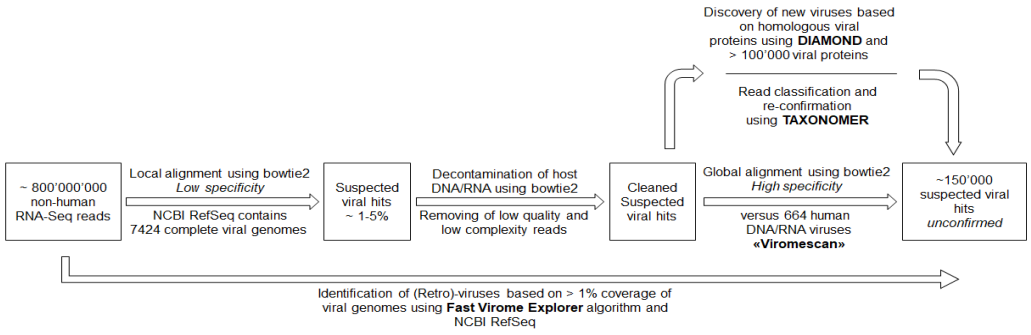

A
Vaccinated
100
75
50
25
0
percentage volunteers (%)
48.4
37.5
62.5
51.6
protected
protected
HPgV (-)
HPgV (+)
B
HPgV (-)
HPgV (+)
10^6
10^4
10^2
10^0
anti-CSP IgG titres (O.D 1)
0.95
0.22
non-protected
protected
non-protected
protected


A
p = 0.8
Pf parasite multiplication rate (fold change per 48h)
25
20
15
10
5
0
HPgV (-)
HPgV (+)
B
p = 0.08
Days to positive blood slide by microscopy (TBS)
20
18
16
14
12
10
HPgV (-)
HPgV (+)
C
HPgV-1 viral load (RNA copies/ml)
10^9
10^8
10^7
10^6
10^5
10^4
CHMI
CHMI+28days
Timepoints of detected viremia
Both timepoints
CHMI only
CHMI+28days only

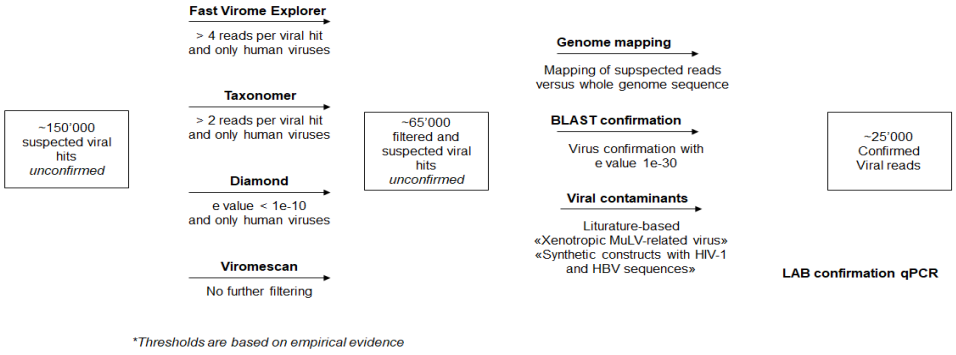
A.



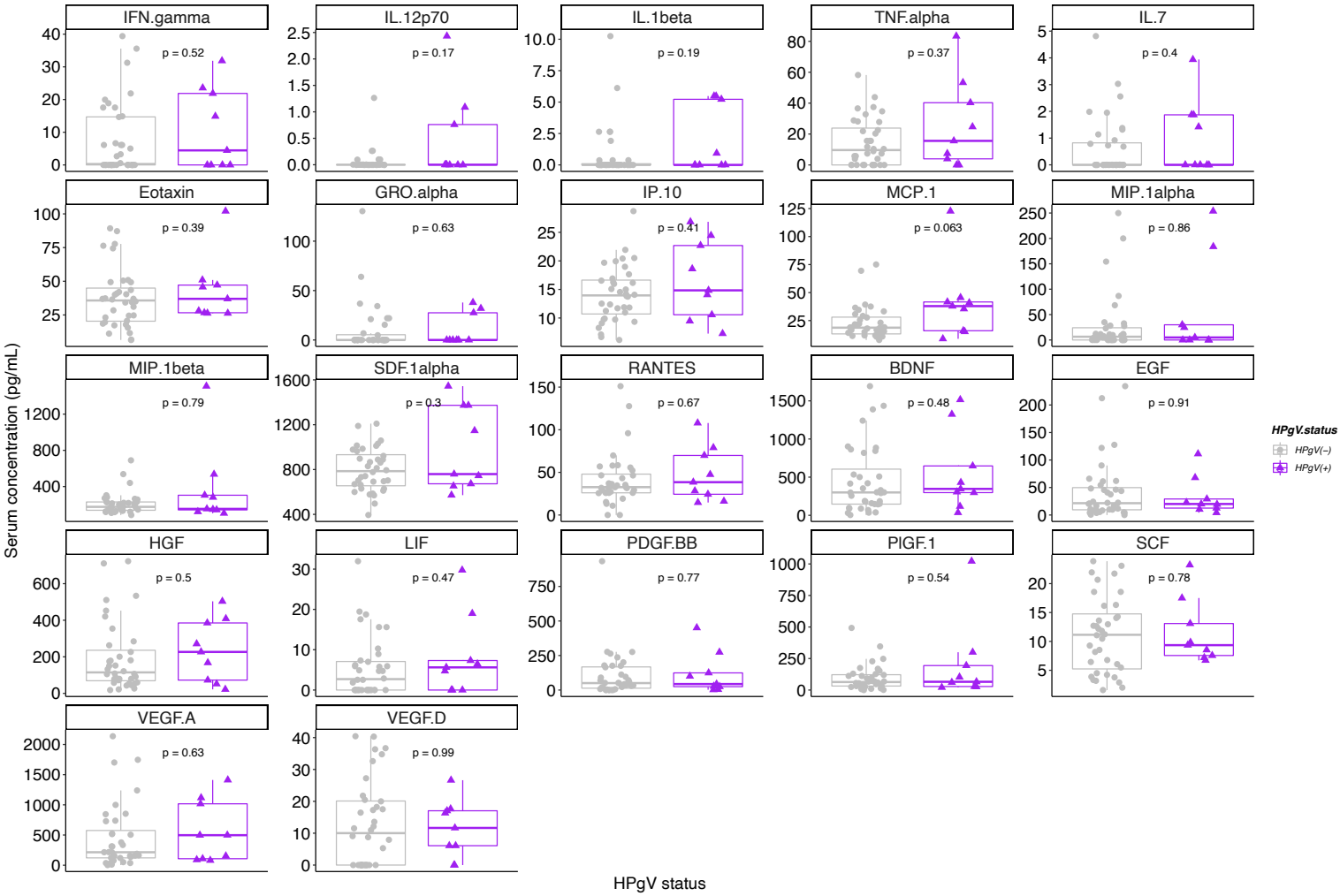
B.



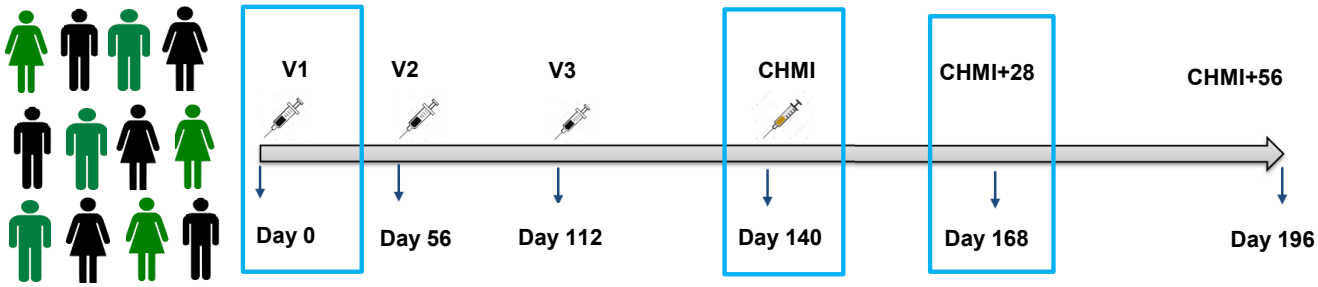
C.

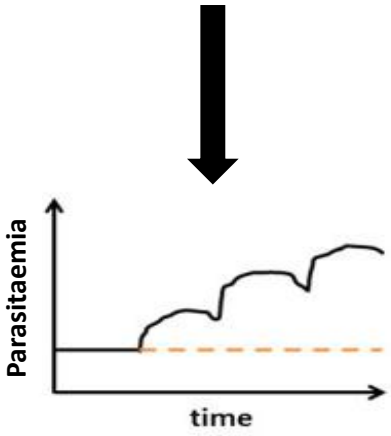


**Thresholds are based on empirical evidence*


IFN.gamma
p = 0.52
IL.12p70
p = 0.17
IL.1beta
p = 0.19
TNF.alpha
p = 0.37
IL.7
p = 0.4
Eotaxin
p = 0.39
GRO.alpha
p = 0.63
IP.10
p = 0.41
MCP.1
p = 0.063
MIP.1alpha
p = 0.86
MIP.1beta
p = 0.79
SDF.1alpha
p = 0.3
RANTES
p = 0.67
BDNF
p = 0.48
EGF
p = 0.91
HGF
p = 0.5
LIF
p = 0.47
PDGF.BB
p = 0.77
PlGF.1
p = 0.54
SCF
p = 0.78
VEGF.A
p = 0.63
VEGF.D
p = 0.99
Serum concentration (pg/mL)
HPgV status
HPgV.status
HPgV(−)
HPgV(+)

Vaccine and placebo groups





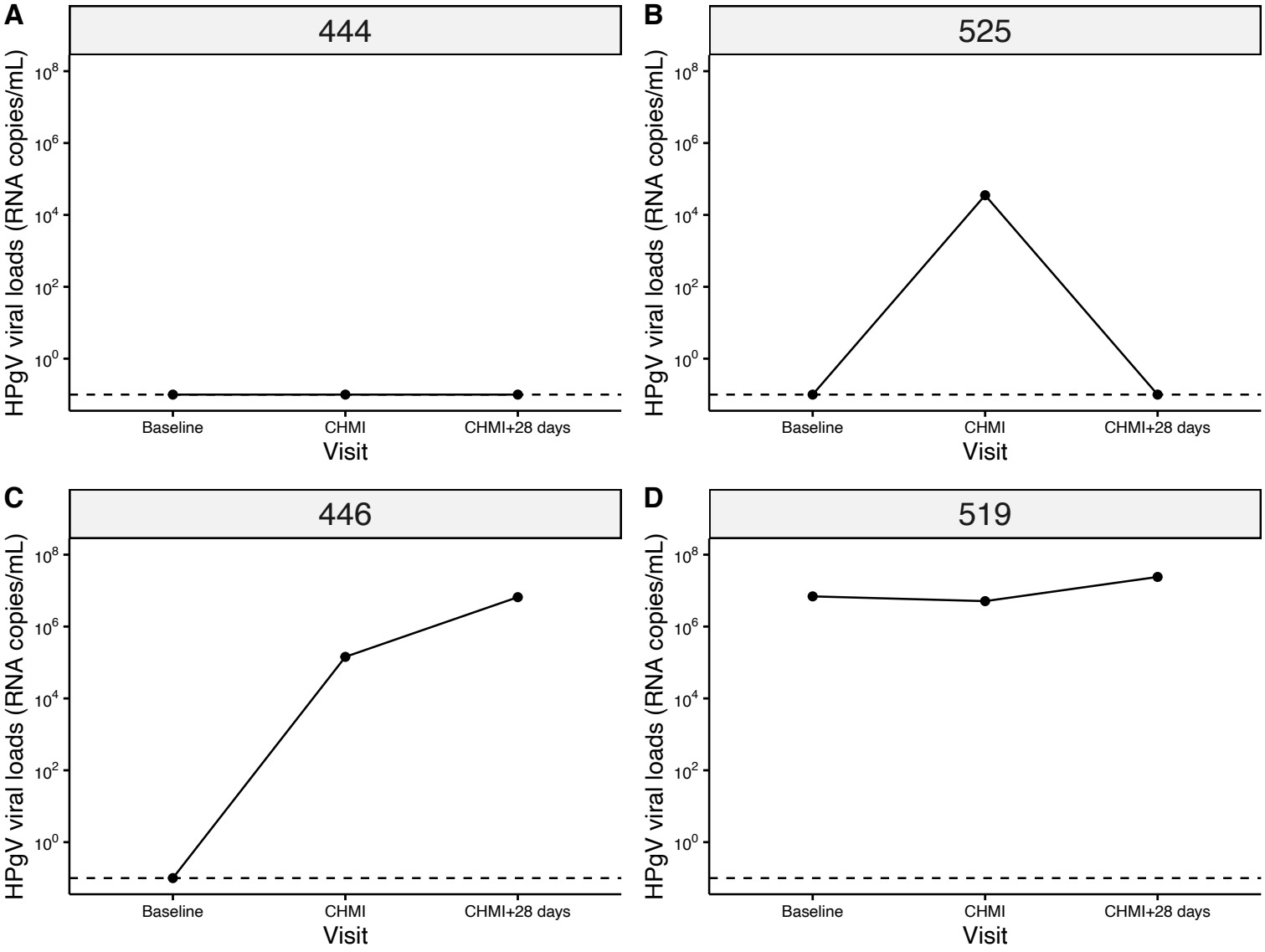
CHMI
Challenge with 3200 fully infectious sporozoites

Malaria positive: Not protected

Malaria negative: Protected

Endpoint: Protection

V: Vaccination 1,2,3
CHMI: Controlled Human Malaria Infection


A
444
B
525
C
446
D
519
HPgV viral loads (RNA copies/mL)
10^8
10^6
10^4
10^2
10^0
Baseline
CHMI
CHMI+28 days
Visit

Chapter 6

Discussion

For several decades, infections with *Plasmodium* spp have had severe negative health and economic effects particularly in the Sub-Saharan region [1]. Years of international development efforts have resulted into the development of a single advanced malaria vaccine candidate, RTS,S that moderately protects infants and children against clinical malaria. RTS,S-induced humoral immune responses wane out rapidly starting from six months following initial vaccination [213], [214]. The ultimate goal of malaria elimination depends on identification and evaluation of next generation, higher efficacious malaria vaccine candidates and approaches to be used in conjunction with currently available control tools: [52] . These next generation, improved malaria vaccines rely on better understanding of naturally acquired immunity against malaria together with vaccine-induced immune mechanisms that potentially confer sterile protection against *Plasmodium* spp [138]. However, some of these protective immune mechanisms are incompletely described [138]. Malaria vaccine development efforts are further compounded by lack of understanding of observed individual and population heterogeneity towards vaccination outcomes [215] . The characterization of immune correlates or surrogates of protection and factors that limits or supports optimal efficacy of malaria vaccines will not only improve vaccine design and immunization strategies but will enhance therapeutics as well as diagnostics [215]. In this thesis, we have evaluated the safety, immunogenicity and protective efficacy of whole sporozoite based malaria vaccine approaches in different populations and age groups residing in malaria endemic countries. We further attempted to elucidate the potential role of chronic viral infections on vaccine-induced protection against homologous CHMI. Our key findings include:

I. We found the PfSPZ Vaccine to be safe and well tolerated in healthy individuals of different age groups. However, immunogenicity and protective efficacy differed according to age, dosage, regimen and immune status with HIV positive individuals showing least protection.

II. Comparisons of two whole malaria sporozoite vaccine approaches (PfSPZ Vaccine and PfSPZ-CVac) in Equatorial Guinean adults (EGSPZV2 study) revealed lower protection levels in PfSPZ vaccinees compared to PfSPZ-CVac vaccines against homologous CHMI. In PfSPZ vaccine inoculated volunteers higher levels of PfCSP specific antibodies were induced when compared to PfSPZ-CVac inoculated volunteers.

III. The unbiased assessment of PfSPZ Vaccine induced humoral immunity using protein microarrays probed with serum samples of HIV positive and HIV negative volunteers revealed a tendency of lower antibody breadth in HIV positive volunteers. Immuno-dominant antigens were PfCSP and PfMSP-5 which were preferably recognized by IgG and IgM, respectively. The personalized antibody profiles observed in volunteers at the baseline did not change upon PfSPZ vaccination, thus confirming our previous observations of the possible antigenic imprinting in malaria.

IV. Co-infection with HPgV-1 seems to result in higher circulating levels of IL-2 and IL-17A. A tendency of higher protection levels following homologous CHMI was measured in the HPgV-1 positive volunteers.

4.1 Safety, immunogenicity, and efficacy of radiation attenuated whole sporozoite vaccine (PfSPZ Vaccine) in African populations of different ages

We conducted two clinical trials in Bagamoyo, the BSPZV1 (NCT02132299) and the BSPZV2, (NCT02613520) studies that evaluated the PfSPZ Vaccine in different age groups [169] 171] [172]. Intravenous administration of PfSPZ Vaccine proved safe and well tolerated by all individuals regardless of age**.** However, PfSPZ-induced antibody response against PfCSP and

protection against homologous CHMI varied widely [169] [171] [172]. In the BSPZV1 study, we enrolled healthy male volunteers that were vaccinated five t

imes with PfSPZ Vaccine and challenged with homologous CHMI. Significantly, lower protective efficacy was seen in Tanzanian volunteers (20%) when compared to US American volunteers (93.4%) and Malian volunteers (29%) that received similar immunization regimens. However, different CHMI approaches were used for assessment of vaccine efficacy [169] [176] [170] [182]. Malaria infected mosquitos were used to challenge the adult US volunteers and in Tanzania intravenous administration of 3200 infectious sporozoites were conducted. In Mali, vaccinees were exposed to malaria field strains representing a heterologous challenge delivered by [169] [176] [170] [182]**.** Vaccine induced anti-PfCSP antibodies and T cell responses were lower in Tanzanian compared to the US volunteers, but considerably higher than in Malian volunteers [169] [176] [170] [182]**.** Even lower anti-PfCSP antibody titres have been reported in adults from the first PfSPZ Vaccine trial in Equatorial Guinea (EGSPZV1) who were vaccinated with the same regimen compared to the study conducted in Mali and Tanzania [183]. In the BSPZV2 study, we enrolled adults, adolescents, infants and children [171]. The adult volunteers were challenged with homologous CHMI and an improved protective efficacy was seen after an increase in the PfSPZ Vaccine dosage to $9X10^5$ (100%, 5/5) but not to 1.8 $X10^6$ (33%, 2/6). The latter results contrasted with a follow up trial in Mali where 100% (30/30) protection was observed in adult volunteers vaccinated with 1.8 $X10^6$ PfSPZ Vaccine and challenged with field exposure during the rainy season (Sissoko et al., unpublished). The lower protection level observed in the Tanzanian group inoculated with 1.8 $X10^6$ PfSPZ Vaccine was somewhat surprising since previous PfSPZ Vaccine studies had anticipated improved protection with increasing PfSPZ vaccine doses. Therefore, these findings suggest possible population based vaccine dose-dependent immunity and protection. In depth analysis of

vaccine induced immunogenicity across different age groups in this cohort included for the first time adolescents, infants and children [171]. When we compared a dose of $9X10^5$ PfSPZ Vaccine in all age groups, higher $CD4^+$ T-cell responses and higher PfCSP-antibody titers were seen in children (older and younger), respectively, when compared to the adult group [171] . These results are possibly linked to the level of malaria pre-exposure before vaccination. Adults residing in malaria endemic countries have most likely encountered several rounds of malaria infections, and children and infants have been less exposed because of their younger age [76] [77]. In this study, only vaccine specific $CD4^+$ T cells but no $CD8^+$ T cells were found in peripheral blood in children, adolescents and adults. No T-cell responses were detected in infants [171].. It is likely that the absence of $CD8^+$ T cells is due to the localization of PfSPZ Vaccine induced T cells in the liver or lymphoid tissues as demonstrated in non-human primate and murine studies [216]. It is difficult to assess the liver resident cellular immunity after PfSPZ vaccination in humans due to lack of access to this tissue. It remains unclear if PfSPZ Vaccine induces $CD8^+$ T cells that recognize Pf infected liver cells during the pre-erythrocytic life cycle stage or if we are technically unable to measure these cells using peripheral blood mononuclear cells. PfSPZ specific $CD4^+$ and $CD8^+$ T cells producing IFN-γ have also been detected in peripheral blood of volunteers after first PfSPZ vaccine inoculation [217] . Depletion of $CD8^+$ T cells in mice compromised the protection against sporozoite challenge while the depletion of $CD4^+$ T cells did not, further suggesting the importance of $CD8^+$ T cells in PfSPZ vaccine induced protection [218], [219]. $CD8^+$ T cells most likely contribute to protection against malaria by recognizing replicating schizonts on the surface of hepatocytes by binding to MHC class I molecules.

The variation in PfSPZ vaccine induced immune responses and protection is not unexpected as heterogeneity in vaccine responses between individuals and populations has also been previously reported in other widely deployed vaccines such as BCG, hepatitis B, influenza,

oral cholera, tetanus, diphtheria, yellow fever, typhoid fever, measles and polio [220], [221] . However, immunological mechanisms for less optimal immunogenicity and protective efficacy of these vaccines in individuals residing in developing countries compared to those living in economically stronger countries remains unclear [222]. In malaria vaccines, among potential factors that contribute to variation in vaccine outcome is *Plasmodium*-induced immune regulation as result of extensive pre-exposure to *Plasmodium* parasites [223]. The mechanisms involved in immune dysregulation resulting in impaired innate and adaptive immune responses include aberrant development of B cells subsets and production of ineffective antibodies [85] [87]

Age and gender are also among widely studied factors that influence vaccine [224]. Infants and neonates often show inadequate protection against infectious diseases and less protective vaccine induced immune responses compared to adults most likely due to the immature status of their immune system or in some cases cross-reactivity with maternal [225], [226]. The fact that cellular responses were not detected in infants in our BSPZV2 study cohort is in line with these observations. However, high antibody titers observed in infants contrasts these expectations and as highlighted above this could be due less malaria pre-exposure. The protective efficacy of PfSPZ Vaccine in these groups is yet to be determined in the field studies. However, differences in protective outcomes against clinical and severe malaria between children and infants immunized with RTS,S have been previously reported [141].Less than desired immune responses have also been documented in the elderly possibly based on immune senescence [227] . The assessment of PfSPZ Vaccine in older adults up to 65 years of age has been conducted in Equatorial Guinean volunteers but these results are yet to be published (EGSPZV2 study, Jongo et al., unpublished). In some vaccines, women are known to mount higher antibody response and are more likely to experience adverse events compared to men [228], [229]. These gender differences are not fully explored in the context of PfSPZ Vaccine

but have been so far described in the leading malaria candidate, RTS,S, [229], where higher mortality was seen in girls despite inducing higher antibody titers [229]. Other factors influencing variation in vaccine responses include geographic location [169] [176] [170] [182] [230], genetics [231], [232], nutritional status [230], [233] , stress [234], gut microbiota [235], [236] , comorbidities [237], [238] and co-infections [184] [185] [186] . Notably, co-infections with bacterial, helminth, viral infections are known to induce immune activation or immune regulation that potentially impacts vaccination outcomes [184] [185] [186] [221], [239]. Our study volunteers were screened for active infectious diseases like tuberculosis, HIV, hepatitis B, hepatitis C and intestinal helminthes prior to immunization. However, the presence of other, less pathogenic chronic infectious diseases was not excluded and could contribute to the variation of PfSPZ vaccination outcomes as highlighted in *Chapter 5*. It is also possible that Tanzanian volunteers (as their Malian and Equatorial Guinean counterparts) enrolled in PfSPZ Vaccine studies had an activated immune microenvironment in the liver at baseline compared to the US volunteers. The impact of activated immune micro-environment on PfSPZ Vaccines has not been extensively studied, but has been shown in Ugandans vaccinated with yellow fever vaccine [221]. Comparative studies involving naïve and pre-exposed individuals immunized with PfSPZ Vaccine are needed to understand the impact of malaria pre-exposure on vaccine responsiveness and mitigate poor response in populations [223]. Earlier comparisons of immune responses in Tanzanian and Dutch volunteers that received similar PfSPZ challenge protocol revealed skewing of Tanzanian immune responses towards higher Pf-specific humoral profile, and lower IFN-γ producing T and natural killer cells than Dutch volunteers [223]. Interestingly, detection of Pf antibody titres at baseline impacted CHMI outcome by delaying prepatent periods and reducing parasite loads in malaria pre-exposed Tanzanian compared to their Dutch counterparts. These analyses further allowed the distinction of individuals with better PfSPZ-induced responses [223]. Similar approaches if expanded in future trials might

help to gain better understanding of the observed heterogeneity of immune responses at an individual and population level.

Collectively, we have established that the PfSPZ Vaccine has excellent safety and tolerability profiles in different populations. With currently deployed vaccine regimens, PfSPZ Vaccine is potentially more efficacious in adolescent and children than in adults. Due to ethical reasons CHMI is not conducted in children and therefore, passive or active, longer term follow up of vaccinees under field exposure will be important to test PfSPZ Vaccine efficacy in children [171]. Thus, optimization of vaccine regimens for different age groups might be a way to ensure that high levels of sterile protection could be achieved across all groups. This is currently being addressed by several ongoing trials in Africa, Europe and US (unpublished).

4.2 Immunogenicity and protective efficacy of radiation-attenuated and chemo-attenuated PfSPZ vaccines in Equatoguinean adults

As highlighted in 6.1, one of the major challenges facing PfSPZ Vaccine is the generation of suboptimal, less protective immune responses in malaria endemic regions. In an attempt to achieve higher levels of sterile protection, alternative whole sporozoite vaccination approaches are currently being pursued [167]. One promising strategy in place is the use of fully infectious, purified sporozoites that are given intravenously under chloroquine chemoprophylaxis (PfSPZ-CVac). Until recently, the PfSPZ-CVac approach has been studied only in malaria naïve populations [173]. In chapter 3 we report for the first time a side by side comparison of two whole sporozoite based vaccine approaches (Jongo et al., in press). In particular, we found that three consecutive doses of PfSPZ-CVac (1 $X10^5$) and PfSPZ Vaccine (2.7 X 10^6) to be safe, well tolerated and immunogenic in African volunteers. Higher protection levels were seen in the PfSPZ-CVac group (55%, 8/13) compared to the PfSPZ Vaccine group (27%, 6/16) against homologous CHMI (Jongo et al., in press). In PfSPZ Vaccine it is thought that the injected

sporozoites arrest early in their development in the liver, mostly between day 1 and day 2 after liver cell invasion. In PfSPZ-CVac, the parasite undergoes a complete liver stage development, thus exposing the immune system to a wide array of different liver stage antigens at higher concentrations which potentially confer better protection [149], [240]. Interestingly, despite the high amount of vaccine dose used (3 times a dose of each $1X10^5$) the PfSPZ-CVac -induced protection in Equatorial Guineans (55%, 8/13) was lower compared to a similar study conducted in Tübingen, Germany. In Tübingen, volunteers were immunized thrice with the dose of $5.12\ X10^4$ of sporozoites and they were 100 % protected against homologous challenge [173]. This protection was linked to PfSPZ CVac- induced effector mechanisms including liver resident $CD8^+$ T cells, $CD4^+$ T cells, neutralizing antibodies and γδ cells [173]. Hence, based on these findings, higher PfSPZ-CVac doses might be required to achieve high levels of protection in malaria pre-exposed volunteers. Studies in Mali have partly addressed this hypothesis and sporozoite doses as high as $2\ X10^5$ have been evaluated; and found to be suboptimal against heterologous field strains (Thera and Lowen, manuscript in preparation).

The low protection level seen in individuals immunized three times with doses of $2.7\ X\ 10^6$ of PfSPZ Vaccine was not surprising. Similar results have been found in the high dose group of the BSPZV2 study. Despite the lower protection levels, the PfSPZ Vaccine inoculated group had 2.9 times higher median PfCSP antibody responses compared to the PfSPZ-CVac group (Jongo et al., in press). This could be attributed to the amount of administered vaccine antigen, with that of PfSPZ Vaccine being higher than PfSPZ-CVac. Together, these findings suggest that increase of PfSPZ Vaccine dose beyond a distinct threshold will not lead to an increased level of protection [173]. Although immunization with PfSPZ Vaccine induced high PfCSP antibody titres, these may not necessarily correlate with protection since they did not differ between protected and non-protected volunteers [241]. One possible factor that have been recently linked to poor antibody function is antibody feedback that potentially masks antigen

epitopes following repeated immunization with PfSPZ vaccine [242]. Despite lower protection levels achieved by whole sporozoite based vaccine approaches in malaria pre-exposed volunteers, these results have been important in guiding optimization of vaccine regimen and dosing. A single dose of 9 $X10^5$ purified sporozoites seems to be an optimal dose for PfSPZ Vaccine in sub-Sahara Africa.

4.3 Humoral immunity in PfSPZ vaccinees assessed by protein microarray analysis

HIV infected individuals are among the high risk groups for development of clinical malaria, [243]. HIV infection has been associated with generation of lower antibody titers to several infectious diseases and after vaccinations [244], [245]. Mechanisms proposed to contribute to inadequate immune responses include dysfunctional B and T cell response, diminished T follicular cell help, impaired T cell activation, reduction of CD4 T cell numbers, B cell exhaustion and expansion [246], [247]. The control of *P. falciparum* infection relies on the generation of effective antibodies against pre-erythrocytic and asexual blood stage parasite antigens [241], [248]. Understanding distinct PfSPZ Vaccine induced humoral immune profiles in HIV positive volunteers is important, as it will help to explain the observed increased malaria susceptibility in this population and the lack of protection after PfSPZ Vaccine inoculation.

We used a protein microarray spotted with 262 pre-selected *P. falciparum* antigens to profile PfSPZ Vaccine induced humoral immune responses in serum collected from HIV positive and HIV negative Tanzanian individuals participating in the BSPZV3a study. While similar analyses have been previously conducted in malaria naïve and malaria pre-exposed individuals immunized with PfSPZ Vaccine and PfSPZ-CVac, respectively [173] [249], [250] this is the first time such comparisons are made in HIV infected individuals. Generally, the breath of antigens recognized at baseline seemed lower in HIV positive individuals albeit it did not reach

statistical significance. Antibodies binding to PfCSP and PfMSP5 were increased in both groups after PfSPZ vaccination. Differential antibody expression patterns were also seen in HIV positives and negative groups, where higher levels of MSP2, LSA1 and PHISTb were seen in HIV positives and MSP8, ApiAP2, CryPH, Phil and COG4 in HIV negatives. Together, these findings indicate that all study volunteers had been exposed to diverse *P. falciparum* antigens prior to immunization. The tendency for a lower antibody breadth highlights the possible negative impact of HIV co-infection on the acquisition of natural immunity to malaria [247]. Similar levels of anti-PfCSP and PfMSP5 antibody responses were elicited in both groups of volunteers. This observation was a little surprising because of the stark difference in the protection levels observed after homologous CHMI in the HIV positive (no protection. 0%) versus 80 % protection in the HIV negative group. Our malaria protein microarray analyses detected total IgG and IgM but we did not examine the immunoglobulin (Ig) subclasses. It is possible that differences might exist in the development of malaria specific Ig subclasses after PfSPZ vaccination, as field studies in Kenya have associated the vulnerability of HIV infected individuals to Pf infections with the shift of ratio of IgG1 and IgG3 subclasses, despite having overall comparable antibody titres measured by multiplex bead arrays [251]. Therefore, inclusion of measurement of Ig subclasses, together with their respective antibody binding affinity and functionality will likely provide more information on antibody mediated protection in malaria [252].

Inoculation of PfSPZ Vaccine resulted in induction of IgG and IgM antibodies binding predominantly to PfMSP5 and PfCSP regardless of HIV status or vaccine dose. The antibodies remained highly present in the vaccinees after homologous CHMI. These findings are consistent to our previous observations in healthy malaria pre-exposed Tanzanian adult volunteers immunized with PfSPZ Vaccine (BSPZV1 study) where IgG targeting PfCSP was the immune-dominant antigen [169] . Zenklusen et al., demonstrated the induction of IgM and

IgG antibodies binding to PfCSP after PfSPZ vaccination in the BSPZV1 study [175]. PfCSP specific IgM antibodies were able of inhibiting *P. falciparum* invasion of the hepatocytes *in vitro* and activated the complement system to lyse sporozoites [175]. PfMSP5 antibodies measured by enzyme-linked immunosorbent assay (ELISA) were also seen in a fraction of volunteers from the same cohort at 14 days after 5th dose [169]. No further assessment was made on the role of these antibodies as analyses focused on PfCSP binding antibodies induced by majority of the volunteers [169]. The expression of PfMSP5 specific antibodies in our cohorts mirror the results obtained in natural malaria transmission studies in Brazil and Papua New Guinea. There, the presence of anti-PfMSP5 antibodies were associated positively with protection from clinical symptoms [253], [254]. In murine malaria studies with *P. yoelii*, antibodies targeting the MSP4/5 homologue have been also associated with protection against malaria challenge [255], [256]. PfCSP is known to be localized on the parasite surface and play major role parasite motility and invasion into hepatocytes [257]. PfMSP5 is predominantly expressed in asexual blood stage merozoite stages. Transcription of the PfMSP5 gene has been described in both, the sporozoite and the pre-erythrocytic hepatocyte stage [258]. PfMSP5 seems not to be important for parasite survival as demonstrated in knock-out experiments [259]. The biological role PfMSP5 specific antibodies in the context of PfSPZ Vaccine warrants a closer investigation. We found a highly personalized pattern of humoral immunity among individuals prior to immunization which did surprisingly not change dramatically after PfSPZ immunization. This result was consistent with our previous findings in adult volunteers from Tanzania in the BSPZV1 study [250]. Similar observations, sometimes described as original antigenic sin has been described in other infectious diseases and vaccines including influenza, pneumococcal vaccines and human cytomegalovirus [260], [261]**.** As in influenza vaccines, it is possible that immunization with PfSPZ Vaccine boosts the memory responses directed towards epitopes that have been encountered previously and thereby preventing the

generation of novel, potentially protective antibody and T cell responses [260]. Volunteers in our cohort were vaccinated multiple times with the same vaccine regimen, whether or not these vaccination regimens influence clonal imprinting has yet to be explored. More research is required to understand underlying mechanisms that influence antigenic imprinting in the context malaria vaccination. Taken together, these findings in the BSPZV3a study show that induction of IgM and IgG binding to PfCSP and PfMSP5 are observed regardless of the HIV infection status and reconfirm previous observation of the immune-dominance of PfCSP after whole sporozoite vaccination.

A number of PfCSP-specific monoclonal antibodies have been isolated from Tanzanian and US volunteers after PfSPZ vaccination that were protected against homologous CHMI [248], [262]. The description of distinct regions in the N-terminus of PfCSP protein recognized by these antibodies and that mediate high levels of sterile protection in an humanized mouse model have provided description of novel sites of attack in the PfCSP protein [248], [262]. Similar studies resulting in the establishment of malaria specific human monoclonal antibodies from HIV positive individuals inoculated with PfSPZ vaccine might shed new light on the potential negative impact of HIV co-infection on antibody maturation and function.

4.4 Role of Pegivirus infections in whole *Plasmodium falciparum* sporozoite vaccine induced humoral immunity and controlled human malaria infections in African volunteers

The fact that *Plasmodium* spp. share large geographical overlap with chronic, often asymptomatic virus infections might deserve more attention. Little is known on how these co-infections might influence the pathogenesis of *Plasmodium* spp. or malaria vaccination outcomes. Human pegivirus is a lymphotrophic virus with immune modulatory properties that involve reduction of immune activation and pro-inflammatory cytokine expression in HIV and

Ebola co-infections [263], [264]. We have investigated for the first time the prevalence and genotype distribution of human pegivirus in East and West African healthy volunteers and have assessed the impact of pegivirus co-infection on PfSPZ Vaccine induced humoral immune responses and protection against homologous CHMI. Consistent with previous studies, we found a high prevalence (29.2%, 28/96) of HPgV-1 in our volunteers from East and West Africa and the most prevalent genotypes were 1, 2, and 5 [265]. These results reflect circulation of wide range of HPgV-1 genotypes in East and West Africa. Whether the virus is more wide spread in this population was not addressed since we did not examine antibodies binding to the HPgV-1 envelope protein 2 (E2) as a marker of previous exposure and [266], [267]. Interestingly, two distinct groups with high and low viremia levels were seen in our HPgV-1 positive volunteers. A similar pattern has been observed in Mexico among HIV positive and HIV negative people with HPgV-1 co-infections [263].

We think this pattern likely represent different replication rates of the virus in our volunteers and could influence clinical outcomes as reported in other viral studies [268]. For example, in HIV and influenza, differences in replication rates are associated with variation in clinical progression, response to treatment as well as cure and relapse [268], [269]. Further assessment on the implication of HPgV-1 high and low vireamia status in the context of an ongoing malaria infection could be interesting to pursue.

Amongst the 23 cytokine, chemokines and growth factors with measurable concentrations analysed by Luminex bead arrays, we found significantly higher concentrations of IL-2 and IL-17A in serum samples of HPgV-1 positive persons. This is in contrast to previous reports that demonstrated reduced IL-2 levels in vitro and in HPgV-1 positive US individuals [270]–[272]. These studies mostly enrolled HIV infected while our cohort was comprised of clinically healthy individuals. The role of IL-2 is documented in the context of HPgV-1/HIV co-infection

but that of IL-17A remains less explored. High levels of IL-2 and IL-17A in our cohort could either imply antiviral responses as described previously in other viral infections including HIV [273], [274]. Alternatively, these increased levels of IL-2 and IL-17A might be based on immunomodulatory mechanism of HPgV-1 supporting long-term survival in the host. The detailed mechanisms that allows HPgV-1 persistence in immune cells are currently unknown.

IL-2 is known to be important for differentiation of $CD4^+$ T cell subsets including Th1, Th2, T_{FH} and Th17 and the maintenance of T regulatory cells [275]. HPgV-1 infection might be associated with reduced B cell activation demonstrated after *in vitro* stimulation of PBMC with soluble CD40 ligand that lead to reduced expression of the CD86 marker on B cells [276]. In recent years, a subset of T regulatory cells (Tregs) designated as T follicular regulatory (T_{FR}) cells has been associated with limiting B cell responses in the germinal centers [277]. Tregs might be better expanded and maintained through IL-2 in the HPgV-1 positive volunteers which might then lead to lower, albeit not statistically significant, median PfCSP antibody titres measured post last PfSPZ vaccination. The PfCSP titres remained lower in the HPgV-1 positive group regardless of protection status against homologous CHMI. Increased levels of circulating IL-17A might be indicative for the presence of Th17 cells whose development is thought to rely on the induction of TGF-β that is also linked to presence of T regulatory cells [278]. IL-17A producing gamma delta T cells might be another contributing source of the IL-17 observed as reported in some acute infections [279]. These cells have been associated with early production of IL-17 in inflammatory settings and their survival and plasticity is thought to be shaped by IL-2 [280].

In this study, we describe a potential beneficial effect of HPgV-1 infection on CHMI outcomes, as a higher proportion of protected individuals were HPgV-1 positive (62.5%) than HPgV-1 negative (51.6%). The exact mechanisms driving this first time observation remains elusive. We have observed a slightly extended asexual blood stage pre-patent period in HPgV-1 positive

volunteers. It might be that HPgV-1 induced immune-modulation results in slowing the development of liver stage parasites resulting into the observed tendency of longer pre-patent periods. Similar observations have been described in Hepatitis C and Pf malaria co-infections [281]. The presence of HPgV-1 infection did not influence Pf parasite multiplication rates. Thus, it could be interesting to investigate if HPgV-1 alters Pf malaria associated morbidity in the field. Our finding of the lack of impact of asexual blood stage malaria infection on HPgV-1 viremia is important since it underscores that the conduct of CHMI is safe in HPgV-1 infected individuals. Taken together our findings in chapter 5 have shed first light on the potential role of chronic viral infections on variation in PSPZ Vaccine induced humoral responses and protection outcomes. These results also demonstrate the feasibility of using CHMI as a valuable platform for identifying and understanding interactions between malaria and other co-infections (Tumbo et al.,

Chapter 7

Outlook

Two whole sporozoite based vaccination approaches, namely PfSPZ Vaccine and PfSPZ CVac, have been evaluated over the past six years by our consortium in several malaria endemic countries including Tanzania and Equatorial Guinea, [169] [171] [172] [183] [282] (Jongo et al., in press). Both approaches have shown that higher vaccine dosages and distinct vaccination regimens, including the intravenous application, are needed to induce higher levels of sterile protection against homologous CHMI in malaria pre-exposed populations compared to malaria naïve populations [169] [171] [172] [183] (Jongo et al., in press). The malaria vaccine technology roadmap of WHO has outlined that by 2030, the international malaria research community should strive to develop and license a malaria vaccine that has a protective efficacy of at least 75 % against clinical disease and lasts longer than two years [283].

To achieve this goal, the PfSPZ vaccine regimen needs further adjustments to tailor for different age groups and to include immunocompromised individuals like HIV infected persons [171] (Jongo et al., manuscript in preparation). To address these gaps, on-going studies in Africa, Europe, US, and Asia are focusing on testing different dosages, fewer immunizations and shorter intervals between immunizations (Equatorial Guinea trial EGSPZV3, NCT03590340; BSPZV3a NCT03420053). It is envisioned that these ongoing studies will provide more information on standard regimen to be used in larger field trials including the planned phase III study planned on Bioko Island in Equatorial Guinea [282] . The phase III trial will help to further evaluate vaccine safety, efficacy under field conditions, provide the data required for vaccine licensure with FDA and EMA and to assess the feasibility of using PfSPZ vaccine as an additional intervention for malaria elimination in combination with already implemented control measures)[282] (Thomas Richie, ASTMH 2019, personal communication).

Many experimental malaria vaccine studies based on PfSPZ Vaccine used an intravenous application of purified, non-attenuated sporozoites as a homologous CHMI to evaluate vaccine efficacy. One published exception is the trial conducted in Mali in which vaccine efficacy was assessed by field exposure. In this trial the vaccine efficacy by proportion analysis was (29%), whereas for at least 24 weeks [169] [171] [172] [183] (Jongo et al., in press). As next steps, it will be interesting to explore vaccine efficacy using defined heterologous challenge strains like Pf7G8 which so far has been evaluated in malaria naïve individuals only [217] . The Pf7G8 strain originates from Brazil and the level of cross-protection after 3D7 based PfSPZ Vaccine application varies widely (8% -83%), depending on the vaccination regimen used [170] [217]. These protection levels were assessed at 24 and 33 weeks post last immunization, respectively. Other Pf strains that have been evaluated including the NF166.C8 (Guinea) and NF135.C10 (Cambodia) albeit these have shown less protective outcome in malaria naïve volunteers [48].

Alternative attenuation approaches of whole sporozoites are followed including genetic manipulation of the parasite through deletion of distinct parasite genes or gene combinations to halt parasite development at different stages of the pre-erythrocytic cycle [240] [284]**.** Genetically engineered *P. berghei* parasites that express *P. falciparum* circumsporozoite protein (PbVac) have also been tested in malaria naïve volunteers [285]. Parasites generated through these approaches are advantageous in that they are homogeneous and most likely have lower batch-to-batch variations compared to PfSPZ Vaccine that is attenuated by irradiation inducing DNA strand breaks [240] Both approaches induce modest immune responses and limited protection against homologous CHMI [284], [285]. Once optimized, it will be valuable to investigate if these attenuation approaches might have result in improved immunogenicity and vaccine efficacy in malaria pre-exposed individuals.

Besides increase of vaccine dose and change of vaccination regimens, administration of vaccine derived from regional circulating malaria strains or eventually using a cocktail of Pf

strains could potentially lead to increase of vaccine efficacy against the vast range of genetically diverse *P. falciparum* strains in malaria endemic regions [48]. The approach of using a cocktail of different vaccine strains has proven successful in veterinary medicine and is used to immunize cattle against the apicomplexan parasite *Theileria parva* [286]. However, for whole sporozoite based vaccines this might be a hurdle due to high production and development costs [48]. Alternatively, the development of subunit vaccines carrying multiple immune-dominant antigens from different Pf stages and formulated with a potent adjuvant might be an alternative approach to the whole sporozoite vaccines discussed [48] [287]. Lack of detailed understanding of the interplay between malaria parasites and the human host represents still a stumbling block for vaccine development [48]. It would be important for future malaria vaccine trials to use systems biology approaches to comprehensively investigate vaccine induce immune responses and increase chances to identify signatures that will explain the discrepancies in outcomes in different age groups and malaria exposure status [230], [288], [289].

Apart from these active immunization approaches mentioned above, there is growing interest in the use of GCP produced human monoclonal antibodies for passive immunization against malaria [72] [290]. Currently, a Phase 1 trial is ongoing in US American volunteers aged between 18-50 years to test an antibody targeting CSP (CIS43LS) that could prevent malaria infection (NCT04206332). The information generated from this study will be invaluable for conducting similar studies in malaria endemic countries. These monoclonal antibodies would be useful particularly for vulnerable population including children, pregnant women, HIV infected persons or during focal malaria outbreaks. Other potential immediate beneficiaries of this approach could be travelers, military and medical personnel staying in malaria endemic countries for a shorter period time [291].

In chapter 4 we found that despite poor protection, HIV positive volunteers under ART seem to mount PfSPZ Vaccine induced humoral response profiles similar to that of HIV negative individuals. A next, logic step to understand the discrepancy in vaccine induced protection would be to investigate the affinity and functionality of the antibodies generated by the PfSPZ-immunized HIV positive volunteers. Addressing potential underlying dysfunctionalities in adaptive immune cellular mechanisms including analyses on B cells and T follicular helper cells could provide more insight [292]. In future, the accessibility to lymph node and liver biopsies of immunized individuals could also potentially improve the knowledge on PfSPZ Vaccine induced tissue resident immune responses. These types of biopsies have been previously employed to understand vaccine induced responses in the context of HIV infection and malaria vaccines employing prime-boost strategies, respectively (Spencer et al., Unpublished, oral presentation at ASTMH, 2019). We and others demonstrated that it is possible to isolate lymph nodes from HIV infected people in malaria endemic rural settings [293], further opening possibilities to extend these techniques in investigating malaria vaccine specific immunity [293]. Future immunization strategies could also consider the use of adjuvants alongside PfSPZ vaccine to improve the quality of humoral responses in the HIV affected population [294].

To extend our findings in Chapter 4, comparative protein array analyses are underway to compare humoral immune responses between adult volunteers immunized with PfSPZ vaccine versus PfSPZ-CVac in Equatorial Guinea. This will be extended to a comparison of humoral immunity in malaria naïve versus malaria pre-exposed PfSPZ-CVac immunized volunteers from Tübingen, Germany and Malabo, Equatorial Guinea, respectively. These analyses aim at unveiling biomarkers of protective immune responses in individuals with different history of malaria pre-exposure. The systematic evaluation of PfSPZ Vaccine in HIV positive volunteers, adults, adolescents, children and infants in East and West Africa is one of our most important

milestone achieved. Using the unbiased protein microarray platform further will enable to evaluate if the proposed hypothesis of antigenic sin and the personalized immune antibody profiles can be observed across all age groups [250]. Moreover, these analyses might be helpful in determining distinctive PfSPZ immunization induced markers potentially associated with protection in different geographic location.

In Chapter 5, we describe the prevalence of HPgV-1 in 96 adult volunteers that participated in our PfSPZ vaccine studies. As a follow up study, we could determine the prevalence and genotype distribution of HPgV-1 in infants, children, adolescents and the older-adults in our cohorts. One of the most interesting outlook could be to investigate the cellular subsets in which the HPgV-1 preferentially replicates. This is currently approached by using the RNA flow method and advanced flow cytometry techniques [295]. These studies might help to explain the immune modulatory mechanisms induced by this virus in general. Elucidation of these immune modulatory mechanisms could also possibly pave a way to the use HPgV-1 virus or some of its components to improve survival outcomes in individuals co-infected with HIV and HPgV-1 or Ebola and HPgV-1 [296]. Interestingly, the E2 protein of HPgV-1 has been found to inhibit HIV infections *in vitro* by inducing cross-reactive antibodies [297]. The fact that HPgV-1 infection seems to confer a certain degree of vaccine independent protection against CHMI in our cohorts raises the intriguing questions whether HPgV-1 might also influence different malaria disease states ranging from asymptomatic, clinical and severe malaria. These analyses could be extend to other routinely used vaccines in HPgV-1 endemic regions.

The availability of the CHMI platform provided us with an unique opportunity to investigate and demonstrate for the first time the role of co-infections like HIV on PfSPZ Vaccine induced protection [187]. Co-infections with pathogenic or nonpathogenic organisms have been partially addressed but the impact of chronic asymptomatic or emerging infectious diseases on malaria immunity and widely deployed routine vaccines remain explored [298]. Often-

infectious diseases are studied in isolation and do not necessarily reflect real life situation where an individual can be co-infected with multiple pathogens [298]. Future studies should aim at addressing co-infections in the context of immunization studies, as it would help in understanding determinants of variations of vaccination outcomes and the design and development of hopefully better vaccines. In addition, the number of individuals suffering from immunosuppressive co-morbidities like cancer and *diabetes mellitus* is also rising sub-Saharan Africa [298].

There is still a paucity of information on the directionality and interactions between non-communicable and communicable diseases, including malaria that might result in changed immunity. Controlled human infection models beyond malaria could provide a platform to evaluate these interactions under controlled conditions [187] (https://www.hic-vac.org). To explore so far potentially under researched co-infections between viruses and malaria in sub-Saharan Africa, we have used an unbiased approach to screen whole blood transcriptome data for RNA molecules encoding for viruses circulating in peripheral blood. These *in silico* searches have become possible based on public availability of large databases containing sequencing data of organisms from all kingdoms [299]. Such resources and approaches might be particularly important in resource poor settings where the majority of infectious diseases exist [300].

In conclusion, it is clear that the goals expressed in the malaria global technical strategy 2016-2030 and the malaria vaccine technology roadmap of WHO are faced with challenges. Apart from biological impediments to malaria vaccine development, scale up of vaccine manufacture, regulatory mechanisms and roll out at large scale in the field are also only partially defined. Information gained from the currently ongoing pilot implementation study of the most advanced malaria vaccine, RTS,S, in Kenya, Malawi and Ghana will be important for guiding the implementation of follow up malaria vaccine candidates currently under development

[301]. The continued interdisciplinary collaborations among different malaria research and control stakeholders have to be fostered and maintained to reach the aim of malaria elimination and its associated benefit for mankind [183] [282].

Chapter 8

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Chapter 9

Curriculum Vitae

Tumbo, Anneth-Mwasi Nasson **Curriculum Vitae**

ADDRESS

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PROFFESIONAL/ RESEARCH EXPERIENCE

01/2021-Present **Research Scientist/Immunology:**

- Supervise projects implementing activities in the field and research laboratory
- Manage all data generated from the projects and liase with project collaborators.
- Develop and maintain networks between IHI and other laboratories within and outside the country.
- Development.optimisation and execution of cellular assays
- Training of new staff and visiting students.
- Participate in relevant scientific meetings and training activities including: seminars, conferences, workshops, forums etc.
- Write new research grants
- Publish/Disseminate project findings to the communities

09/2016-01/2021 **Research Scientist/PhD Researcher:**

University of Basel/ Swiss Tropical and Public Health Institute Basel, Switzerland and Ifakara Health Institute, Bagamoyo Tanzania

- Heterogeneity in *Plasmodium falciparum* whole sporozoite vaccine induced humoral immune responses and protection in African volunteers: The role of age, human pegivirus and human immunodeficiency virus co-infections

05/2019 **Visiting Scientist/Institute of Tropical Medicine, University of Tubiengen, Germany**

- Immune profiling of whole sporozoite-vaccine induced humoral immune responses in african volunteers using *Plasmodium falciparum* protein arrays.

04/2017-08/2017 **Visiting Scientist/ Equatorial Guinea Malaria vaccine Initiative, Malabo, Equatorial Guinea**

- Laboratory preparation and analysis of biological samples from clinical trial participants
- Training and supervision of new laboratory staff and visiting students

06/2014-08/2014 **Visiting Research fellow, Allergy/Immunology group of Prof. Giuseppe Pantaleo: Centre Hospitalier Universitaire Vaudois, (CHUV) Lausanne, Switzerland.**

- Analysis of Mycobacterium specific immunity in Tanzanian latent and active TB patients with *diabetes mellitus* co-morbidity

09/2011-06/2014 **Research Intern (2011-2012); Research officer (2012-2014); Ifakara Health Institute, Bagamoyo, Tanzania and Swiss Tropical and Public Health Institute**

- Laboratory preparation and analysis of biological samples from clinical trial participants
- Preparation of standard operating procedures
- Laboratory supply chain management
- Assisting in vaccine formulation and dosage preparations during vaccine and controlled

human malaria infection studies

- Training and supervision of new laboratory staff and visiting students

10/2012-12/2012 **Visiting Research Scientist, Immunology Department of HIV Vaccine Trials Network group at Fred Hutchison Cancer Research Centre, Seattle, USA.**

- Comprehensive training in Flow Cytometry techniques (Intracellular cytokine staining, Phenotyping of peripheral blood mononuclear cells, Thawing of cells, Standardization of the flow cytometric assays, antibody titrations, data analysis based on Facs Diva and Flow Jo

2010 **Biomedical intern, Histopathology, Cytology, Microbiology and Molecular Detection Laboratories: Muhimbili National Hospital, Dar-es-Salaam, Tanzania.**

- Cancer screening: collection of tissue biopsies for cancer screening and tested them for different forms of cancers.
- Performed culture media preparations, culturing of various microorganisms and antibiotic sensitivity testing.
- Performed diagnosis of HIV by PCR in children less than 18 months of age.

2009 **Biomedical Intern, Haematology and Clinical Biochemistry Laboratories: University Teaching Hospital, Lusaka, Zambia**

- Laboratory quality control of haematological and biochemical analysers
- Sickle cell screening of new sickle cell patients and monitoring the old patients.

2008 **Research assistant, Microbiology and Molecular Biology Laboratories: Zambia AIDS and Tuberculosis Research Project (ZAMBART), Lusaka, Zambia**

- Performed diagnosis of latent TB among HIV patients using TB Gold Quantiferon assay.

EDUCATION

09/2016- 11/2020 PhD candidate, Department of Medical Parasitology and Infection Biology, Clinical Immunology Unit, University of Basel/Swiss Tropical and Public Health Institute Basel, Switzerland and Ifakara Health Institute, Bagamoyo Tanzania.
Supervision: Prof. Claudia Daubenberger and Prof. Dr. Marcel Tanner; Department of Medical Parasitology and Infection Biology, Clinical Immunology Unit, University of Basel/ Swiss TPH
PhD thesis: Heterogeneity in *Plasmodium falciparum* whole sporozoite vaccine induced humoral immune responses and protection in African volunteers: The role of age, human pegivirus and human immunodeficiency virus co-infections

09/2014-02/2016 MSc. Infection Biology, University of Basel/ Swiss TPH, Basel, Switzerland**.**
Supervision: Prof. Claudia Daubenberger and Dr. Damien Portevin; Department of Medical Parasitology and Infection Biology, Clinical Immunology Unit, University of Basel/ Swiss TPH.
MSc. Thesis: Mycobacterium specific immunity in Tanzanian latent and active TB patients with *diabetes mellitus* co-morbidity

04/2008-07/2011 BSc. Biomedical Sciences, University of Zambia-School of Medicine, Lusaka, Zambia
Supervision: Dr Hamakwa Mantina, Department of Pathology and Microbiology, University Teaching Hospital, Lusaka Zambia.
BSc. Thesis: Classification of Leukaemia using Flowcytometry at University Teaching Hospital (UTH)

SKILLS:

Language: Excellent command of Kiswahili (Mother tongue), English (Fluent), German (Beginner)

Analytical Skills: R, Graph Pad, Flow jo, Basic bioinformatics

AWARDS:

2017: Winner, Presentation, Host directed therapeutic strategies for infectious diseases, International Immunological Workshop organised by International Center for Genetic Engineering and Biotechnology and South African Research Foundation.

2016-2020 PhD Fellowship at University of Basel and Ifakara Health Institute, funded by Swiss Government through ESKAS program

2012: Session winner, poster presentation Haematology/Immunology.19th International Student Congress of (bio) Medical sciences (ISCOMS-12) University Medical Centre Groningen, Netherlands

MEMBERSHIPS:

African Society for Laboratory Medicine

Swiss Society of Tropical Medicine and Parasitology (SSTMP)

INTERESTS/EXTRACURRICULAR ACTIVITIES:

One Young World ambassador for Tanzania: Focus areas Global health, Inter-faith dialogue, Education and Environment

Young Life Switzerland

Cooking and reading various books on different cultures

REFERENCES:

Upon request

PUBLICATION LIST

1. **Tumbo AM**, Schindler T, Dangy JP, Orlova-Fink N, Bieri JR, Mpina M, Milando FA, Juma O, Hamad A, Nyakarungu E, Chemba M, Mtoro A, Ramadhan K, Olotu A, Makweba D, Mgaya S, Stuart K, Perreau M, Stapleton JT, Jongo S, Hoffman SL, Tanner M, Abdulla S, Daubenberger C. Role of human Pegivirus infections in whole Plasmodium falciparum sporozoite vaccination and controlled human malaria infection in African volunteers. Virol J. 2021

2. Jongo SA, Urbano V, Church LWP, Olotu A, Manock SR, Schindler T, Mtoro A, Kc N, Hamad A, Nyakarungu E, Mpina M, Deal A, Bijeri JR, Ondo Mangue ME, Ntutumu Pasialo BE, Nguema GN, Owono SN, Rivas MR, Chemba M, Kassim KR, James ER, Stabler TC, Abebe Y, Saverino E, Sax J, Hosch S, **Tumbo AM**, Gondwe L, Segura JL, Falla CC, Phiri WP, Hergott DEB, García GA, Schwabe C, Maas CD, Murshedkar T, Billingsley PF, Tanner M, Ayekaba MO, Sim BKL, Daubenberger C, Richie TL, Abdulla S, Hoffman SL. Immunogenicity and Protective Efficacy of Radiation-Attenuated and Chemo-Attenuated PfSPZ Vaccines in Equatoguinean Adults. Am J Trop Med Hyg. 2021.

3. Hill DL, Carr EJ, Rutishauser T, Moncunill G, Campo JJ, Innocentin S, Mpina M, Nhabomba A, **Tumbo A**, Jairoce C, Moll HA, van Zelm MC, Dobaño C, Daubenberger C, Linterman MA. Immune system development varies according to age, location, and anemia in African children. Sci Transl Med. 2020

4. Jongo SA, Church LWP, Mtoro AT, Chakravarty S, Ruben AJ, Swanson PA, Kassim KR, Mpina M, **Tumbo AM**, Milando FA, Qassim M, Juma OA, Bakari BM, Simon B, James ER, Abebe Y, Kc N, Saverino E, Gondwe L, Studer F, Fink M, Cosi G, El-Khorazaty J, Styers D, Seder RA, Schindler T, Billingsley PF, Daubenberger C, Sim BKL, Tanner M, Richie TL, Abdulla S, Hoffman SL. 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. Am J Trop Med Hyg. 2019.

5. Jongo SA, Church LWP, Mtoro AT, Chakravarty S, Ruben AJ, Swanson Ii PA, Kassim KR, Mpina M, **Tumbo AM**, Milando FA, Qassim M, Juma OA, Bakari BM, Simon B, James ER, Abebe Y, Kc N, Saverino E, Fink M, Cosi G, Gondwe L, Studer F, Styers D, Seder RA, Schindler T, Billingsley PF, Daubenberger C, Sim BKL, Tanner M, Richie TL, Abdulla S, Hoffman SL. Increase of dose associated with decrease in protection against controlled human malaria infection by PfSPZ Vaccine in Tanzanian adults. Clin Infect Dis. 2019.

6. Boillat-Blanco N, **Tumbo, A. N**, Perreau, M, Amelio, P, Ramaiya K. L, Mganga, M, Schindler C, Gagneux S, Reither K, Probst-Hensch N, Pantaleo G, Daubenberger C, Portevin D. (2018). Hyperglycaemia is inversely correlated with live M. bovis BCG-specific $CD4^+$ T cell responses in Tanzanian adults with latent or active tuberculosis. *Immunity, inflammation and disease*.

7. Amelio P, Portevin D, Hella J, Reither K, Kamwela L, Lweno O, **Tumbo A**, Geoffrey L, Ohmiti K, Ding S, Pantaleo G, Daubenberger C, Perreau M. HIV Infection Functionally Impairs Mycobacterium tuberculosis-specific CD4 and CD8 T-cell responses. Journal of virology. 2018.

8. Jongo S. A, Shekalaghe S. A, Church L, Ruben A. J, Schindler, T, Zenklusen I, Rutishauser, T, Rothen J, **Tumbo, A,** Mkindi C, Mpina, M, Mtoro A. T, Ishizuka A. S, Kassim K. R, Milando F. A, Qassim M, Juma O. A, Mwakasungula S, Simon B, James E. R, Abebe Y, KC N, Chakravarty S, Saverino E, Bakari B. M, Billingsley P F, Seder R. A, Daubenberger C, Sim, B, Richie T. L, Tanner M, Abdulla S, Hoffman S. L. (2018). Safety, Immunogenicity, and Protective Efficacy against Controlled Human Malaria Infection of *Plasmodium falciparum* Sporozoite Vaccine in Tanzanian Adults. *The American journal of tropical medicine and hygiene*, *99*(2)

9. Amelio. P, Portevin, D, Reither K, Mhimbira F, Mpina M, **Tumbo A**, Nickel B, Marti H, Knopp S, Ding S, Penn-Nicholson A, Darboe F, Ohmiti K, Scriba TJ, Pantaleo G, Daubenberger C, Perreau M. Mixed Th1 and Th2 *Mycobacterium tuberculosis*-specific CD4 T cell responses in patients with active pulmonary tuberculosis from Tanzania. PLoS Negl Trop Dis. 2017

10. Mwakasungula S, Schindler T, Jongo S, Moreno E, Kamaka K, Mohammed M, Joseph S, Rashid R, Athuman T, **Tumbo AM**, Hamad A, Lweno O, Tanner M, Shekalaghe S, Daubenberger CA. Red blood cell indices and prevalence of hemoglobinopathies and glucose 6 phosphate dehydrogenase deficiencies in male Tanzanian residents of Dar Es Salaam. Int J Mol Epidemiol Genet. 2014.

11. Immune activation and magnitude and breadth *Plasmodium falciparum* antigens in malaria pre-exposed volunteers with or without HIV infection during PfSPZ vaccination and controlled human malaria infection. (Tumbo, AT etal., *ASTMH conference*, 2019

MANUSCRIPTS IN PREPARATION

12. HIV-1 positive and HIV-1 negative Tanzanian adults undergoing whole irradiation attenuated Plasmodium falciparum sporozoite vaccination mount antibody responses targeting the circumsporozoite protein and merozoite surface protein 5. **Tumbo AN**, Lorenz FR, Mordmüller B, Fendel R, Daubenberger CA et al., (manuscript under review in EMBO molecular Medicine)

13. Humoral immunity in PfSPZ-Vaccine and PfSPZ-CVac vaccinees assessed by protein microarray analysis in malaria exposed adults. **Tumbo AN**, Lorenz FR, Mordmüller B, Fendel R, Daubenberger CA et al. (to be submitted to Frontiers in Immunology)
14. Systemic Immune activation in HIV positive versus HIV negative volunteers during experimental malaria vaccination and controlled human malaria infection and correlation to magnitude, breadth and longevity of malaria specific humoral immunity. **Tumbo AN**, Daubenberger CA et al., (to be submitted to Immunity, Inflammation and Disease)
15. Safety, immunogenicity and protective efficacy of whole sporozoite based malaria vaccine in Tanzanian HIV positive and HIV negative individuals. Jongo S et al., **Tumbo AN et al**., (Manuscript in preparation)

Declaration:

☒ I hereby declare that *all the above information is correct* and *accurate*

Place Bagamoyo, Tanzania Date 25.01.2022 Signature