EVALUATION OF THE MALARIA TRANSMISSION-REDUCING ACTIVITY OF Pfs25-IMX313/MATRIX-M VACCINE AND NATURALLY ACQUIRED ANTIBODIES IN TANZANIAN VOLUNTEERS

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ABSTRACT

Malaria is still a public health challenge in Tanzania, control relies on the use of artemisinin-based combination therapies and insecticide-treated materials. The effectiveness of these interventions is threatened by drug and insecticide resistance in parasites and vectors respectively. Transmission-blocking interventions are therefore needed to supplement the current interventions. This study evaluated the ability of vaccine-induced Pfs25 antibodies, to block malaria parasite development in mosquitoes, following a Phase1 vaccine trial in Bagamoyo, Tanzania, between 2021 and 2023. The transmission reducing activity (TRA) was evaluated via Standard Membrane Feeding Assays (SMFAs), where laboratory mosquitoes were fed with a mixture of test or control antibodies and cultured *Plasmodium falciparum* gametocytes. The TRA was determined as the reduction in the number of oocysts by test antibodies compared to a negative control lacking blocking antibodies. In addition, a survey was conducted to determine the seroprevalence of natural antibodies to malaria antigens; Pfs25, Pfs230D1M, and Pfs48/45, as well as the malaria prevalence in 467 participants from five villages in Bagamoyo district. The malaria and natural antibody survey were conducted as a baseline for phase1 evaluation of Pfs25-IMX313/Matrix-M vaccine candidate. Trial results indicated that the Pfs25-IMX313/Matrix-M vaccine induced long-lasting antibodies, which promoted significant TRA in 18 of the 20 vaccinated participants. Survey findings showed that 23.5% (110/467) of the participants tested positive for malaria, and 24% of the malaria-positive participants had the sexual form of malaria (gametocytes) responsible for transmission. Regression analysis showed that gametocytes were more likely to be present among male participants than female participants [ORa: 2.79 (95% CI: 1.19 – 6.59) p=0.019]. The survey results further indicated that the seroprevalence of Pfs230D1M IgG was 56% (157/281), whereas that of Pfs48/45 IgG was 49% (141/291). Seroprevalence for anti-Pfs230 and anti-Pfs48/45 IgG increased significantly with participants' age, with adults more likely to have antibodies than children; for Pfs230 [adjusted OR 3.18, (95% CI: 1.85 - 5.57), p=<0.0001], and Pfs45/48 [OR 3.11, (95% CI: 1.83 - 5.29), p = <0.0001]. When Pfs48/45 and Pfs230 seropositive serum was tested in the SMFAs, only two (2) of the 10 participants demonstrated significant TRA. We conclude that a transmission-blocking vaccine will be a great addition to the current malaria interventions, and children as well as adults should be targeted, if malaria elimination to be achieved. The Pfs25-IMX313/Matrix-M vaccine in this study should be further developed in combination with another transmission-blocking target such as Pfs230D1M or Pfs48/45.

DECLARATION

I, Charles Mulamba, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the Nelson Mandela African Institution of Science and Technology, Arusha. It has not been submitted before for any degree or examination at this or any other University.

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CERTIFICATION

The undersigned certify that, they have read and hereby recommend for acceptance by the Nelson Mandela African Institution of Science and Technology a thesis titled ***"Evaluation of the malaria transmission-reducing activity of Pfs25-IMX313/Matrix-M vaccine and naturally acquired antibodies in Tanzanian volunteers"*** in partial fulfilment of the requirements for the Doctor of Philosophy in Life Sciences degree of the Nelson Mandela African Institution of Science and Technology.

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DEDICATION

This thesis is dedicated to the loving memory of my dear parents; Silverno Byakika Maganda and Tedora Kyaliki.

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LIST OF ABBREVIATIONS AND SYNONYMS

An.	Anopheles
bp	Base Pair
DNA	Deoxyribonucleic Acid
hrs	Hours
IgG	Immunoglobulin G
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Bed Nets
KM	Kilometres
LLITNs	Long Lasting Insecticide Treated Nets
mL	Millilitres
MoH	Ministry of Health
ºC	Degrees Celsius
P.	*Plasmodium*
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
Pfs	*Plasmodium falciparum* surface antigen
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
s.l	*Sensu lato* (in the broad sense)
s.s	*Sensu stricto* (in the strict sense)
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel
SP ID	Species Identification
TAE	Tris-acetate-EDTA buffer
TRA	Transmission-Reducing Activity
UK	United Kingdom
V	Volts
WHO	World Health Organisation
w/v	Weight/Volume
µL	Micro Litres
µM	Micro Molar

CHAPTER ONE: INTRODUCTION

1.1. Background of Research problem

Malaria remains a major public health and socio-economic problem in 85 endemic countries and territories in sub-Saharan Africa, the Amazon basin, and Asia. (WHO, 2019, 2022, 2023). The global malaria burden is still high; 249 million cases and 608,000 deaths were reported in 2023. More than 90% of the malaria cases and deaths occur in the Sub-Saharan Africa (WHO, 2020, 2021, 2022, 2023), and Tanzania alone accounted for 4% of the global malaria deaths reported in 2022 (WHO, 2023).

Malaria mostly affects populations in low-resource settings and is most prevalent in children and pregnant women. Five species are known to cause human malaria but *Plasmodium falciparum* (*P. falciparum*) is responsible for majority of infections and deaths. The malaria parasite growth cycle is complex, involving different stages between humans and female *Anopheles* mosquitoes. The parasite is well adapted to develop in different forms, including infectious forms to the human liver/pre-erythrocytic (sporozoites) and erythrocytes (merozoite), as well as sexual/sporogonic stages (gametocyte/gametes/oocysts, etc.) in humans and mosquitoes. The gametocytes are responsible for the spread of the disease. (Florens et al., 2002).

While the available malaria interventions have led to substantial reductions in morbidity and mortality worldwide, malaria decline has stalled due to numerous factors, including drug resistance, low-scaling up of available control tools to those who need them the most, and most recently, the covid-19 pandemic. Anti-malaria drug and insecticide resistance have been reported in malaria endemic areas, with resistance to artemisinin-based combination therapies (ACTs)and pyrethroids being the biggest concern because ACTs and pyrethroids are the cornerstones of many national malaria control programs (WHO, 2021, 2022, 2023). The persistence of malaria in many endemic areas suggests that the current interventions may not be targeting all the sources of transmission, and therefore new tools are required to supplement the available interventions. Transmission-blocking vaccines are among novel tools being pursued for malaria control and elimination. This study aimed to evaluate the ability of a transmission-blocking vaccine candidate, Pfs25-IMX313/Matrix-M, to induce antibodies capable of interrupting malaria transmission between mosquitoes and humans. The study also set out to characterize the distribution of gametocyte carriage and naturally

acquired antibodies to selected gametocyte antigens, which are of great importance for developing malaria transmission-blocking interventions.

1.2. Statement of Research problem

The available malaria control tools in Tanzania rely heavily on the use of anti-malarial drugs and insecticide treated materials. However, standard malaria drugs do not readily kill malaria stages infectious to mosquitoes. Furthermore, the efficacy of the present control interventions is heavily threatened by the widespread of drug and insecticide resistance in malaria parasites and vectors. (Hemingway & Ranson, 2000; Killeen & Sougoufara, 2023; Kisinza et al., 2017; Muller et al., 2008; Ramphul et al., 2009; Tungu et al., 2023; Wondji et al., 2009). The evolution of insecticide resistance has been shown to impact mosquito fitness in malaria vectors (Djogbenou et al., 2008; Lynd et al., 2005), with resistant mosquitoes thought to be more susceptible to *P. falciparum* infection than insecticide sensitive mosquito strains (Alout et al., 2013). Addressing malaria transmission within the context of antimalarial and insecticide resistance is essential for malaria control and elimination in Tanzania and other endemic areas of sub-Saharan Africa. Malaria elimination will require multiple efforts including Transmission-blocking interventions to thwart transmission through mosquitoes.

While clinical malaria results from parasite replication in human erythrocytes, it is the gametocytes, which are solely responsible for the spread of the disease (R. Sinden, 2015)**.** There is very limited literature on gametocyte burden and anti-gametocyte responses, as well genetic variation of parasite target antigens in Tanzania. Efforts for developing and evaluating malaria transmission-blocking vaccine tools require thorough understanding of human gametocyte reservoirs so that the most important hosts for the disease transmission are targeted (J. Bousema et al., 2007; T. Bousema et al., 2010). In addition, regular monitoring for polymorphs in the parasite target antigen in field is important for ensuring vaccines efficacy.

1.3. Rationale of the Study

The main objectives in the WHO Malaria Vaccine Technology Roadmap ("Malaria Vaccine Technology Roadmap ", 2006; "Malaria Vaccine Technology Roadmap," 2013) include developing effective vaccines against *P. falciparum* species by 2030 and enable substantial reduction of malaria transmission to levels that can enable malaria elimination in multiple settings. Alongside the current control strategies, transmission-blocking vaccines offer a new approach by targeting developing parasites in the mosquito host —a bottleneck in the malaria parasite lifecycle(Smith, Vega-Rodríguez, & Jacobs-Lorena, 2014) and will contribute significantly to malaria control and elimination. Compared to vector control interventions, TBVs are ecologically safer, cost-effective, and can readily enable high coverage of populations. In Tanzania, the Malaria National Strategic Plan 2021-2025, targets to reduce the malaria burden in moderate and high-risk areas from 15% to less than 7.5 % prevalence in 2025 and to further reduce transmission in low prevalence areas to less than 0.5% *P. falciparum* parasite rate (pfpr) in 2025. Vaccination is considered as one of the important strategies in the fight against malaria in Tanzania(MoHSW, 2014), and therefore vaccine research and development is essential to the country.

Several transmission-blocking candidate vaccines are in the clinical pipeline (Coelho, Doritchamou, Zaidi, & Duffy, 2017) (Doumbo, Niaré, Healy, Sagara, & Duffy, 2018). In the current study, the ex-vivo efficacy of *P. falciparum*-based vaccine candidate, *Pfs*25-IMX312 in Matrix-M adjuvant was assessed following vaccination in adult volunteers from Bagamoyo. The distribution of gametocyte carriage and naturally acquired anti-gametocyte antibodies to a set of selected gametocyte antigens were also characterized because they are of paramount importance for the development and evaluation of transmission-blocking tools in endemic settings.

1.4. Study Objectives

1.4.1. General Objective

To evaluate the ability of Pfs25-IMX313 vaccine candidate, to induce antibodies that prevent the development of malaria parasites in mosquitoes and block malaria transmission.

1.4.2. Specific Objectives

The following specific objectives were pursued:

i. To assess the current *P. falciparum* positivity in humans and field mosquitoes
ii. To investigate naturally acquired transmission-blocking responses against *P. falciparum* gametocytes
iii. To determine the levels of vaccine-induced anti-Pfs25 total IgG responses in healthy individuals naturally exposed to malaria
iv. To determine the malaria transmission-reducing activity of vaccine-induced anti-Pfs25 IgG and naturally acquired anti-gametocyte IgG in laboratory-reared mosquitoes
v. To assess genetic polymorphism in Pfs25 transmission-blocking target protein in field isolates of *P. falciparum*

1.5. Research Questions

The study sought to answer the following questions:

i. What is the current malaria prevalence in humans and field mosquitoes in Bagamoyo?
ii. Is there evidence of naturally acquired malaria transmission-blocking immunity in Bagamoyo?
iii. Does Pfs25-IMX313 induce long-lasting anti-Pfs25 responses in people naturally exposed to malaria?
iv. To what extent can vaccine-induced anti-Pfs25 IgG reduce malaria oocyst development in mosquitoes?
v. What is the level of genetic variation in the Pfs25 transmission-blocking target protein in Bagamoyo?

1.6. Significance of Study

This study provides formative insights into ideal field dose of the vaccine candidate Pfs25-IMX313 in endemic settings as well as guidance on the choosing other ideal vaccine targets in malaria endemic areas. The study findings highlight the importance of targeting children and adults to achieve malaria elimination. The anti-gametocyte immunological and parasitical parameters from this study are useful indicators for considering demographic characteristics when developing and evaluating transmission-blocking vaccines in endemic areas.

The study provides information on any changes in the genetic makeup of transcripts for TBV targets to ensure that the vaccine tool remains efficacious.

1.7. Delineation of the Study

The vaccine candidate evaluated in this study was based on *Plasmodium falciparum* zygote and ookinete surface antigen (Pfs25) fused to nanoparticle IMX313, and was administered in a single syringe to study participants. The Pfs25-IMX313/Matrix-M candidate vaccine targets only *P. falciparum* and this was the first time it was tested in healthy adults, living in malaria endemic area. The study focused on Pfs25, Pfs230 and Pfs48/45 proteins as these are the most clinically advanced targets for transmission-blocking vaccine candidates. Recombinant Pfs25, Pfs230, and Pfs48/45 antigens were used to determine total IgG levels for respective antigens, and antibody functionality was assessed by ex-vivo methods, using strains of *P. falciparum* parasites and mosquitoes grown in a controlled environment. The presence of gametocyte and naturally acquired malaria transmission-blocking immunity to *P. falciparum* was assessed in school-age children and adults from five (5) villages in the Bagamoyo district. The prevalence of other malaria species was also determined.

CHAPTER 2: LITERATURE REVIEW

2.1. Overview of Malaria

Malaria is a very old disease, believed to have been around since 3200 BC. Its name dates back to the ancient Romans, who considered the disease to be airborne and highly associated with swamps (Garnham, 1966). In 1880, Charles Laveran showed that malaria is caused by protozoan parasites of the genus *Plasmodium*, and later, Sir Ronald Ross confirmed that malaria parasites are transmitted by mosquitoes(Garnham, 1966; Harrison, 1978). There are five parasite species known to cause malaria in humans, including *Plasmodium falciparum* (*P. falciparum*), *Plasmodium vivax* (*P. vivax*), *Plasmodium ovale* (*P. ovale*), *Plasmodium malariae* (*P. malariae*) *(*Manske *et al.) and Plasmodium knowlesi*. *P. falciparum* is the deadliest and predominant species in many endemic areas. *P. vivax* and *P. ovale* are traditionally thought to occupy complementary niches, with *P. ovale* predominating in Sub-Saharan Africa and *P. vivax* in the other areas; however, their geographical ranges overlap. These two species are not always distinguishable based on morphological characteristics alone. *P. malariae* has wide global distribution, being found in South America, Asia, and Africa. Most of the malaria cases in Malaysia are caused by *P.knowlesi* and also in the South East Asian countries (Sato, 2021) .

2.1.1. Human-to-mosquito malaria transmission

Malaria is transmitted in tropical and subtropical areas because climatic factors such as temperature, humidity, and rainfall allow Anopheles mosquitoes to survive and multiply. The tropical environment is also suitable for malaria parasites to complete their development cycle in the mosquitoes (Figure 1). Ambient temperature is particularly critical for malaria transmission; for example, at temperatures below 20°, *P. falciparum* cannot complete the cycle in the *Anopheles* mosquito, and thus cannot be transmitted.

Transmission of *P. falciparum* from humans to mosquitoes depends on the sexual phase of the parasite's life cycle. The sexual cycle in humans starts with the activation of asexual schizonts to express the Apatella2-gene (AP2-G) and the production of sexual progeny, which become gametocytes (Kafsack et al., 2014; Sinha et al., 2014). *P. falciparum* gametocytes undergo five morphologically distinct forms (stages I-V) over a period of 10-12 days. The gametocyte stages I-IV sequester primarily in the bone marrow and spleen (Joice et al., 2014), before finding their way into blood circulation to complete the final maturation

steps (Aguilar et al., 2014). Stage V gametocytes can circulate for several weeks after clearance of asexual parasites (Reuling et al., 2018), both male and female gametocytes must be taken up by a female *Anopheles* mosquito for the parasite sexual cycle to complete. Many factors influence the likelihood of gametocytes being transmitted to mosquitoes and the establishment of a successful mosquito stage infection (T. Bousema & Drakeley, 2011). General parasite characteristics associated with differences in transmission potential and infectivity include; gametocyte density (Bradley et al., 2018; K. A. Collins et al., 2018; Dari F Da et al., 2015), concurrent asexual parasite density (Lin Ouédraogo et al., 2016), male-to-female gametocyte ratio (Bradley et al., 2018; Paul, Brey, & Robert, 2002), and duration of infection (Johnston, Smith, & Fidock, 2013; Lin Ouédraogo et al., 2016). Host factors including, age, anaemia, immunity, and mosquito factors also influence gametocyte infectiousness (Smith et al., 2014).

In the mosquito midgut, *Plasmodium* gametocytes egress from the host erythrocyte and develop into gametes. Gametogenesis is induced by conditions in the mosquito midgut including; reduction in temperature, increase in pH and exposure to xanthurenic acid (Billker et al., 1998; Billker, Miller, & Sinden, 2000). Male gametocytes exflagellate producing up to eight motile microgametes; whereas, female gametocytes "round-up" to form one immotile macrogamete (R. Sinden, 1983a, 1983b). Fertilization of a macrogamete by a microgamete result in the formation of a zygote, which then develops into a mature motile ookinete that traverses the midgut wall and forms an oocyst. Approximately 10-12 days after blood meal ingestion the rupture of oocysts results in the release of sporozoites, which invade the mosquito salivary glands completing the mosquito stage of the *Plasmodium* life cycle (Meis, Wismans, Jap, Lensen, & Ponnudurai, 1992).

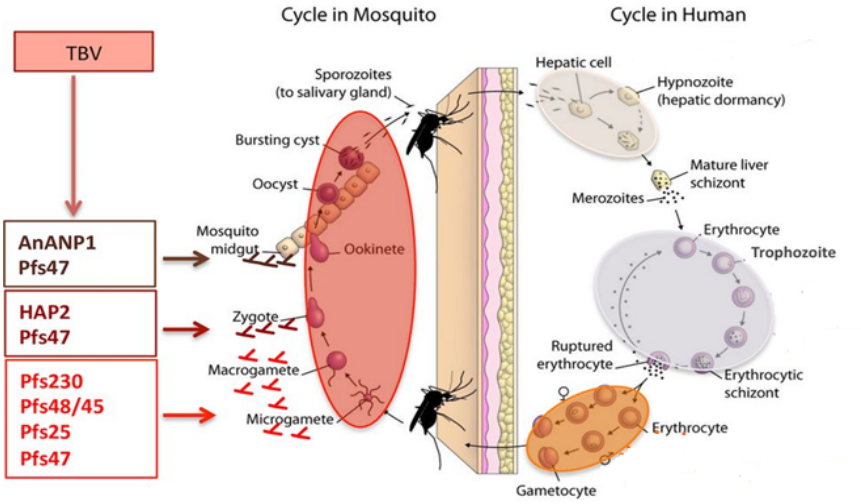

Figure 1: Potential targets for TBVs in the malaria transmission cycle

Figure was adapted from Acquah, Festus K., *et al*., 2019 (Acquah, Adjah, Williamson, & Amoah, 2019)

2.2. Malaria in Mainland Tanzania

Tanzania is one of the 11 countries with the high malaria burden (WHO 2022, 2023). There were approximately 4.5 million cases and 25,000 malaria deaths reported in 2022, and more than 60 million people are at risk of the disease (WHO 2022). The malaria case incidence increased from 123 per 1000 at risk in 2020, to 126 per 1000 in 2021. The prevalence varies by region from <1% in the highlands of Arusha to as high as 15% in the Southern Zone and 24% along the Lake and Western zones. *P. falciparum* is responsible for more than 90% of malaria infections in Tanzania, while *P. malariae* and *P. ovale* account for less than 5% of all the reported cases. The primary malaria vectors in include; *Anopheles gambiae* (*An.gambiae*) *sensu stricto*, *An. arabiensis* and *An. funestsus sensu stricto* (Killeen & Sougoufara, 2023; Kisinza et al., 2017).

2.2.1. Malaria control in Tanzania

Malaria control takes a significant share of the annual health budget, with approximately 40% national health expenditure devoted to malaria. At least $100 million, mainly from external donations and non-government organizations, was spent annually on the fight against the disease in period between 2019 and 2023 alone (MoHSW, 2014). Tanzania's Malaria National Strategic Plan (MNSP) revises its targets every five years. The MNSP 2020 – 2025 aims to reduce the average malaria prevalence in children under 5 years, from 7% in 2017 to < 3.5% in 2025. Furthermore, there are targets to reduce malaria burden in moderate and high-risk areas, from 15% prevalence in 2017 to < 7.5 % in 2025 and to further reduce transmission in low prevalence areas from 1% *P. falciparum* parasite rate (pfpr) in 207 to < 0.5% (pfpr) in 2025. The MNSP implements a number of strategies to reach the set targets; the first strategy involves malaria case identification using mRDT and/or microscopy, and treatment with ACTs, quinine, and intermittent preventive treatment in pregnancy (IPTp) with sulfadoxine-pyrimethamine (SP) to pregnant women from high transmission areas. The second strategy is integrated vector control, which involves determining the geographic distribution and insecticide resistance profiles of malaria vectors, as well as the use insecticide-treated materials such as insecticide-treated mosquito net (ITNs) and indoor residual spraying (IRS). Other cross-cutting strategies include:

i. improving the supply chain to ensure timely availability of safe and quality malaria commodities and supplies

ii. surveillance, monitoring, and evaluation (SME) to provide timely and reliable information for assessing progress toward the global and national targets as well as ensuring cost-effective use of resources; and accounting for investments made in malaria control in the country; and

iii. social and behavior (SBC) change to create and strengthen an environment where the population at risk are empowered to protect themselves from malaria and seek proper and timely malaria treatment

Malaria vaccines are also regarded as an important component in the fight against malaria but there is limited research and development for malaria vaccines in the country yet some malaria vaccines including RTS,S and R21 are currently recommended in Tanzania (MoHSW, 2014).

2.2.2 Drug and insecticide resistance in Tanzania

In 2001, chloroquine (CQ) resistance forced Tanzania to replace CQ with sulphadoxine-pyrimethamine (SP) as first-line treatment (Mohammed et al., 2013). Due to high resistance, SP lasted for only five years and was replaced with the current ACTs (artemisinin-combination therapies) (Baraka et al., 2015; Mohammed et al., 2013). However, SP is still being used for intermittent preventive treatment during pregnancy (IPTp-SP). A high prevalence of haplotypes conferring SP resistance has been reported in some areas in Tanzania including; Kagera, Tanga, and Lindi; and this could undermine the use of IPTp-SP(Baraka et al., 2015; Moser et al., 2021).The extended use of ACTs in malaria control and elimination programs has resulted to the emergence of *P. falciparum* resistant to artemisinin in neighboring Kenya (Makau et al., 2024), Uganda (Conrad et al., 2023), and Rwanda (Uwimana et al., 2020). Resistance to artemisinin alone rarely leads to treatment failure but resistance to both artemisinin and the partner drug within the ACTs can lead to high treatment failure rates, as reported in parts of Southeast Asia. To date, there are no reports of resistance to ACT partner drugs in Africa. However, the limited data and contradictory reports on ACT efficacy as well as diagnostic resistance (Conrad et al., 2023; Makau et al., 2024; Uwimana et al., 2020), are of great concern.

Insecticide resistance among malaria-vector species is now a universal occurrence, affecting malaria-endemic areas worldwide (Killeen & Sougoufara, 2023). Entomological surveillance conducted in Tanzania, between 2009 and 2020 (Tungu et al., 2023), showed that insecticide resistance in principle malaria vectors had increased in prevalence and intensity, and has been spreading geographically (Figure 2). Wide spread resistance to permethrin, deltamethrin, and lambda-cyhalothrin has been observed in a significant number of areas across Tanzania (Tungu et al., 2023).

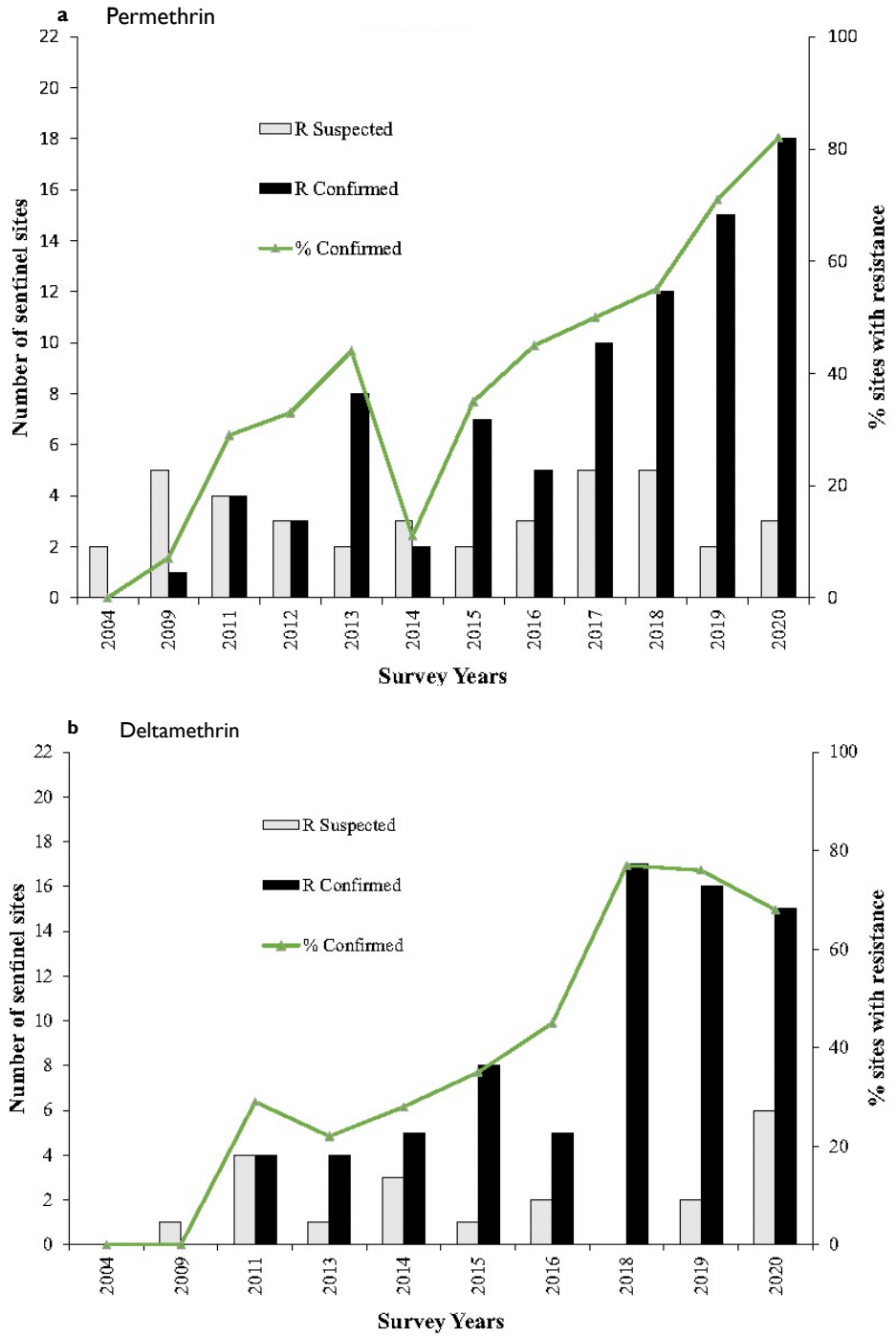

Figure 2: Spatiotemporal trend of insecticide resistance in Tanzania

A) Spatiotemporal trend of permethrin phenotypic resistance in *Anopheles gambiae sensu lato* mosquitoes. B) Spatiotemporal trend of deltamethrin phenotypic resistance in *Anopheles gambiae sensu lato* mosquitoes. The solid bars indicate number of sites with resistance and the line graph indicates the percentage of sites with resistance (Tungu et al., 2023)

2.3. Overview of Malaria vaccine development

Vaccine development generally starts with the identification and characterization of target antigens through scientific research, which usually occurs in laboratories with extensive infrastructure and cutting-edge technologies. Potential vaccine candidates are then evaluated preclinically in animal models followed by clinical trial phases I–III in humans, to determine the safety, immunogenicity, and efficacy of the candidate vaccine. The clinical trials are followed by regulatory approval and field deployment. The development of the Pfs25-IMX313 malaria vaccine candidate is following the same process.

Malaria vaccines in the pipeline are designed to target specific asexual or sporogonic/sexual stages of the malaria parasites in humans or mosquitoes. Sporozoite and liver stage vaccines like RTS-S and R21 are designed to elicit protection and prevent sporozoites from infecting hepatocytes and liver stage parasites to develop into blood stage parasites (Reyes-Sandoval et al., 2011; Vanderberg, Nussenzweig, Most, & Orton, 1968) respectively. Blood stage candidate vaccines such as reticulocyte homolog 5 (RH5) induce protection which reduces parasitemia and therefore severity or episodes of the disease (Fried & Duffy, 2015). Malaria candidate vaccines designed to target the sexual stages of malaria parasites are commonly known as transmission-blocking vaccines (TBVs), and they induce antibodies which neutralize parasites in the mosquitoes after their infectious blood meal, consequently blocking onward transmission (Coutinho-Abreu & Ramalho-Ortigao, 2010). TBVs do not directly protect immunized individuals from clinical malaria but can reduce the number of circulating infectious mosquitoes below a threshold that can sustain transmission when deployed at the community level. Malaria vaccine research initially focused on the parasite stages leading to human infection (sporozoites/ pre-erythrocytic stages) and clinical disease (asexual stages), but as TBV development gained pace, the biological understanding of sexual stages (gametocyte/gametes) has improved dramatically (de Jong et al., 2020).

2.4. Characterization of Transmission-blocking antigens

The observation of blocking immunity against malaria infection in mosquitoes was first reported in 1958 (Huff, Marchbank, & Shiroishi, 1958) and later confirmed in 1976 to be antibody-mediated inhibition of the development of sexual stages in the female mosquitoes (R. Carter & Chen, 1976); the 1958 studies were conducted using the avian *P. gallinaceum* and *P. fallax* in chickens and turkeys and the outcome showed that birds immunized with

killed parasites had a significant and rapid fall in the infectivity of gametocytes compared with controls, supporting the role of active immunity (Huff et al., 1958). Similar experiments were reproduced in 1976 and demonstrated that antibodies were responsible for the inhibition of sexual parasite development in the mosquito midgut (R. Carter & Chen, 1976). Transmission-blocking monoclonal antibodies (mAbs) were later generated following immunization of BALB/c mice with *Plasmodium,* facilitating the characterization of the first set of sexual-stage antigens for transmission-blocking antibodies (Rener, Graves, Carter, Williams, & Burkot, 1983). These antigens included P230 and P48/45, and later P28 and P25 were also characterized; "P" refers to genus *Plasmodium* and the number refers to the antigen molecular weight on SDS-PAGE (Kaushal et al., 1983; Rener et al., 1983).

2.4.1. Transmission-blocking target antigens

Malaria transmission-blocking antigens are broadly classified into two groups: pre-fertilization and the post-fertilization antigens. Both groups have homologs in all malaria species, and have been the focus of gametocyte research for decades as well as forming the basis of malaria transmission-blocking vaccine (TBV) development (R Carter et al., 1984; Vermeulen et al., 1985).

i. **Pre-fertilization antigens:** they are expressed during gametocyte development in humans, and contribute to the viability of mosquito stage parasites as well as playing a crucial role in fertilization (Eksi et al., 2006; van Dijk et al., 2001). Some pre-fertilization antigens are gametocyte-specific while others are shared by the two sexes. Gametocyte-specific proteins remodel the human host cell to support gametocyte morphological development and maturation (Tibúrcio et al., 2012)**.** Pre-fertilization antigens shared by two gametes are involved in processes necessary for parasite colonization of the mosquito midgut. *P. falciparum* surface antigens 230 and 48/45 **(**Pfs230 and Pfs48/45) are the most studied pre-fertilization antigens, belonging to the 6-cysteine protein family (Gerloff, Creasey, Maslau, & Carter, 2005).

ii. **Post-fertilization antigens**: these are expressed solely in the mosquito vector; though transcription may occur in circulating gametocytes (Miao et al., 2013). The environment in mosquito midgut triggers the expression of post-fertilization antigens (Mair et al., 2006). *P. falciparum* surface antigens 25 and 28 (Pfs25 and Pfs28) are the most advanced post-fertilization antigens (Nikolaeva, Draper, & Biswas, 2015; Yimin Wu, Sinden, Churcher, Tsuboi, & Yusibov, 2015). Pfs25 is a cysteine-rich 217-amino acid composed

of four tandem epidermal growth factor (EGF)-like domains and encoded by a 0.65-kb gene. Pfs25 is predicted to be a 25-kDa glycosylphosphatidylinositol (GPI)-anchored protein belonging to a 13-member P25 family of proteins (Kaslow et al., 1988). The protein is involved in ookinete formation, survival in the mosquito midgut, and a possible role in parasite traversal of the mid-gut epithelium (Baton & Ranford-Cartwright, 2005; K. Miura et al., 2007; Tomas et al., 2001). The Pfs25 molecule is thought to be triangular and flat, and extensively expressed on the ookinete surface, forming a protective interlocking sheet (Saxena et al., 2006; Stowers, Keister, Muratova, & Kaslow, 2000). Pfs25 expression starts from the point when gametes egress from ingested red blood cells in the mosquito midgut, through the zygote and ookinete stages, with evidence of continued expression in the oocyst. The female gametocyte contains abundant Pfs25 transcripts, but its translation is repressed until transmission to the mosquito vector and egress from the gametocyte infected red blood cell (Mair et al., 2006). Lack of expression in the human host means that Pfs25 has not come under the same level of immune pressure as many other potential malaria vaccine antigens (Kaslow, Quakyi, & Keister, 1989). Large-scale deep sequencing of P. falciparum field isolates from diverse geographic regions indicates that the Pfs25 gene is highly conserved, revealing only one synonymous mutation (Manske et al., 2012). It is therefore hypothesized that Pfs25 vaccine–induced antibody responses should have transmission reducing activity against diverse parasitic strains. Da, et al. demonstrated that antibodies against recombinant Pfs25 had significant transmission reducing activity against diverse isolates from Burkina Faso and Thailand (D. F. Da et al., 2013).

2.5. Transmission-blocking immune mechanisms

Immune responses to pathogens generally involve humoral and cellular mechanisms. Humoral responses to pre-fertilization antigens of malaria parasites can be naturally acquired in the human host(T. Bousema et al., 2011; Drakeley et al., 2004; Mulder et al., 1999; Premawansa et al., 1994), while humoral responses against post-fertilization antigens expressed solely in the mosquito do not occur naturally in humans but can be induced by vaccination. Natural transmission-blocking antibodies occur because most of the gametocytes die in the human host, releasing intracellular proteins into the host circulation (Kengne-Ouafo et al., 2019). The released proteins are processed and presented to the human immune system resulting in induction of antibodies, which can cause a reduction or interruption of parasite

development (fertilization) in the mosquitoes when taken up in a blood-meal containing gametocytes (Mendis, David, & Carter, 1990). Antibodies against surface antigens on the gamete surface may prevent fertilization by opsonization resulting in immune cell-mediated lysis (Ranawaka, Alejo-Blanco, & Sinden, 1994) or agglutination of gametes (Tachibana, Ishino, Tsuboi, & Torii, 2018), or by direct lysis of gametes and activation of the complement system (Grotendorst, Carter, Rosenberg, & Koontz, 1986; Healer et al., 1997). Antibody activity against malaria parasite development in mosquitoes was first observed in 1958 in studies conducted using the avian *P. gallinaceum* and *P. fallax* in chickens and turkeys, with results showing that birds immunized with killed parasites had a significant and rapid fall in infectivity of gametocytes, supporting the role of active immunity (Huff et al., 1958). These experiments were reproduced in 1976, and demonstrated that antibodies were indeed responsible for preventing parasite development in the mosquitoes midgut (R. Carter & Chen, 1976).

Naturally acquired antibody responses to *P. falciparum* gametocytes were first reported in individuals from malaria endemic sites in Papua New Guinea, where up to a 95% reduction in mosquito infectivity was observed during mosquito feeding experiments with cultured gametocytes (Graves, Carters, Burkot, Quakyi, & Kumar, 1988). The presence of naturally occurring antibodies in endemic areas has since been observed in studies from different countries including; Tanzania (J. Bousema et al., 2007; T. Bousema et al., 2010), Gambia, Kenya, Cameroon (T. Bousema et al., 2011; Drakeley et al., 2004; Mulder et al., 1999), Sri Lanka (Premawansa et al., 1994) and Burkina Faso (Ouédraogo et al., 2011). Published data sets strongly suggest that recent gametocyte exposure is associated with a strong and effective transmission-blocking immunity (TBI) (T. Bousema et al., 2011; Drakeley et al., 2004; Ouédraogo et al., 2018). TBI has been significantly associated with antibody responses to pre-fertilization antigens; Pfs230, Pfs48/45, PfsHAP2, and to other novel gametocyte proteins (Jones et al., 2015; Stone et al., 2018). There have been conflicting observations regarding age as a factor of natural seroprevalence even in similar settings; in some studies, higher responses have been observed in older individuals (Stone et al., 2018) while this has not been the case in other studies even in similar study areas (Drakeley et al., 2006; Ouédraogo et al., 2011).

The cellular immune mechanisms involved in the clearance of circulating gametocytes are not well understood. Since the red blood cells lack major histocompatibility complex molecules, direct targeting of gametocyte-infected erythrocytes by T lymphocytes is not

possible. However, CD4 + T cells can respond to gametocyte antigens (Goodier & Targett, 1997; Riley et al., 1990) and appear capable of inducing long-lasting gametocytocidal immunity in rodent models (Harte, Rogers, & Targett, 1985).

Malaria parasite neutralization inside the mosquito is not only enabled by human-derived molecules but also by mosquito cellular immune components already reviewed in (Kengne-Ouafo et al., 2019). Mosquito infection requires the ingestion of both male and female gametocytes, whose microgametes and macrogametes must meet inside the midgut for fertilization. Studies have extensively characterized the population bottlenecks facing the malaria parasites in the mosquito midgut, indicating that only a small proportion of gametocytes ingested in the blood meal by the mosquito develop into oocysts; and about 38% of mosquitoes that take gametocytemic blood become infected (Gouagna et al., 1998; R. E. Sinden & Billingsley, 2001; Smith et al., 2014). The parasite bottleneck is largely attributed to formation of a parasite physical barrier (peritrophic membrane or matrix), surrounding the blood meal after ingestion (Dinglasan et al., 2009; Shao, Devenport, & Jacobs-Lorena, 2001). The membrane prevents direct contact between the parasite and the midgut epithelium thereby interrupting the mosquito-midgut invasion (Shao et al., 2001); although ookinetes are able to bleach this barrier through chitinase enzymes (Vinetz et al., 1999). In addition, there are peroxidase and nitric oxide synthase present in epithelium, which leads to nitration of the gut epithelium with subsequent tagging of ookinetes for destruction by the complement system (Garver, de Almeida Oliveira, & Barillas-Mury, 2013; Kumar, Gupta, Han, & Barillas-Mury, 2004).

2.6. Status of Pfs25-based Vaccines

Pfs25 is the most advanced transmission-blocking target protein (Coelho et al., 2017) in the clinical pipeline, with Pfs230 the only other protein to be clinically evaluated. Because Pfs25 is not expressed in the vertebrate host, it is not subjected to natural immune selection pressure hence reduced risk of development of resistance parasite strains. However, Pfs25 cannot benefit from the natural boosting following vaccination.

The first clinical evaluation of the Pfs25 antigen was done in a Phase I/II trial that tested a multi-stage vaccine containing attenuated recombinant vaccinia viral vector encoding the sporozoite targets CSP and PfSSP2, the liver-stage target LSA1, and blood-stage antigens MSP1, SERA and AMA1 alongside Pfs25. The trial assessed the high and low doses of the vaccine and found the vaccine to be well tolerated. The Pfs25 component of the vaccine was

highly immunogenic, but the anti-Pfs25 antibodies did not show any transmission-blocking activity (Ockenhouse et al., 1998).

In another study, the recombinant Pfs25 and the *P. vivax* homolog Pvs25, formulated with Montanide ISA 51 adjuvant (a water-in-oil emulsion) were tested in a single blinded, dose escalation-controlled Phase Ia trial. There was observed systemic reactogenicity, which was associated with the antigen/adjuvant combination. There had been no reports of previous severe systemic reactions for Montanide ISA 51 (Malkin et al., 2005). Nevertheless, 5/5 volunteers who had completed the vaccination before cessation of the trial developed substantial antibody responses to Pfs25, and the antibodies blocked parasite development in *Anopheles stephensi* mosquitoes; TRA correlated with antibody titres, with serum from the best responder showing greater than 90% reduction in oocyst intensity (Y. Wu et al., 2008).

Pfs25 has also been evaluated as protein-in-adjuvant formulation Pfs25-EPA in Alhydrogel in a phase Ia trial (Talaat et al., 2016). Pfs25-EPA is a recombinant Pfs25 expressed in *P. pastoris*, chemically conjugated to detoxified ExoProtein A from *Pseudomonas aeruginosa*. The Pfs25-EPA/Alhydrogel vaccine was evaluated in a two-dose, three-dose, four-dose regimen, administered at 0, 2, 4 and 10 months, in thirty US healthy volunteers. The vaccine demonstrated a favorable safety profile in thirty healthy adult volunteers. At the highest dose, specific IgG responses were seen following the second and third vaccination, peaking two weeks after the 4th booster. In the two-dose regime, seroconversion rates were generally low with exception of one volunteer demonstrated high Pfs25 specific antibody titres after receiving a third booster dose. Antibody avidity was also shown to increase over successive vaccinations. In standardized membrane feeding assay (SMFA), significant TRA (greater than 50%) was demonstrated in the highest dose group after the fourth vaccination. Significant TRA was not detected in the majority of sera from timepoints following the second or third vaccination (Talaat et al., 2016).

A Phase I dose-escalation study in the US recently evaluated the safety and immunogenicity of virus-like particle (VLP) candidate Pfs25 VLP-FhCMB. Pfs25 VLP-FhCMB consist of Pfs25 genetically fused to the Alfalfa mosaic virus coat protein and is produced in Nicotiana benthamiana plants (Chichester et al., 2018). Safety and immunogenicity of Pfs25 VLP-FhCMB, formulated in Alhydrogel, was evaluated in a three-dose regimen, administered on days 0, 56 and 168, in 44 healthy volunteers. The vaccine was well tolerated at all doses and specific IgG responses were induced one month after the second and third vaccinations. The

two lower doses were generally not immunogenic. Statistically significant TRA was detected in the highest dose group, inhibiting oocyst intensity at close to 80% in a subset of 2/8 individuals receiving the 100μg dose; however, overall, TRA was weak. Pfs25-based candidates in clinical developments are summarized in Table 1 (Doumbo et al., 2018).

Early phase testing of TBV efficacy requires biological endpoints that can be used to estimate human-to-mosquito transmission. These endpoints include transmission-blocking activity (TBA) and Transmission-reducing activity (TRA) which are measured via functional assays known as standard membrane feeding assays (SMFA) (Kazutoyo Miura, Stone, et al., 2016). In the SMFA, high densities of cultured *P. falciparum* gametocytes are fed to mosquitoes in combination with test or control samples. TBA is the ability of a vaccine to reduce the proportion of infected mosquitoes. In most cases, vaccines do not have the ability to completely inhibit parasite development in mosquitoes but reduce the infection burden in the mosquitoes. The achieved reduction of parasite density in test samples compared to controls is termed as TRA(Kazutoyo Miura, Stone, et al., 2016). SMFA are optimized to achieve high parasite (i.e., oocyst) densities in control mosquitoes to maximize precision and reproducibility (Churcher et al., 2012). Because of this high infection intensity in control mosquitos, even highly potent samples (antibodies) may not prevent oocyst formation completely and TRA is the common readout of SMFA(Kazutoyo Miura, Stone, et al., 2016).

Table 1: Pfs25-based Candidate Vaccines in Clinical pipeline

Vaccine candidate	Type	Stage of development	Trial Identification number/reference
Pfs25M-EPA/AS01	Subunit vaccine	Phase 1	NCT02942277
Pfs25EPA/Alhdrogel	Subunit vaccine	Phase 1	NCT02334462
Pfs25EPA/Alhdrogel	Subunit vaccine	Phase 1	NCT01867463
Pfs25 VLP-FhCMB	VLP vaccine	Phase 1	NCT02013687
ChAd63 Pfs25-IMX313+/- MVA Pfs25-IMX313	Viral vector & nanoparticle vaccine	Phase 1	NCT02532049
Pfs25 /Montanide ISA 51	Subunit vaccine	Phase 1	(Y. Wu et al., 2008)
Pfs25-Pfs25	Conjugate vaccine	Phase 1	NCT00977899

The early Pfs25-based vaccine clinical trials have yielded modest and short-lived antibody responses with poor transmission-blocking activity, some have shown significant reactogenicity attributed to adjuvant formulations. Several strategies are being pursued to overcome this hurdle; including advances in vaccine expression systems, delivery platforms, and adjuvant formulations. Expression in a variety of recombinant systems, including yeast (Barr et al., 1991; Stowers et al., 2000), plants (Farrance, Chichester, et al., 2011), and algae (Patra et al., 2015), have been successful. Monoclonal antibodies raised against correctly folded recombinant Pfs25 antigens, such as the highly effective 4B7, have been found to achieve potent transmission-blocking activity (Cox et al.) at low concentrations (Barr et al., 1991; K. Miura et al., 2007), and MAb 4B7 is used as a reference and positive control for mosquito-feeding assays (Li et al., 2016; Kazutoyo Miura, Stone, et al., 2016; Kazutoyo Miura, Takashima, et al., 2013). A number of approaches to vaccine particle development have also been pursued to increase immunogenicity. Conjugation to carriers is one of the advanced methods to improve vaccine immunogenicity where carrier proteins such as *Pseudomonas aeruginosa* exoprotein A (EPA) (Qian et al., 2007) and bacterial outer membrane protein complex (OMPC) have been used. Another method is fusion to partners that form complexes, generating particles [such as C4bp oligomerization domain (IMX313)] expressed in *E. coli* or modified lichenase carrier (LiKM) produced in *Nicotiana benthamiana*. Viral vector vaccines, such as Chad63/Modified Vaccinia Ankara, are also

being assessed to improve immunogenicity (Li et al., 2016; Ogun, Dumon-Seignovert, Marchand, Holder, & Hill, 2008; Yimin Wu et al., 2006) (Ogun et al., 2008). Adjuvants, such as Alhydrogel® and Montanide®, have been used for clinical trials of TBVs with some reactogenicity issues observed, and recently, the saponin-based Matrix-M adjuvant used for formulating the pre-erythrocytic vaccine R21 (Datoo et al., 2021), this would simplify future efforts to combine products (Doumbo et al., 2018).

2.6.1. Pfs25-IMX313 vaccine development

Pfs25-IMX313 is a protein-nanoparticle vaccine for which the Pfs25 antigen is genetically fused to the IMX313 oligomerization domain (Ogun et al., 2008). The Pfs25 protein is based on the sequence from the 3D7 *P. falciparum* strain, with three potential N-linked glycosylation sites (112, 165 and 187) mutated. The recombinant protein-nanoparticle is expressed and secreted in the *Pichia pastoris* expression system (Li et al., 2016).

IMX313 is a hybrid of the oligomerization domain of chicken complement inhibitor C4-binding protein (C4bp), with 21% homology to the sequence of the human protein (11 identical residues in an overlap of 52 amino acids) (Ogun et al., 2008). It is thus unlikely that vaccination with an antigen fused to IMX313 would generate an immune response against hC4bp. IMX313 forms homogenous, self-assembling heptamers of the antigen fused to it (Ogun et al., 2008). This C4bp oligomerization domain has been shown to spontaneously form soluble heptameric structures (termed nanoparticle in this study) when expressed in *E. coli*, and protein antigens fused to these domains induce higher antibody responses compared to the same amount of monomeric antigen. In addition, mice immunized with the blood-stage malaria vaccine candidate antigen MSP119 fused to IMX313 (expressed in *E. coli*) were protected against challenge with a lethal dose of *P. yoelii* parasites (Ogun et al., 2008). Immunization of mice and non-human primates with the *Mycobacterium tuberculosis* antigen 85A fused to IMX313 in both DNA vaccines and viral vectors also demonstrated that IMX313 enhances T-cells response, with a consistent increase in both CD4+ and CD8+ T cell responses.

Clinically, IMX313 was first assessed in the recombinant MVA85A-IMX313 tuberculosis vaccine candidate, administered intradermally, in a Phase Ia trial at the Jenner Institute in Oxford. In this first-in-human dose escalation study a total of 18 volunteers received either MVA85A-IMX313 or MVA85A. There were no safety concerns and no significant

difference in the number of adverse events (AEs) between the MVA85A-IMX313 group and the MVA85A group. While there were no significant differences in immunogenicity between MVA85A and MVA85A-IMX313 groups (Minhinnick et al., 2016).

Pfs25-IMX313 has been clinically tested using ChAd63 prime and MVA boost (De Graaf et al., 2021). Both vaccines had a favorable safety profile and induced both antibody and T-cell responses, but no significant TRA was observed.

2.6.2. Pre-clinical evaluation of Pfs25-IMX313

Pfs25-IMX313 protein-in-adjuvant vaccines are immunogenic in BALB/c mice: Pfs25-IMX313, formulated in Alhydrogel, induced higher Pfs25-specifc IgG responses than monomeric Pfs25 in Alhydrogel. These vaccine-induced antibodies were demonstrated to recognize native parasite proteins by immunofluorescence microscopy (Li et al., 2016). Vaccination with Pfs25-IMX313/Alhydrogel has been shown to induce antibody responses in BALB/c mice with significantly higher TRA compared to IgG from mice immunized with monomeric Pfs25/Alhydrogel ($p < 0.02$ at all concentrations of IgG tested). A similar effect was observed following vaccination with ChAd63/MVA Pfs25-IMX313, compared to viral vectors encoding Pfs25. Based on functional activity as assessed in SMFA, the quality of the anti-Pfs25 antibody response induced by immunization with the Pfs25-IMX313 protein-nanoparticle was significantly improved in comparison to that induced by monomeric Pfs25 in Alhydrogel. (Li et al., 2016). In addition, antibody responses induced following immunization with Pfs25-IMX313 had higher avidity than antibodies induced by soluble Pfs25 (Li et al., 2016).

2.6.3. Pfs25-IMX313 in Matrix-M

The clinical development of Pfs25-IMX313 in Matrix-M adjuvant is aimed towards the production of an effective transmission-blocking malaria vaccine for individuals in malaria endemic regions. Matrix-M is a potent saponin-based adjuvant, comprising partially purified extracts of the bark of the *Quillaja saponaria* Molina tree, phosphatidylcholine and cholesterol, formulated as a 40nm-sized complex. Matrix-M has been shown to efficiently activate and recruit immune cells to the draining lymph node, including T-cells, B-cells, NK-cells, dendritic cells and granulocytes, which may lead to enhanced antigen presentation (Bengtsson et al., 2016).

Matrix-M has demonstrated a favourable safety profile and enhanced cellular and humoral immune responses to a range of vaccines (Bengtsson et al., 2016; Cox et al., 2011; Venkatraman et al., 2017). Available clinical data demonstrate that the Matrix-M adjuvant is well tolerated and that there have been no reported serious unexpected adverse reactions or adverse reactions of special interest (Bengtsson et al., 2016; Cox et al., 2011; Datoo et al., 2021; Lövgren Bengtsson, Morein, & Osterhaus, 2011). Pre-clinically, Pfs25-IMX313 administered both with and without the Matrix-M adjuvant has been demonstrated to be immunogenic in BALB/c mice, and the addition of Matrix-M adjuvant led to significantly higher antibody responses (Li et al., 2016).

CHAPTER 3: METHODS AND MATERIALS

3.1. Description of study area and population

The study was conducted in the Bagamoyo district, which is in Pwani region, Tanzania (Figure 3). The district represents low malaria transmission, with prevalence in the general population estimated at 10% (MoHSW, 2011). Bagamoyo is one of the 6 districts of Tanzania's coast region, with a population of about 345,000. It has a humid tropical climate with seasonal average temperature ranging from 13˚C to 30˚C and relative humidity of as high as 98%. Malaria remains a public health problem in the district; and transmission tends to be highest between March-May and October-November (T. Bousema et al., 2010).The transmission intensity is usually high during and after the long rainy season (T. Bousema et al., 2010), which usually occurs from March to June. Majority of the reported cases are caused by *Plasmodium falciparum*, but *Plasmodium malariae* and *Plasmodium ovale* have also been reported. (Hofer et al., 2023; Z. Mwangonela, 2023; Popkin-Hall et al., 2023; Tarimo et al., 2022).

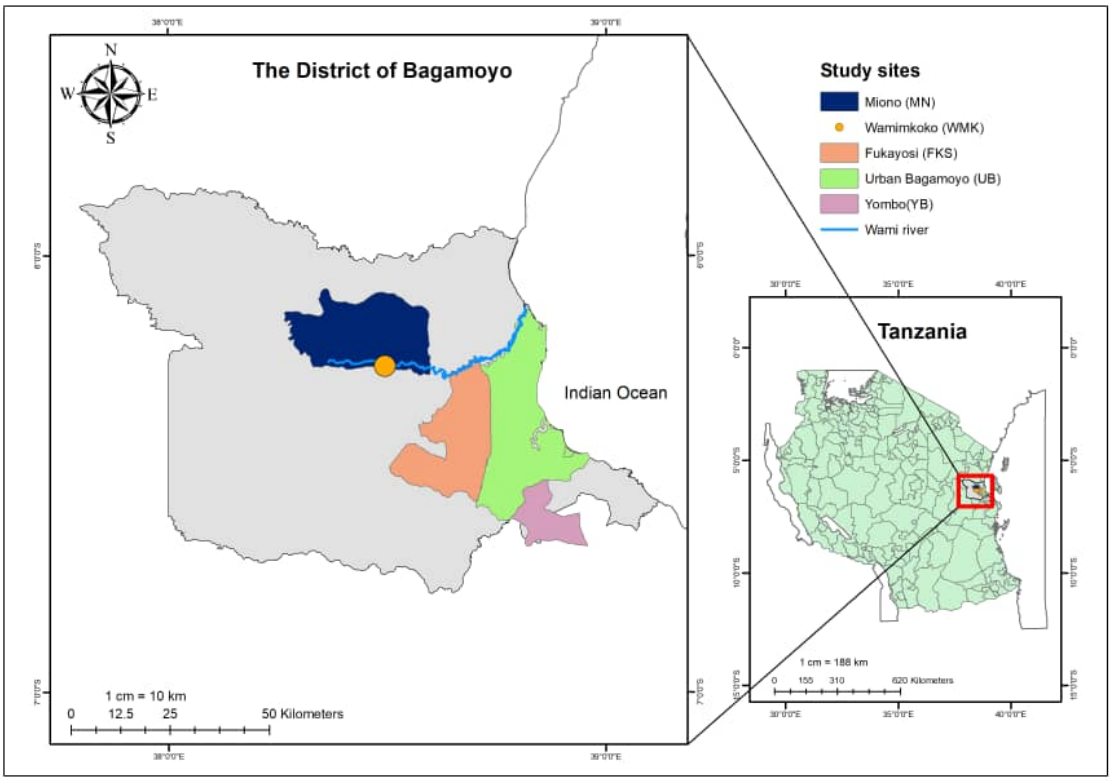

Figure 3: Map of the Bagamoyo district in Tanzania indicating the location of the Study sites

3.2. Study design

3.2.1. Malaria, Natural blocking antibody and Field mosquito surveys

To determine the malaria prevalence in humans and the evidence of naturally acquired malaria transmission-blocking immunity, a survey was conducted between May 2022 and August 2022. The survey was conducted in five villages that were selected based on malaria transmission intensity, including Bagamoyo township, Fukayosi, Yombo, Wami-Mkoko and Miono, with the first three sites having lower malaria transmission intensity than the last two sites based on malaria prevalence data available in the national Health Management Information System (HMIS) for Tanzania. Four hundred and sixty-seven (467) asymptomatic participants aged 5–45 years, were enrolled from primary schools, peripheral dispensaries and community-based malaria testing camps. The sample size was calculated using the formula for prevalence studies (Charan & Biswas, 2013) as follows: n = (Z^2 P (1 - P))/d^2; where n is sample size, Z is a standard normal variate of 1.96 at confidence level of 95%, and d is a 0.05 precision. P is the previous malaria prevalence (10%) in Bagamoyo district at 95% confidence interval. Using the formular above, 139 participants were required for the malaria and natural antibody survey but 467 participants were recruited to improve the power of the survey. Of the 467 participants, 59 were randomly selected for testing the persistence of anti-gametocyte antibodies at one month (Day 28, D28), two months (D56), four months (D112), six months (D168), and ten months (D280) after baseline testing.

Participants who tested positive for malaria were treated with artemether-lumefantrine (ALU) within 24 h of diagnosis, as per the national guidelines (MoHSW, 2011). A total of 109 malaria parasite isolates from positive survey participants were analysed using the sanger sequencing method to assess if there is any genetic polymorphism in the Pfs25 transmission-blocking target protein.

A field mosquito surveillance was also conducted to determine malaria positivity rates in field mosquitoes. Field mosquitoes were collected from Wami Mkoko village twice a month for two consecutive nights; in April 2022 and in May 2023. Wami Mkoko village was selected because it was easily accessible and it was logistically feasible to collect mosquitoes there. Indoor mosquitoes were collected using the CDC Light traps ((Model 512; John W. Hock Company, Gainesville, FL, USA), which were positioned indoors next to children's or adults' sleeping spaces in 85 households randomly selected. The mosquito traps were set between 17.00 and 18.00 and mosquitoes were collected at 07.00 and 08.00 the following

morning. The households had bed nets and the selection criteria included presence of household head/adult to consent and having people currently sleeping inside the house on the nights of collection.

3.2.2. Induction of Pfs25 IgG responses by vaccination with Pfs25-IMX313

To determine whether Pfs25-IMX313 vaccine candidate is able to induce long-lasting anti-Pfs25 responses in people naturally exposed to malaria, the concentration of vaccine-induced antibodies was measured in human volunteers who were vaccinated with the Pfs25-IMX313 during a phase1 vaccination trial. The Pfs25-IMX313 vaccine was administered along with 50 μg of Matrix-M adjuvant to healthy Tanzanian adults (18-45 years). A total of 100 volunteers from Bagamoyo township were screened, and 20 were randomly selected for vaccination. The selected individuals were divided into two groups of 10. The inclusion criteria included giving oral and written consent, abstaining from unprotected sex throughout the study period. The exclusion criteria included testing positive for malaria prior to vaccination, a positive pregnancy test (for female participants) at any point during the study period, and having any underlying illness like HIV or terminal illnesses. The study participants were recruited on the basis of first come first served. One group (three females and seven males) received a series three doses of 10 μg of Pfs25-IMX313. The other group (all males) received a series of three doses of 50 μg of Pfs25-IMX313. Allocation to a vaccination group was based on the order that the volunteers presented for vaccination. The three vaccinations in each group were given on days; Day 0 (D0), D28, and D56. Vaccination in the first group started on 24th May, 2021 and completed on 7th August, 2021. Vaccination in the second group started on 7th October, 2021 and completed by 30th April, 2022. Vaccine-induced Pfs25 IgG responses were measured at baseline (Day 0, D0), one week (D7, D35, D63), two weeks (D14, D42, D70), one month (D28, D56, D84), after each vaccination, and three months (D140), 6.5 months (D240), 12 months (D336) and 24 months (D672) after the last vaccination. The vaccination trial was designed to balance the chance to detect any possible untoward reactions against the desire to limit the number of volunteers involved for safety purposes, hence a small sample size. In addition, similar studies evaluating Pfs25 candidate vaccine used between 6 and 20 participants per groups.

3.2.3. Evaluation of Transmission-reducing activity of Pfs25-IMX313 vaccine-induced and Natural antibodies

To evaluate the ability to reduce malaria parasite development in mosquitoes by vaccine-induced and natural antibodies, peak vaccine-induced Pfs25 IgG or naturally acquired Pfs230D1M IgG and/or Pfs48/45 IgG were mixed with cultured *P. falciparum* gametocytes and fed independently to a group of laboratory mosquitoes via artificial membranes (SMFAs) (Kazutoyo Miura, Deng, et al., 2013). The fed mosquitoes were analyzed 7 days post-feeding, to determine the number of oocysts (malaria parasites) in the infected mosquitoes. The TRA was determined as the reduction in the number of oocysts by test antibodies compared to a negative control (naïve serum from people who had never been exposed to malaria) lacking blocking antibodies. SMFAs are the gold standards for evaluating malaria transmission-blocking tools including new vaccines and drugs.

The research framework is summarized in Figure 4. Overall, the study was conducted for approximately 2.5 years.

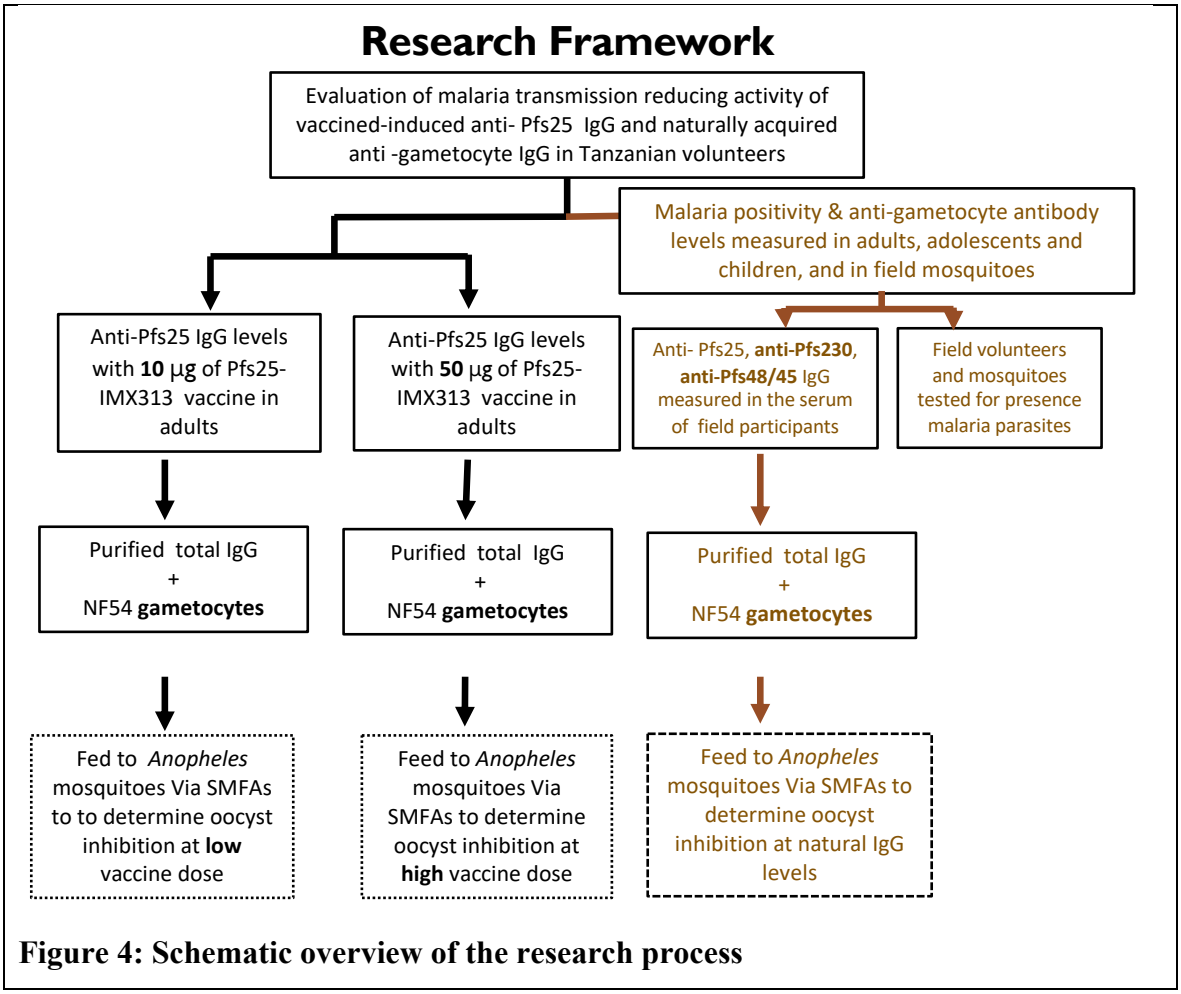

Figure 4: Schematic overview of the research process

3.3. Sample Collection and Preservation

Blood samples used for malaria and natural antibody testing, and for assessing Pfs25 genetic polymorphism, were collected into 2.0 mL tubes containing EDTA preservative to prevent the collected blood from clotting. Of the 2 mL in the EDTA tube, 20 µL were used for malaria detection using rapid diagnostic test (mRDT) and light microscopy, 200 µL was immediately preserved in 600 µL of 1 × DNA/RNA Shield™ (Zymo Research, Irvine, USA) for nucleic acid extraction to detect and quantify circulating parasites as well as amplifying the genes for Pfs25 and assess genetic variation. Plasma from the remainder of the 2 mL EDTA blood sample was used for detecting antibodies against Pfs25 and gametocyte antigens Pfs230D1M, and Pfs 48/45. An extra 2 mL of blood from unvaccinated participants were collected into plain vacutainers (with no preservative). Serum from this extra 2 mL of blood sample was used for measuring transmission-reducing activity of natural gametocyte IgG via SMFAs.

Field mosquitoes were collected and preserved individually in 1.5 mL Eppendorf tubes containing silica gel, before identification and analysis for malaria infections.

Blood samples used for measuring the levels and transmission-reducing activity of vaccine-induced Pfs25 IgG were collected into 10.0 mL plain vacutainers. Of the 10 mL, 5 mL were used for measuring Pfs25 IgG levels and other immunological parameters and the remaining sample was used for evaluating transmission-reducing activity of Pfs25 IgG via SMFAs.

3.4. Sample analysis

3.4.1. Malaria testing in human volunteers and mosquitoes

i. Malaria testing in human volunteers

Each survey participant was tested for malaria using three different methods including; malaria rapid diagnostic testing (mRDT), light microscopy (LM) and quantitative polymerase chain reaction (qPCR) methods.

a. Malaria detection by mRDT

The Bioline™ Malaria Ag P.f/Pan (HRPII/pLDH), Standard Diagnostic, South Korea), was used for malaria rapid testing as described previously (Hofer et al., 2023). Approximately 10 µL blood was added into the sample well, including two drops of the standard buffer in the developer well, and left for 15 minutes before the result was read independently by two technicians and recorded per each test run.

b. Malaria detection by Light microscopy

Blood thick smears were prepared and stained for 30 min with 10% Giemsa stain as described previously (Iqbal, Hira, Al-Ali, Khalid, & Sher, 2003). The smears were examined at 100 × magnification to detect the presence of asexual and sexual malaria parasites. In malaria positive smears, asexual parasites were counted per 200 white blood cells (WBCs) and gametocytes were counted per 500 WBCs. Malaria parasite densities were determined by assuming 8000 white blood cells per µL of blood. Smears were considered microscopy-positive for gametocytes when at least one gametocyte was observed. Smears were considered negative if no *Plasmodium* parasites were detected in 100 consecutive (Conway et al., 2001) high-power fields. Two microscopists examined the slides independently while a third microscopist re-examined in case of any discordant smears.

c. Malaria detection and quantification by qPCR

To test and quantify general parasitaemia, qPCR was performed on the blood samples preserved in the DNA/RNA Shield™ (Hofmann et al., 2015; Kamau, Alemayehu, Feghali, Saunders, & Ockenhouse, 2013). Genomic DNA was extracted from whole blood using Quick-DNA miniprep plus kit (Zymo research, USA), and eluted in 50 µL elution buffer. A single qPCR reaction contained 2 µL parasite DNA and 8 µL reaction master mix containing 1 × Luna Universal Probe qPCR Master Mix (New England Biolabs, USA). The qPCR assays were run in duplicate along with negative controls (DNA material from known *P. falciparum* negative sample) and *P. falciparum* NF54 DNA as positive control. The qPCR measurement was performed on a CFX96 real-time qPCR thermocycler (Bio-Rad, Singapore) and analyzed with CFX Manager Software (v2.2). The following thermal profile was used; polymerase activation at 95C for 60 s, 45 cycles of denaturation at 95°C for 15 s and annealing and elongation for 45 s at 57°C. Parasites per µL were calculated against the WHO international standard for *P. falciparum* DNA quantification techniques (Kamau et al., 2013).The standard was serially diluted from 100,000 parasites/µL—0.0001 parasites/µL and run in triplicate. To investigate other *Plasmodium* species infections in the study sites, all *Plasmodium*-positive samples were further analysed using the qPCR assay previously described (Schindler et al., 2019). The primers and probes used in Schindler *et al*, 2019 (Schindler et al., 2019), were used as described in Hofer *et al,* 2023 (Hofer et al., 2023). Sample analysis was done using the same qPCR system, reaction volumes, and materials as described above, except the cycling conditions used for the assay were as follows: activation for 5 min at 95°C; 45 cycles of denaturation 15 s at 95°C and annealing and elongation for 45 s at 57°C. Appropriate controls for at all *Plasmodium* species were included.

Gametocyte detection was done by qRT-PCR using ribonucleic acid (RNA) extracted from all qPCR-positive samples using the Quick-RNA™ MiniPrep Plus kit (Zymo Research). *P. falciparaum* gametocytes were detected and quantified using a *multiplex* qRT-PCR assay described previously in Meerstein-Kessel *et al*, 2018 (Meerstein-Kessel et al., 2018). The assay combined two independent *Plasmodium* targets; the female gametocyte-specific marker (CCp4) and the male-gametocyte specific marker (*Pf*MGET), as detailed in Appendix 1. Reverse transcription, amplification and qPCR measurements were performed using a CFX96 real-time qPCR thermocycler (Bio-Rad, Singapore) as above with the following thermal profile: 15 min at 55 °C; 60 s at 95 °C; 40 cycles of 10 s 95 °C and 60 s at 60 °C. Each reaction contained 4 µL RNA and 10 µL reaction master mix containing Luna Universal

1 × One-Step qRT-PCR Kit (New England Biolabs, USA). The standard curves for gametocyte quantification were prepared using RNA standards (obtained from Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, The Netherlands) for sex-sorted gametocytes [26, 28]. A tenfold dilution series in the range of 106/mL to101/ mL were made for each standard and used for standard curve preparation. The reaction efficiency for *Pf*MGET was 95.7% and 80.0% for CCp4, as shown in Appendix 2 and 3, respectively.

ii. **Field mosquito identification and examination for Malaria**

The collected anopheline mosquitoes were identified taxonomically to species level where possible. The morphological criteria according to the established taxonomic keys(Gillies & Coetzee, 1987; Gillies & De Meillon, 1968) was followed during mosquito dentification. Genomic DNA was extracted from head and thorax using the Livak protocol described in (F. H. Collins et al., 1987). A standard PCR according to protocol by (Koekemoer, Kamau, Hunt, & Coetzee, 2002) was performed to confirm the mosquito in the *An. funestus* group, while a multiplex PCR protocol previously described (Scott, Brogdon, & Collins, 1993), was performed to confirm species in the *An. gambiae* group. The *An. gambiae* universal forward primer, GGTTGCCCCTTCCTCGATGT, reverse primer CTGGTTTGGTCGGCACGTTT, and *An. arabiensis* reverse primer, AAGTGTCCTTCTCCATCCTA, were used for identification of *An. gambiae* species, while *An. funestus* universal forward (ITS2) primer TGTGAACTGCAGGACACAT and reverse primer CGATCGATGGGTTAATCATG were used for the identification of *An. funestus* s.s. The amplification conditions for *An. gambiae* were; initial denaturation at 94˚C for 3 minutes followed by 30 cycles of 94°C for 30 seconds, 42°C for 30 seconds, 72 °C for seconds and a final extension at 72 °C for 10 minutes and held at 10°C. The PCR amplification conditions for *An. funestus* were as follows: initial denaturation at 94˚C for 5 minutes, 35 cycles of denaturation at 94˚C for 30 sec, annealing at 45˚C for 30 sec, primer extension at 72˚C for 40 seconds, final extension at 72˚C for 5 minutes and held at 10°C. A set of known mosquito DNA from laboratory strains were used, as well as blank well and internal negative controls. All PCR amplicons were electrophoresed on 2% w/v agarose and viewed with Bio-Rad imaging system.

P. falciparum parasites were tested in individual mosquito DNA using the qPCR protocol previously described (Schindler et al., 2019). The qPCR protocol consisted of *Plasmodium* and mosquito targets combined in a multiplex assay. The *Plasmodium* primers and probes used were similar to ones described for detecting *Plasmodium* in human samples in section

3.4.1.i.c, above. The assays included a mosquito 28S rRNA (Anspp 28S) sequence which was targeted as an internal control to assess the quality of DNA extraction and qPCR amplification. Sample analysis was done using the same reaction volumes, materials, and qPCR system as described for detecting *Plasmodium* in humans in section 3.4.1. ic, above. Appropriate controls for at all *Plasmodium* species were included.

3.4.2. Natural and Vaccine-induced antibody level measurement

i. Standardized Enzyme-Linked Immunosorbent Assays (ELISA) for measuring Natural and Vaccine-induced and antibodies

Standardized Enzyme-Linked Immunosorbent Assays (ELISA) were performed on serum or plasma samples to measure levels of vaccine-induced total IgG and/or total IgG to Pfs48/45, Pfs230D1M and Pfs25 antigens. The ELISA were performed following the protocol described previously (Kazutoyo Miura et al., 2008). Briefly, ELISA plate wells were coated overnight with 50 µL of either Pfs48/45, Pfs230D1M or Pfs25 recombinant proteins at concentration of 2µg/mL. The plates were blocked with StartingBlock™ T20 (PBS) Blocking Buffer (ThermoFisher Scientific, UK) for 1 hour before 50 µL of the diluted test plasma or serum samples were added in triplicates. The plates were then incubated for 2 hours at room temperature before a detecting antibody (Goat anti-human Horse Radish Peroxidase (HRP)-conjugated IgG antibody) was added. The plates were incubated at room temperature for 1 hour, and 100µl of the pNPP substrate were added in each well. The substrate was left to develop for 25 minutes and ELISA plate was read at 405nm absorbance using a plate reader. Between each incubation step, plates were washed six times with 1 x PBS + 0.05% Tween20. The ELISAs were performed using a standard curve and internal positive controls from reference samples. Known anti-Pfs25 sera from De Graaf *et al* (De Graaf et al., 2021), was used as reference and positive control for Pfs25 ELISAs. The Pfs230D1M and Pfs48/45 reference sera/plasma were prepared as described previously by (Kazutoyo Miura et al., 2008). Briefly plasma samples from 20 donors, highly reactive to Pfs230D1M and Pfs48/45 were pooled and optimized at 1:800 dilution in StartingBlock™ T20 (PBS) Blocking Buffer. For each plate, a total of six wells were incubated with positive control and a panel of negative control sera from unexposed United Kingdom (UK) donors. Wells containing no test sera were used to deduct background reactivity from each sample. A seropositivity cut-off point was set at an OD above the mean + 2SDs of a panel of UK donors. A standard curve

and Gen5 ELISA software v3.04 (BioTek, UK) was used to convert the OD405 of individual test samples into arbitrary units (AU).

ii. Analyzing the quality of vaccine-induced Pfs25 total IgG

Sodium thiocyanate (NaSCN, a chaotropic agent) based avidity assays were conducted to estimate the quality of antibody response. The avidity assay were performed according to protocol previously described (Pullen, Fitzgerald, & Hosking, 1986). Briefly, each individual serum was diluted based on standardized ELISA readouts to reach an OD of 1. The serum was added into ELISA plate wells coated overnight with 50µL/well of 2µg/mL Pfs25 protein. Concentrations of the NaSCN chaotropic agent, ranging from 0-7M were added in increasing concentration down the plate. The plates were incubated at RT for 15min, and a detecting secondary antibody was added followed with pNPP substrate (100µl/well). The plates were allowed to develop before being read at 405nm optical density (OD) on Bio-tek ELx800 Microplate Reader with Gen5 software. The ODs with different NaCSN concentrations were then converted to the percentage of total bound IgGs. The binding of antibodies with lesser avidity to antigen is disrupted at concentrations of NaSCN lower than for antibodies with greater avidity.

iii. Assessing cross reactivity against hc4bp in Vaccinated participants

Human C4bp (hC4bp) is similar to the IMX313 component of Pfs25-IMX313 vaccine candidate. Endpoint ELISA assays were conducted to confirm that IMX313 did not generate immune responses which cross-react with hC4bp. A protocol previously described (De Graaf et al., 2021) was followed. Briefly, each individual serum was duplicated and serially diluted 3 folds down the ELISA plate wells coated overnight with 50µl/well of 2µg/ml hC4bp protein. The anti hC4bp rabbit antibody was used as the positive control and the day 0 serum pool as negative. After an hour incubation at room temperature, detecting antibody (Sigma anti-human), was added to each well except for positive control wells, to which 50uL of Sigma anti-Rabbit IgG was added as detecting antibody. The endpoint titre was defined as the dilution at which the OD of the sample reached 1 plus 3x standard deviation. The background was calculated for each individual using their D0 sample. A fixed cut-off value 0.22 OD was set based on previous trials (De Graaf et al., 2021). Data for each plate was copied into an already established hC4bp Endpoint ELISA template, where endpoint titres for each sample

were calculated in "y=mx+c" and summarized. For the sample data on the plate to be accepted, the negative control OD must have been below 0.22.

3.4.3. Evaluating Transmission-reducing activity of Vaccine-induced and Natural antibodies

The ability to reduce *P. falciparum* infections in mosquitoes by vaccine-induced Pfs25 total IgG and total IgG to Pfs230 and Pfs48/45 antigens were evaluated independently at the National Institute of Allergy and Infectious Diseases (NIAID) laboratory, using standard membrane feeding assays (SMFAs) methodology, previously described (Kazutoyo Miura, Deng, et al., 2013). The samples were kept at -80°C prior to shipment to NIAID. The samples were kept in liquid nitrogen throughout the shipment to NIAID for analysis. The IgG from individual samples were purified using Protein G affinity chromatography and reconstituted to final concentrations of 10 μg/mL in PBS. The Purified human total IgG was then mixed with of NF54 *P. falciparum* gametocytes, and fed to laboratory-reared mosquitoes via a parafilm® membrane. The mosquito midguts were dissected and examined microscopically to assess oocyst intensities in the test and control assays. The SMFAs was performed by Kazutoyo Miura's team at the NIAD laboratory.

i. *P. falciparum* gametocytes and Mosquitoes used for SMFAs

The NF54 laboratory strain of *P. falciparum* gametocytes were cultured following a protocol previously by (Kazutoyo Miura, Deng, et al., 2013). Briefly, the cultures were initiated at asexual parasitemia of 0.15–0.2% and haematocrit of 5% in 10 mL complete medium (RPMI-1640 with 6 g/L of HEPES, 50 mg/L of hypoxanthine, 2.5 g/L of sodium bicarbonate and 10% human serum). The cultures were maintained for 16 days, at conditions: 90% Nitrogen, 5% Carbon dioxide, 5% Oxygen, with a daily media change. More than 3 identical replicates of culture were maintained. For each experiment, at least two replicates were pooled on the basis of stage V gametocytemia and exflagellation levels. The culture medium was replaced with normal human serum, and normal red blood cells (RBCs) at a stage V gametocytemia of about 0.15 %, and 50% haematocrit (1mL serum:1 mL of RBCs). The male-female ratio was stabilized at 1male: 2–3 female. The human serum and RBC used were obtained from Interstate Blood Bank, Inc, USA.

Laboratory colony of female *An. stephensi* mosquitoes (Nijmegen strain, *SDA50*) with reduced heterogeneity were used for the SMFAs. The mosquitoes were reared in cages,

within the insectary equipped with window screening and a double door system to ensure that no infected mosquito can escape. Mosquito rearing was done following standard protocols previously described by (Benedict, 2009). The mosquitoes were maintained at conditions: 26 °C and 80% relative humidity and were starved for at least 1hour prior infective feeding.

ii. Standard Membrane Feeding assays (SMFAs)

Stage V gametocyte cultures were mixed with purified test IgG at 10 mg/mL concentrations without human complement. For each volunteer, 60 µL of the purified IgG from test samples were then mixed with 200 µL of the gametocyte cultures and immediately fed to a group of 50, 4–6-day old starved female mosquitoes via a parafilm® membrane fitted on a feeding apparatus (18 mm diameter; Chemglass Life Sciences, Vineland, NJ). Mouse monoclonal antibody 4B7 at a concentration of 93.8 µg/mL was used as positive control. The negative control was a pool of naïve human serum without test IgG antibodies. At least two rounds of SMFAs were performed for every vaccinated participant.

iii. Mosquito dissection and Oocyst counting to determine TRA

Mosquitoes were kept for 8 days, and midguts were dissected under a stereo-microscope. The midguts were stained with 0.5 % mercurochrome dye for approximately 10 minutes before being transferred into a drop of RPMI on glass slides with cover slips. The midguts were then examined by light microscopy to enumerate the oocysts. Staining enables the oocysts to absorb the dye differentially from the midgut tissue, and the characteristic features of malaria parasites are easily recognized (Musiime et al., 2019) .

Twenty fed mosquitoes per volunteer were dissected for every SMFA round, oocysts counted and recorded. TRA, a measure of percent (%) reduction in oocyst intensity was calculated relative to the respective control IgG tested in the same assay. TRA was calculated as: 100 X {1— (mean number of oocysts in the test assays)/(mean number of oocysts in the control assays)} (Kazutoyo Miura, Swihart, et al., 2016). An assay was acceptable when the negative control group had a mean of 4 or more oocysts per mosquito, regardless of the number of mosquitoes without oocysts in the test group.

3.4.4. Pfs25 gene amplification and Sequencing

The Pfs25 (650bp) gene was amplified from 109 *Plasmodium falciparum* positive genomic DNA aliquots left after qPCR parasite detection in section 3.4.1.i.c. Standard PCR amplifications were performed on C1000™ Thermal Cycler (Bio-Rad, Singapore) using the following primers sequences; Pfs25 forward (ATG AAT AAA CTT TAC AGT TTG) and Pfs25 reverse (CAG TAT GCT TTT TTA TAA TGT AA). The primers were designed to amplify the entire ORF of Pfs25 based on the nucleotide sequence of LR131490.1. The protocol previously described (Mlambo, Vasquez, LeBlanc, Sullivan, & Kumar, 2008)was adapted and used as follows; a final reaction volume of 25 µL was prepared by addition of 5 µL of parasite DNA to a mixture containing 11.5µL of OneTaq Quick load PCR Master Mix (2X), 1µL of forward primer (10µM), 1 µL of reverse primer (10µM) and 6.5µL of DNase free water. of double-distilled water. PCR amplification conditions were as follows: initial denaturation at 94˚C for 3 minutes, 34 cycles of denaturation at 94˚C for 30 sec, annealing at 53˚C for 30 sec, primer extension at 72˚C for 1 min and final extension at 72˚C for 6 min. The annealing temperature was determined by performing a gradient PCR on WHO international standard for *P. falciparum* DNA. Amplification was carried out with the proof-reading enzyme Takaka Ex-Premier, giving a single band at the expected size for each sample. The quality of amplicons was assessed by performing gel electrophoresis in 2% w/v agarose gel after amplification and before sequencing. The PCR reactions were then cleaned up with Exo-SAP-IT before the amplicons were send for sanger sequencing using the same primers as for the original amplification. Forward and reverse sequences were subsequently aligned against the LR131490.1 sequence.

3.5. Data management

Participants and samples were given study identification numbers (IDs) for easy tracking and analysis. The sample IDs were delinked from study participant names and study locations to minimize the risk of loss of confidentiality. In addition, all forms containing the names of study participants were kept under lock and key all the time. Study files were kept in compliance with the principles of good clinical practice and regulatory and institutional requirements, and in compliance with the requirements for the protection of confidentiality of participants. Only study team members were given access to participant files and data records. Only authorized representatives from the ethics committee(s)/regulatory bodies and sponsors, were allowed to inspect study documents and records. Data was recorded in pre-

prepared data collection templates as detailed in Appendix 4.

3.6. Statistical analysis

Data was recorded in Excel (Microsoft, 2016) and analyzed using Stata version 16 (StataCorp., 2019) and GraphPad Prism version 10 (GraphPad Software Inc., California, USA). Descriptive analysis was conducted on the demographic characteristics of the participants as well as the status (negative or positive) in relation to malaria parasite and antibody presence, using proportions and their respective 95% Confidence Intervals (C I). The parasite density and antibody responses were descriptively analyzed using Geometric mean (95% Confidence interval), and presented in tables and box plot, with p-values for log of gametocyte density and antibodies responses using t-test. Pearson correlation coefficient (PCC) was used to assess the correlation between the log of gametocyte and log of antibodies, including the correlation between the two antibodies (Pfs230D1M vs anti-Pfs48/45 IgG). Multinomial logistic regression was used to estimate the association between the presence of gametocytes as well as antibodies on the age groups (5-12, 13-17 and 18-45 years), sex (Male and female) and location (low and high transmission) of the participants. Pearson correlation coefficient (PCC) was also used to assess the correlation between levels of vaccine-induced Pfs25 antibodies and transmission-reducing activity of Pfs25-IMX313/Matrix-M vaccine. The Mann-Whitney U test was used to assess if there is significant difference in the levels of vaccine-induced Pfs25 antibodies and transmission-reducing activity between the low and high dose vaccination groups.

3.7. Ethics approval and participants' consent

This study received ethical clearance by the Institutional Review Board (IRB) of IHI (IHI/IRB/No: 29 -2021) as detailed in Appendix 5, and by the National Institute for Medical Research (NIMR), Tanzania (NIMR/HQ/R.8a/Vol. IX/3716), as detailed in Appendix 6. All participants orally assented to participate in the study and written consent was obtained for all participants from their caregivers. Enrolment was based on written informed consent for adults and caregivers of participants aged 5-17 years, in addition to assent from these group (5-17 years). Households were approached for written consent prior to mosquito collection.

CHAPTER 4: RESULTS AND DISCUSSION

4.1. Results

4.1.1. *P. falciparum* positivity in Humans and Field mosquitoes

i. *P. falciparum* positivity in Humans

A total number of 467 participants including; 41% children (5-12 years), 14% adolescents (13-17 years) and 45% adults (18-45 years), were recruited summarized in Table 2. The average age (years) for each age-group was 9, 15, and 31 years, for children, adolescents, and adults, respectively. The proportion of female participants was 54%. Participant recruitment varied slightly between low (44%) and high (56%) malaria transmission sites.

Table 2: Study participants' demographic characteristics

Demographic variables (N=467)	**n (%)**
Age-group	
Children (5-12)	192 (41.1)
Adolescent (13-17)	65 (13.9)
Adult (18-45)	210 (45.0)
Sex	
Female	252 (54.0)
Male	215 (46.0)
Location	
Low transmission	205 (43.9)
High transmission	262 (56.1)

a. Asexual parasite prevalence

All three malaria diagnostic tools used, detected high asexual parasite positivity in children and adolescents, with values that were distinctly higher than in adults. All age groups combined, qPCR detected the highest number of infections (n = 110), with a mean asexual parasite density of 391.8 parasites/µL. Microscopy detected 73 infections, with mean asexual parasite density of 1170 parasites/µL. Only 67 true asexual positives could be detected by mRDT. Ten false malaria-positive infections were detected by mRDT, as indicated in Figure 5. The ability of the qPCR to detect a lower mean parasite density indicates the sensitivity of molecular assays over the mRDT and microscopy for malaria testing.

Using sensitive qPCR as the reference standard, the majority of malaria- positive participants [93% (102/110)] were infected with P. *falciparum* (*Pf*). There was one participant infected with *P. malariae* only, and the rest of the positive participants had mixed infections of *P. malariae* and *P. falciparum*. The geometric mean asexual parasite density in children (746.1 parasites/µL) was four-fold higher than in adolescents (192.6 parasites/µL), and three-fold higher than that of adults (234.6 parasites/µL). The positivity rate in male participants was higher compared to female counterparts, but a similar mean asexual parasite density was observed in both genders as shown in Table 3.

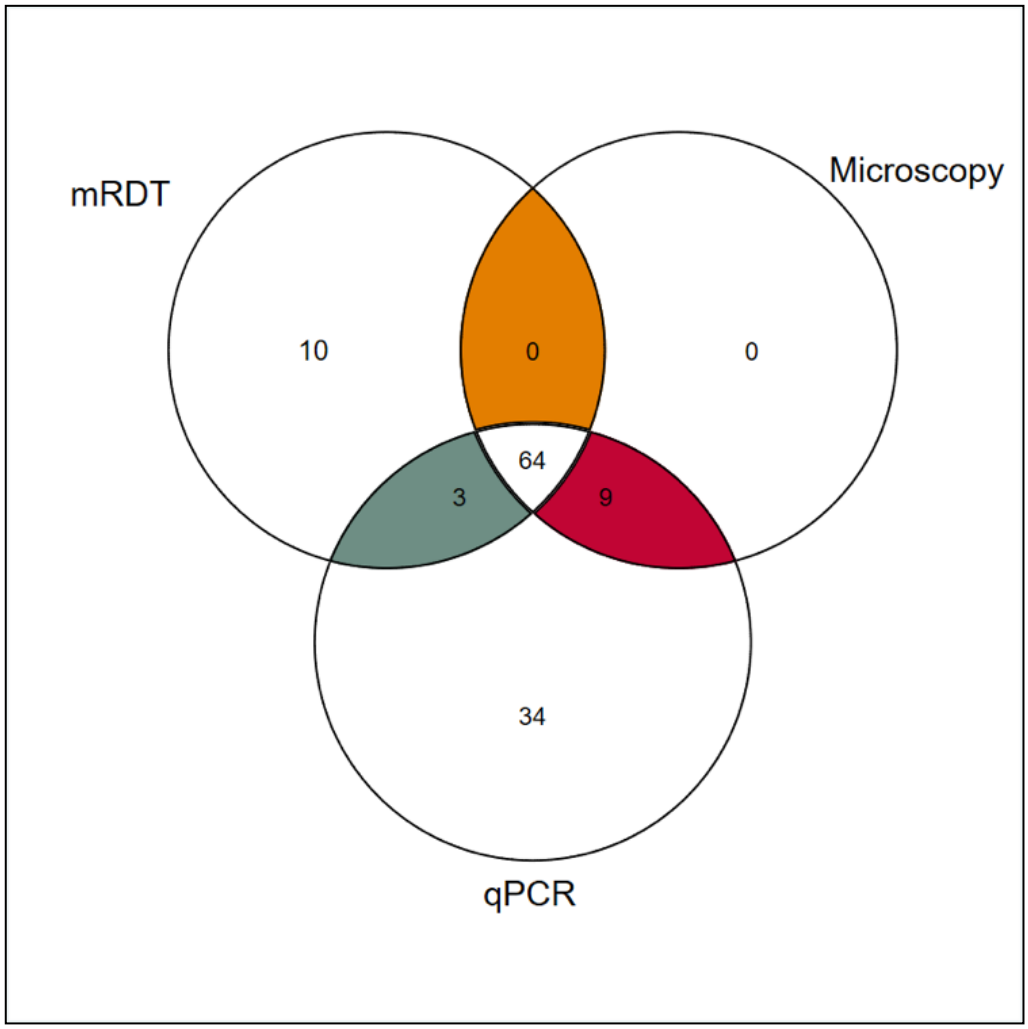

Figure 5: Concordance in malaria testing methods for malaria-positive participants positive, (n = 110)

Table 3: Asexual parasite positivity by qPCR in relation to participants' demographic characteristics

Demographics	Asexual parasites (N=467)			Detected parasite species distribution					
				P. falciparum (*Pf*)		*P. malariae* (*Pm*)		*Pf* & *Pm*	
	n^t	$\%^t$ (95% C I)	G. Mean parasite density (95% CI)	n^f	$\%^e$ (95% C I)	n^m	$\%^m$ (95% C I)	n^d	$\%^h$ (95% C I)
Overall	110	23.6 (19.9 – 27.6)	391.8 (232.1 – 661.4)	102	92.7 (86.0 – 96.3)	01	0.9 (0.1 – 6.3)	07	6.4 (3.0 – 12.8)
Age-group									
Children	52	27.1 (21.3 – 33.8)	746.1 (330.9 – 1682.3)	48	92.3 (81.1 – 97.1)	01	1.9 (0.3 – 12.7)	03	5.8 (1.8 – 16.6)
Adolescent	19	29.2 (19.5 – 41.4)	192.6 (79.1 – 469.2)	17	89.5 (65.9 – 97.4)	00	00	02	10.5 (2.6 – 34.1)
Adult	39	18.6 (13.9 – 24.4)	234.6 (94.5 – 582.6)	37	94.9 (81.4 – 98.7)	00	00	02	5.1 (1.3 – 18.6)
Sex									
Female	52	20.6 (16.1 – 26.1)	386.8 (161.7 – 925.2)	47	90.4 (78.7 – 96.0)	01	1.9 (0.3 – 12.7)	04	7.7 (2.9 – 18.9)
Male	58	27.0 (21.5 – 33.3)	396.3 (209.2 – 750.8)	55	94.8 (85.0 – 98.3)	00	00	03	5.2 (1.7 – 15.0)

N = 467 participants tested for malaria parasites
n^t = total number of participants who tested positive for malaria
$\%t = n^t/N*100$
n^f = number of *Plasmodium falciparum* infections
n^m =number of *Plasmodium malariae* infections
n^d = number of mixed infections with *Plasmodium falciparum* and *Plasmodium malariae*
$\%^f = n^f/n^t*100$
$\%^m = n^m/n^t*100$
$\%^d = n^d/n^t*100$

b. *P. falciparum* gametocyte prevalence by qPCR

The overall gametocyte prevalence by qPCR was 5.6 % (26/467), and the overall gametocyte sex ratio (proportion of gametocytes that are male), was estimated to be 0.18, as indicated Table 4. About 24% (26/110) of participants who tested positive for asexual infections were gametocyte positive. The gametocyte prevalence was highest in children, and higher among the male than the female counterparts as well as among those living in high transmission intensity than the low as shown in Table 4. However, the geometric mean (GM) gametocyte density (gametocytes/μL) was higher in adults (124.6) than in children (71.7), and adolescents (50.5). A similar pattern of higher GM was also seen among those living in the high transmission location (126.0) than the low (31.5). The gametocyte sex ratio in children and adult gametocyte carriers was similar but higher than what was observed in adolescents, as shown in Table 4. About 35% of gametocyte carriers identified by qPCR, were submicroscopic and could not be detected by microscopy. As expected, higher gametocyte positivity rates were detected by qPCR than microscopy for all age groups.

Table 4: Gametocyte positivity by qPCR in relation to participants' demographic characteristics

Demographics	n^g	$\%^g$ (95% CI)	G. Mean gametocyte density (95% CI)	Weighted A. Mean gametocyte sex ratio (95% CI)
Overall	26	5.6 (3.8 – 8.1)	82.2 (44.6 –151.6)	0.18 (0.15 – 0.21)
Age-group				
Children	13	6.8 (4.0 – 11.3)	71.7 (30.8 – 167.0)	0.20 (0.15 – 0.25)
Adolescent	04	6.2 (2.3 – 15.3)	50.5 (6.8 – 376.7)	0.08 (0 – 0.25)
Adult	09	4.3 (2.2 – 8.0)	124.6 (31.0 – 500.9)	0.18 (0.14 – 0.22)
Sex				
Female	08	3.2 (1.6 – 6.2)	89.4 (23.9 – 334.1)	0.14 (0.09 – 0.19)
Male	18	8.4 (5.3 – 12.9)	79.3 (37.0 – 169.6)	0.19 (0.15 – 0.24)
Location (transmission intensity)				
Low	08	3.9 (2.0 – 7.6)	31.5 (15.0 – 66.2)	0.20 (0.16 – 0.24)
High	18	6.9 (4.4 – 10.7)	126.0 (58.2 – 273.0)	0.17 (0.13 – 0.22)

N = 467 participants tested for gametocytes
n^g = number of participants who tested positive for gametocytes
$\%^g = n^g/N*100$
sex ratio = number of male gametocytes/total gametocyte count

The multinomial logistic regression performed to assess if age, sex, and location influence gametocytemia indicated that there was no association between gametocytemia and age (P-value=0.7107) as shown in Table 5. In addition, no significant difference was observed between the log of gametocyte density among the age groups as indicated in Figure 6a, but it was shown that gametocytes were more likely to be present among male participants than female participants [ORa: 2.79 (95% CI: 1.19 – 6.59) p=0.019] (Table 5), the GM of gametocyte density was not significantly different between male and female participants as indicated in Figure 6b. The GM gametocyte density was significantly higher among the participants in the high transmission areas than in the lower-transmission areas as shown in Figure 6c; however, there was no significant difference between transmission setting and gametocyte prevalence as shown in Table 5.

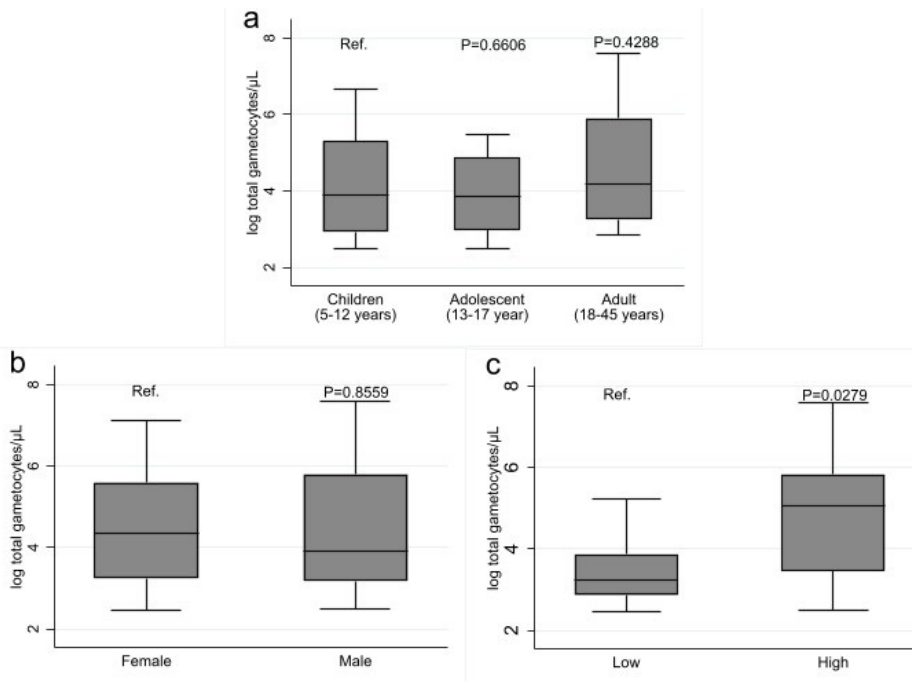

Figure 6: The distribution of Ct-based estimated gametocyte density in survey participants. Gametocyte density by participants' age (a), sex (b) and location (c)

Table 5: Influence of participants' age and sex on gametocyte carriage

	Unadjusted OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value	LR test P-value
Age-group					
Children	1.00		1.00		0.7107
Adolescent	0.90 (0.28 – 2.87)	0.863	0.82 (0.25 – 2.67)	0.743	
Adult	0.62 (0.26 – 1.48)	0.278	0.69 (0.28 – 1.68)	0.412	
Sex					
Female	1.00		1.00		0.0144
Male	2.79 (1.19 – 6.54)	0.019	2.81 (1.19 – 6.65)	0.019	
Location (transmission intensity)					
Low	1.00		1.00		0.1994
High	1.82 (0.77 – 4.27)	0.171	1.72 (0.73 – 4.17)	0.211	

Each adjusted model examines the relationship between each predictor variable and gametocytemia adjusting for other variables (Age, sex, and location).

ii. *P. falciparum* positivity in field mosquitoes

A total of 126 female anopheles field mosquitoes were collected, of which, majority (41.3%), were *An. funestus s.s,* 34.9% were *An. gambiae s.s.*, and the rest were *An. arabiensis*. These species were confirmed using standard PCR as shown in Figure 7. Other mosquito species including culicines were collected but these were not analyzed. Of the 126 field-caught mosquitoes, four (4) were positive for *P. falciparum* parasites (Table 6). *P. falciparum* was the only detected malaria species in positive mosquitoes with *An. funestus s.s* having a higher infection rate (4.5 %) compared to *An. gambiae s.s.* All the tested *An. arabiensis* were negative for malaria parasites.

Table 6: *P. falciparum* positivity in field mosquitoes

Field mosquito species	Number of mosquitoes tested	Number of malaria positive mosquitoes	% positivity	ct-values	
				Lowest	Highest
An. funestus s.s	67	3	4.5	22.45	33.72
An. gambiae s.s	44	1	2.3	31.62	-
An. arabiensis	15	0	0.0	-	-
Total	**126**	**4**	**3.2**	-	-

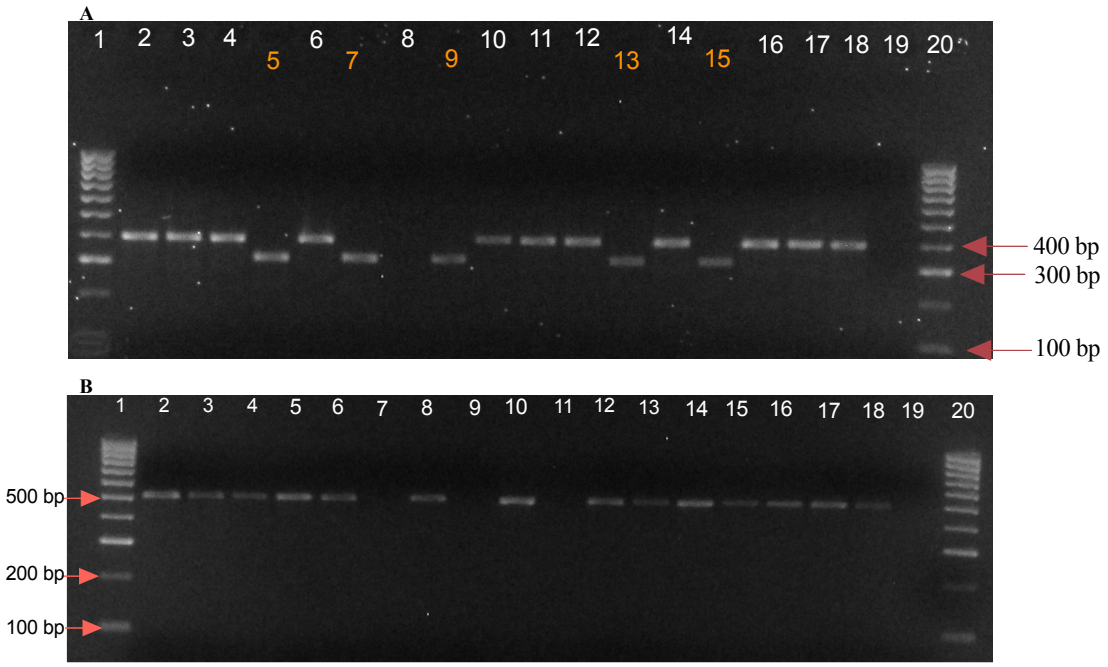

Figure 7: Molecular confirmation of collected field mosquito species

(**A**) Gel electrophoresis of standard PCR amplicons for field *An. gambiae* group. Lanes 1 and 20 were 1000 bp DNA markers. Lane 18 was *An. gambiae s.*s positive control, showing the expected band size of about 390 base pairs (bp). Lane 19 was negative control. Lanes 5, 7, 9, 13 and 15, were field *An. arabiensis*, showing expected band sizes of 300 pb. All other lanes were field *An. gambiae s.s* at expected band sizes of about 390 bp. (**B**) Gel electrophoresis of standard PCR amplicons for field *An. funestus* mosquitoes. Lanes 1 and 20 were 1000 bp DNA markers, Lane 18 was *An. funestus s.s* positive control at the expected band size position of about 500 bp. Lane 19 was a negative control. Lanes 7, 9 and 11, were nonreactive field samples. All other lanes were field *An. funestus s.*s at expected band sizes of about 500 bp.

4.1.2. Evidence of Natural responses to Pfs230D1M, Pfs48/45, and Pfs25 antigens

The seroprevalence and total IgG levels were determined in response to the *P. falciparum* antigens Pfs230D1M, Pfs48/45, and Pfs25. The total number participants tested for antibodies against Pfs230D1M and Pfs48/45 were 281 and 291 respectively, whereas 118 participants were tested for antibodies against all the three (3) antigens.

There was no natural Pfs25 IgG detected among participants tested. Overall, 56% (157/281) participants were categorized as seropositive for anti-Pfs230D1M whereas 49% (141/291) were seropositive anti-*Pfs*48/45 IgG. Approximately 30% (87/291) of the participants were seropositive for both antibody types (**Table 7**). Seroprevalence for anti-Pfs230 and anti-Pfs48/45 IgG increased significantly with participants' age, with adults more likely to have antibodies than children; for Pfs230 [adjusted OR 3.18, (95% CI: 1.85 - 5.57), p=<0.0001], and Pfs45/48 [OR 3.11, (95% CI: 1.83 - 5.29), p = <0.0001], reflecting cumulative exposure to infection. The Pfs230 antibody titres increased with age, but there was no significant difference in antibody tires for Pfs48/45 among the study age groups (Figure **8d**), although Pfs48/45 antibody titres were slightly higher in male participants than in female participants (Figure **8e**).

Table 7: Anti-gametocyte seropositivity in relation to participants' demographic characteristics

Demographics	Pfs230D1M (N^a=281)			Pfs48/45 (N^b=291)			Combination of Pfs230D1M and Pfs48/45 (N^c=291)		
	n^a	% ($n^{a/}$/ N^a)	aOR (95% CI) p-value	n^b	% ($n^{b/}N^b$)	aOR (95% CI) p-value	n^c	% ($n^{c/}N^c$)	aOR (95% CI) p-value
Overall	157	55.9	-	141	48.5	-	87	29.9	-
Age-group									
Children	44	41.9	ref	42	36.8	ref	20	17.5	ref
Adolescent	20	45.5	1.01 (0.48 – 2.11) 0.977	14	31.8	0.76 (0.35 – 1.64) 0.488	7	15.9	0.81(0.31 – 2.11) 0.665
Adult	93	70.5	3.18 (1.85 – 5.47) <0.0001	85	63.9	3.11 (1.83 – 5.29) <0.0001	60	45.1	3.85(2.12 – 7.01) <0.0001
Sex									
Female	79	54.5	ref	64	42.7	ref	37	24.7	ref
Male	78	57.4	1.08 (0.66 – 1.79) 0.755	77	54.6	1.80 (1.09 – 2.96) 0.021	50	35.5	1.82 (1.06 – 3.13) 0.030
Location									
Low	72	62.6	ref	52	41.6	ref	34	27.2	ref
High	85	51.2	0.63 (0.38 – 1.05) 0.076	89	53.6	1.73 (1.05 – 2.86) 0.031	53	31.9	1.33 (0.77 – 2.29) 0.311

Note: No natural Pfs25 IgG was detected in the participants tested. n^a — number of participants who tested positive for anti-Pfs230 IgG, n^b — number of participants who tested positive for anti-Pfs48/45 IgG, n^c— number of participants who tested positive for both anti-Pfs230 IgG and anti-Pfs48/45 IgG

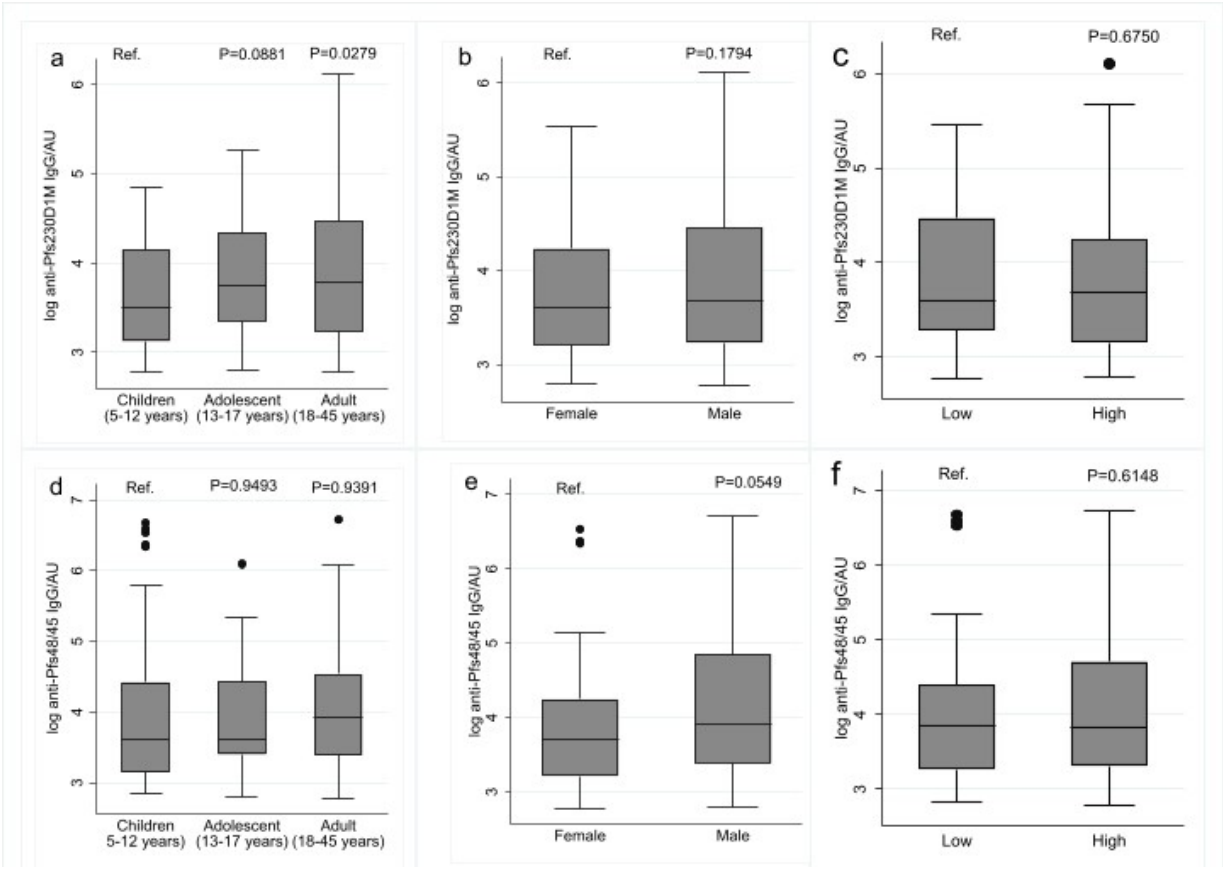

Figure 8: Anti-gametocyte IgG levels by age, sex and location of participants

Anti-Pfs230D1M IgG variation by age (**a**), sex (**b**) and location (**c**). Anti-Pfs48/45 IgG variation by age (**d**), sex (**e**) and location (**f**).

Correlation between antibodies and gametocytes.

There was no evidence of a correlation between gametocyte density and antibody titres for Pfs230 [Correlation: -0.0710, p=0.8093] (Figure 9A). A weak positive correlation [Corr: 0.2013, p=0.4385) between gametocyte density and Pfs 48/45 titres was observed (Figure 9B). There was a weak but significant positive correlation between anti- Pfs48/45 IgG titres and anti-Pfs230D1M titres [Corr: 0.2267, p=0.0347] (Figure 9C).

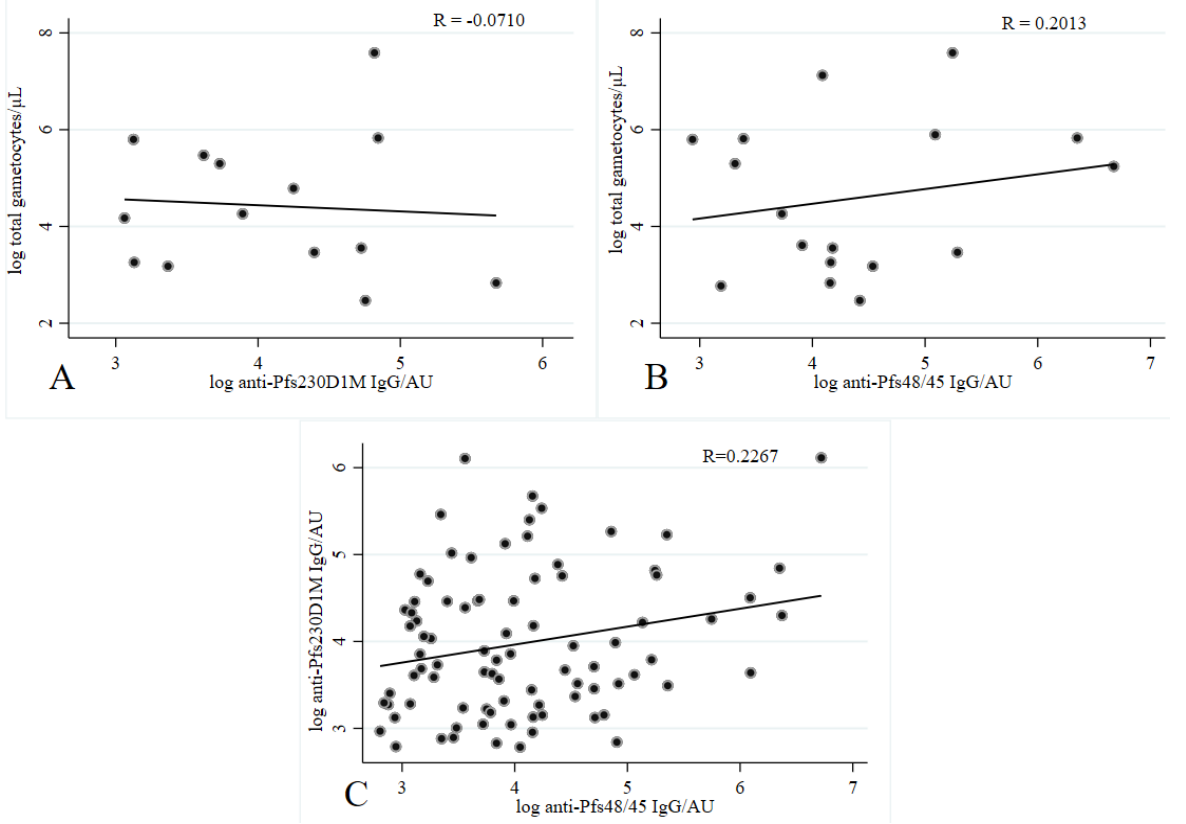

Figure 9: Correlation between gametocyte and anti-gametocyte antibodies
Correlation between gametocyte and Pfs230 antibody in figure (**A**), correlation between gametocyte and Pfs 48/45 antibody in figure (**B**), and correlation between Pfs230 antibody and Pfs 48/45 antibody in figure (**C**). Line is the fitted line.

Total IgG titres for anti-Pfs230D1M and anti-Pfs48/45 were measured at six (6) different time points in the same individuals for a group of 40 adults and 19 children over a period of ten months. Seropositive participants on the first day of sampling (day 0) remained positive at

each time point, and their mean antibody responses are shown in Figure 10. There was no significant difference in antibody responses over time, although a slight increase in titres was observed with an increase in average rainfall between day168 and day 280. Participants who tested negative on day 0 remained negative at almost all timepoints and were not included in analysis.

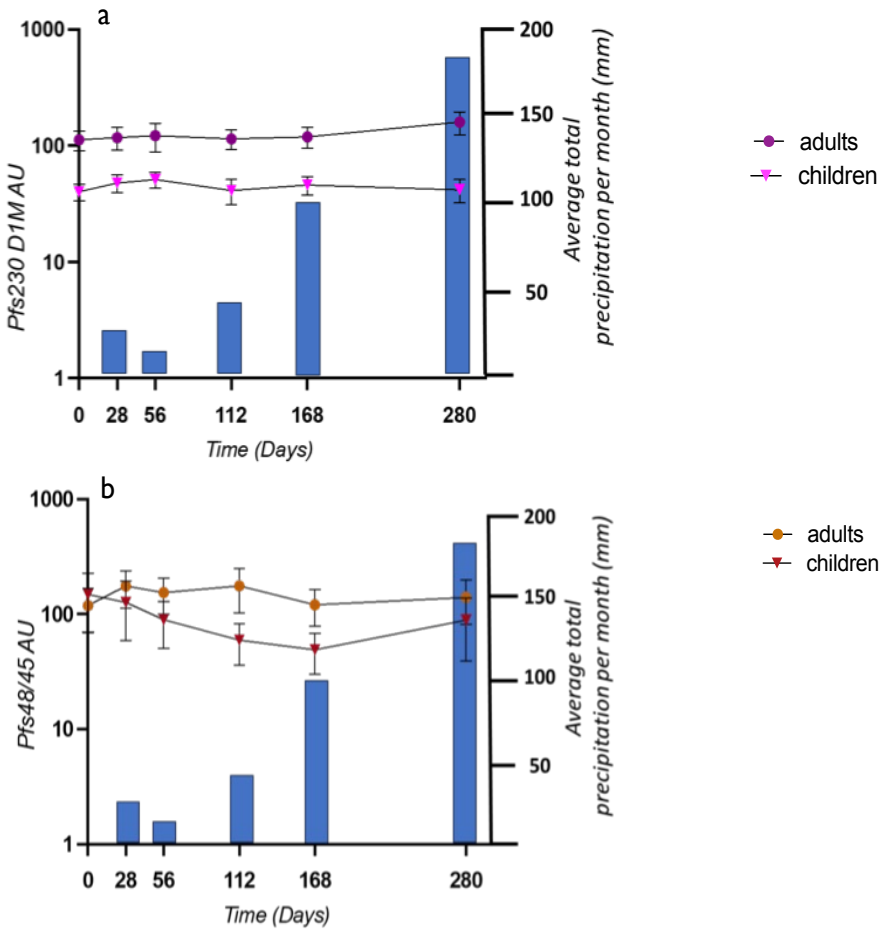

Figure 10: Mean anti-Pfs230D1M and anti-Pfs48/45 antibody variation in seropositive adults and children over 10 months-period

a) anti–Pfs230D1M total IgG responses; n = (10 children and 23 adults), **b**) anti–Pfs48/45 total IgG responses; n = (9 children and 17 adults), antibody responses are shown on the y-axes. The error bars indicate the lowest and highest antibody titre. Average precipitation at each time point is indicated by blue bars (right y axes). The sampling time points were between June 2022 and March 2023.

4.1.3. Levels and quality of Pfs25 total IgG induced by Pfs25-IMX3

i. Pfs25-IMX313 in Matrix-M induced high levels of anti-Pfs25 total IgG responses in Vaccinated volunteers

Vaccination with Pfs25-IMX313 in Matrix-M induced Pfs25-specific IgG responses in all vaccinated volunteers **(Figure 11)**, median IgG titres overtime is shown in Figure **11A** and individual responses in **Figures 11B-F**. All volunteers in both groups seroconverted two weeks (day 14) after the first vaccination with median Pfs25 IgG of 2.4 μg/mL and 4.8 μg/mL for Groups 1 and 2 respectively. The responses increased significantly one week (day 35) after the second vaccination (median IgG titers: 94.8 μg/mL and 150.4 μg/mL for Groups 1 and 2 respectively). Peak responses were recorded two weeks (Day 70) after the third vaccination (median responses of 229.7 μg/mL and 160.1 μg/mL for Groups 1 and 2 respectively). There were insignificant differences in Pfs25 IgG titers between the two groups at D35 ($P = 0.14$) and D70 ($P = 0.32$) following assessment by Mann-Whitney test. Serum antibody responses decreased by day 140 (median: 21.9 μg/mL and 52.5 μg/mL for group 1 and 2 respectively) but were maintained above pre-vaccination levels up to the end of the study period, 24 months after the third vaccination.

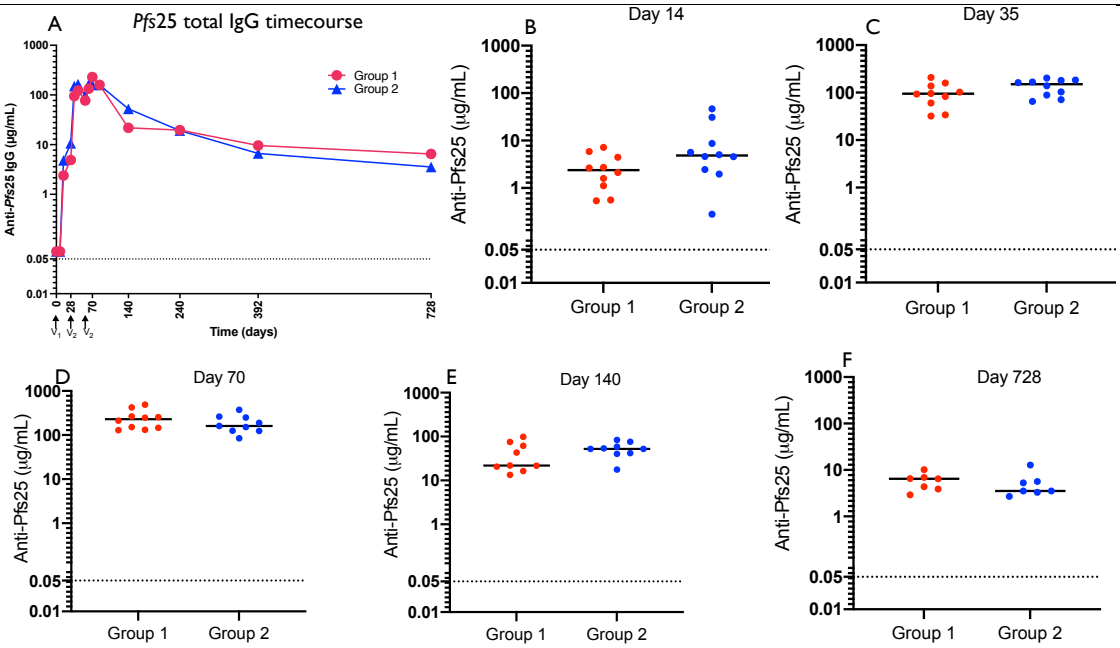

Figure 11: Vaccine-induced Pfs25 antibody responses in vaccinated participants

(A) Median anti-Pfs25 total IgG responses over time. Individual and mean responses are shown at (B) day 14, (C) day 35, (D) day 70, (E) day 140 and (F) day 728, after the first vaccination (V1). Group 1, adults, 18-45 years, received 10 µg of Pfs25-IMX313, N=10; Group 2, adults, 18-45 years, received 50 µg of Pfs25-IMX313, N=10, all groups pooled, N=20. Symbols are color-coded according to group. Baseline = day 0 pre-vaccination time-point shown for both Groups. The horizontal dotted line shows the limit of detection of the ELISA assay. The responses in Groups 1 and 2 were compared using the Mann-Whitney test.

ii. Pfs25-IMX313 in Matrix-M induced good quality Pfs25 total IgG

The avidity ELISAs were performed to further characterize the vaccine-induced antibody responses in the low and high dose vaccine groups. As expected, the avidity of the anti-Pfs25 antibodies increased over time (Figure 12A). Avidity peaked at D140 and remained high for the rest of the test period. There was no significant difference in the avidity of the antibodies induced in low and high dose groups (12B-E)

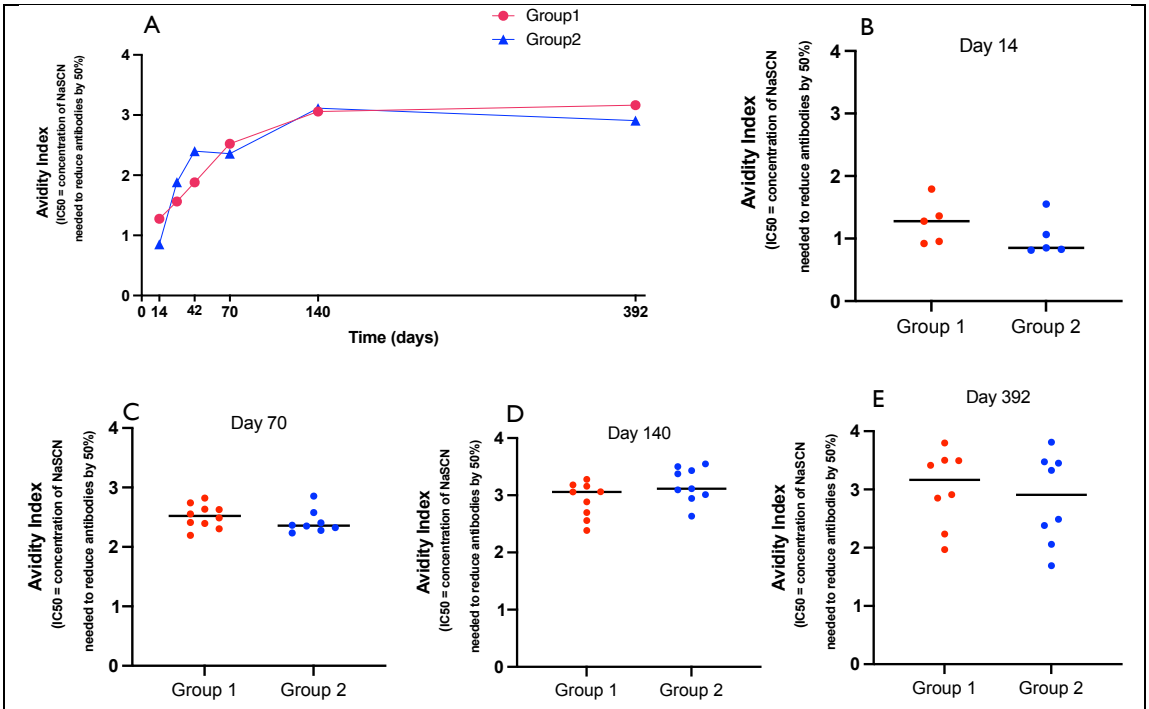

Figure 12: Pfs25 antibody-antigen binding strength in vaccinated participants

Median avidity indices of total Pfs25 IgG in sera from volunteers vaccinated with different doses of Pfs25-IMX313 (A). Individual and median avidity indices at (B) day 14, (C) day 70, (D) day 140 and (E) day 392. Sera were tested individually, and increasing concentrations of NaSCN (0, 1, 2, 3,4,5, 6, 7 and 8 M) were used prior to development of the ELISA reaction.

iii. Pfs25-IMX313 in Matrix-M did not generate Immune responses against hC4bp

The IMX313 component of the Pfs25-IMX313 has less than 20% homology with human C4bp (hC4bp). All participants in both groups were therefore tested for reactivity to hC4bp in endpoint ELISA. Sera on Day 14 (D14), D28, D56, and D84 were tested for the ability react to hC4bp. There was no cross reactivity to hC4bp in any participants from either group (Figure 14).

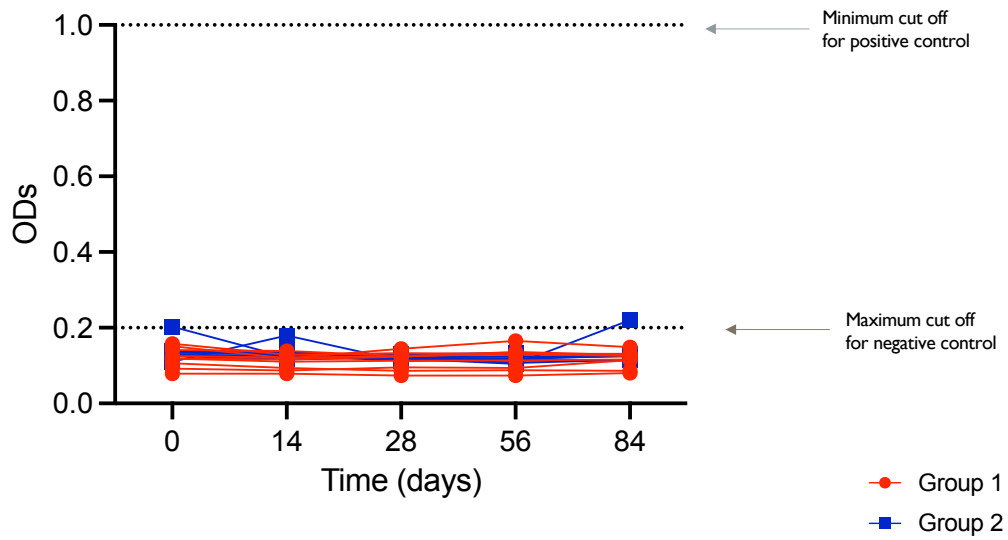

Figure 13: Individual optical density readouts for hC4bp ELISA of participants in Group 1 and 2.

All ODs were below the maximum cut off for the negative control, implying that all individuals from both groups were nonreactive to hC4bp at the indicated time points.

4.1.4. Transmission-reducing activity of Natural and Vaccine-induced transmission-blocking antibodies

i. Total IgG from field sera reactive to Pfs230 and Pfs48/45 antigens showed highly variable TRA

Serum samples from ten (10) field participants were individually tested for TRA via two rounds of SMFA; 2/10 participants demonstrated significant TRA (Table 8). These two participants reduced oocyst development by 90%, and were highly reactive to both Pfs230 and Pfs48/45 antigens. The TRA values for the rest of participants were not significant, highly variable, and were not correlated with antibody concentrations measured by ELISA.

Table 8: Transmission-reducing activity of Natural antibodies from Field participants

Sample ID	Mean oocyst count	%TRA	95%CI Low	95%CI High	p-value
Naive serum	41.7				
4B7	1.4				
N-006*•	1.4	**93.0**	84.7	97.0	0.001
N-008*•	0.1	**99.7**	99.1	99.9	0.001
N-019•	31.2	**7.0**	-96.9	57.4	0.792
N-001•	32.4	**3.6**	-101.6	54.7	0.918
N-021•	36.7	**-9.2**	-137.5	50.2	0.796
N-026*	32.3	**3.9**	-102.8	57.4	0.911
N-055*	33.3	**0.9**	-113.6	55.8	0.935
N-027*	23.0	**31.4**	-50.6	68.0	0.316
N-028*	26.5	**21.2**	-63.9	63.1	0.531
N-036*	34.3	**-2.1**	-120.1	52.7	0.935

*• = reactive to both *Pfs*48/45 and *Pfs*230, • = reactive to *Pfs*48/45 only, * = reactive to *Pfs*230 only

ii. **Vaccine-induced Pfs25 antibodies promoted significant TRA**

Purified total IgG from D0 and D70 serum for each of the 20 vaccinated individuals was used to assess the functional activity of Pfs25 IgG elicited in Groups 1 and 2. At least 20 mosquitoes per test sample were fed with a mixture of purified IgG (10 mg/mL) and in vitro cultured *P. falciparum* NF54 gametocytes. The 10 mg/mL test concentration was chosen as it is the average physiological concentration of total IgGs in adults (Harkness et al., 2020). The mosquitoes were dissected, and number of oocysts per mosquito counted (Figure 14A and B). At least two independent feeds were performed for each test sample, and TRA was estimated as the reduction in average oocyst count in test samples compared to a negative control

lacking blocking antibodies. The combined analysis of two independent SMFAs for each individual showed that 18 out of 20 individuals in Group 1 and 2 combined had significant Pfs25-specific TRA at D70 (Figure 15 A and B). The median TRA in the low dose Group was 67.1% (rage: 34.3% to 92.3%), whereas the high dose Group had a median TRA of 68.1% (range: 33.6% to 94.9%). There was insignificant difference in the TRA of the low and high dose groups when compared using Mann-Whitney test (p-value = 0.5922). As expected in semi-immune individuals, most D0 sera samples (pre-vaccination) showed some TRA at levels which were not as significant as the observed TRA with D70 samples.

The relationship between anti-Pfs25 IgG antibody concentration at day 70 in both groups and TRA was investigated, and a positive correlation was observed [Spearman r = 0.4663; P = 0.0382] (Figure 15C). In particular, Group 2 volunteer ID 054, 043 and 064, showing the topmost TRA, are the top three individuals with the highest levels of Pfs25-specific IgG antibody in the high dose Group. Likewise, Volunteer ID 022 and 020 in Group 1, showing the highest and second highest TRA respectively, are among the top four individuals with highest Pfs25-specific IgG antibodies in the low dose group.

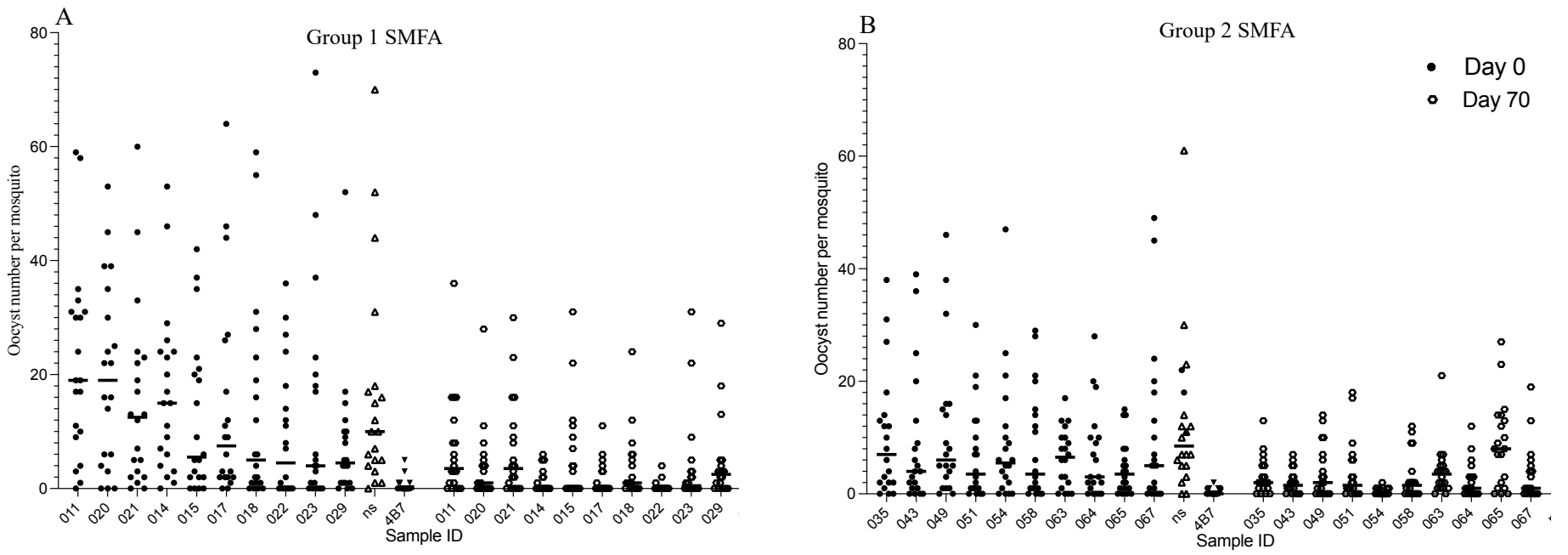

Figure 14: Oocyst intensity after one round of standardized membrane feeding assay with IgG from participants of Group 1 and 2

(**A**) Oocyst counts after assay tests for total IgG from Group 1 participants. (**B**) Oocyst counts after assay tests for total IgG from Group 2 participants. Purified IgG was mixed with *P. falciparum* NF54 cultured gametocytes and fed to mosquitoes (n = 20 per test assay), which were dissected 7 days post feeding to determine oocyst intensity. Purified IgG from a pool of naïve sera (**ns**) was used as negative control. Monoclonal anti-Pfs25 antibodies **(4B7**) was used as the positive control.

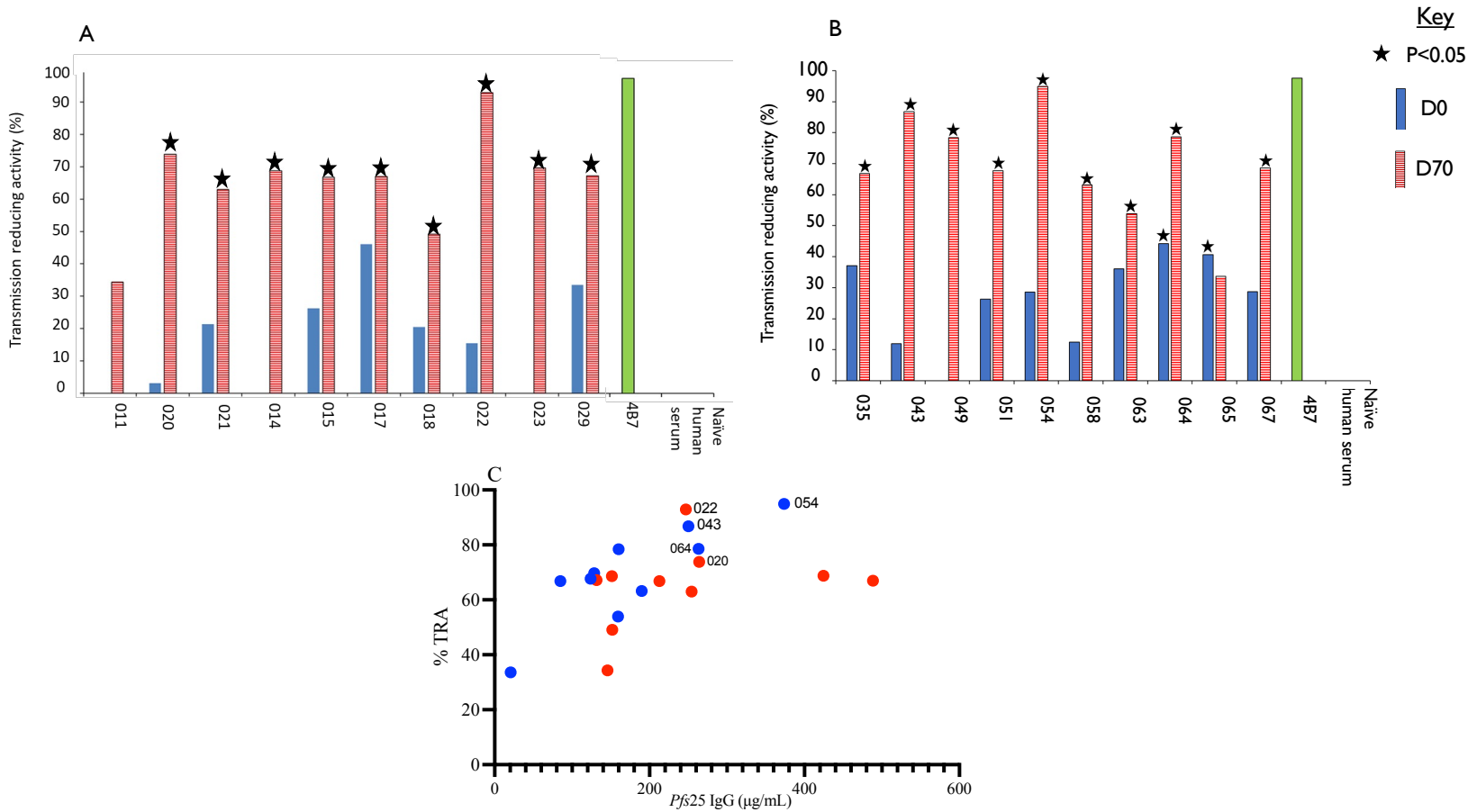

Figure 15: Transmission-reducing activity (TRA) in Groups 1 and 2 as measured by the first round of standardized membrane feeding assay
(**A**) Transmission-reducing activity of IgG from individuals of Group 1. (**B**) Transmission-reducing activity of IgG from individuals of Group 2. (**C**) Correlation between anti-Pfs25 specific IgG concentrations and TRA in individual of Groups 1 (in red) and 2 (in blue); subject ID showing the strongest transmission-reducing activity is indicated. The asterisk ★ on bars means statistically significant difference between TRA by test IgG from D0 or D70 sera and negative control IgG from naïve serum.

4.1.5. Field Pfs25 gene transcripts were identical to Pfs25 reference sequence

To determine if Pfs25 polymorphisms are present at significant frequencies within the study area, Pfs25 transcripts were amplified from 109 *P. falciparum* field samples of which, 85 successfully amplified a PCR band at the approximate size for Pfs25. Aliquots of Pfs25 amplicons were sent for sanger sequencing and the results aligned back to the Pfs25 reference sequence (LR131490.1), which was used in the design of the Pfs25-IMX313 vaccine. All the sequenced field samples (N = 59) gave a 100% sequence identify to the Pfs25 reference sequence, suggesting no or only very limited frequency of polymorphs in the Pfs25 gene sequence.

4.2. Discussion

4.2.1. Malaria infection in Humans and Field mosquitoes

i. Malaria infection in Humans

Identifying the human infectious reservoirs is paramount for successful malaria transmission-blocking interventions. In this study, the asexual and gametocyte prevalence, were assessed in school-age children, adolescents and adults, for the first in the district of Bagamoyo. Unsurprisingly, the malaria positivity and gametocyte prevalence were highest in children and adolescents, as these age groups are more vulnerable to malaria infections compared to adults. In line with common practices in many African countries, we utilized malaria rapid diagnostic tests (mRDTs) and microscopy to detect malaria infections in the study. Additionally, we employed sensitive qPCR assays to ensure the detection of asymptomatic infections that may be missed by mRDTs and microscopy.

The qPCR results showed that microscopy missed more than one-third (33.6%) of infections, whereas mRDT missed nearly 40% of the qPCR-positive infections. These findings corroborate previous studies in Tanzania (Hofmann et al., 2015), Nigeria (Umunnakwe et al., 2019), and Ghana (Opoku Afriyie et al., 2023), where significant proportions of qPCR-positive infections were missed by microscopy and mRDT. The missed infections by microscopy and mRDT in this study could be attributed to submicroscopic parasites, which tend to persist for several months without any symptoms. Furthermore, the observed mRDT false negatives in this study may have been as result of Histidine-Rich Protein 2 (HRP-2) gene deletions, which is prevalent in Tanzania (Z. E. Mwangonela et al., 2023). The missed infections are of great concern since failure to identify malaria-carriers may lead to continued transmission and increase in the malaria burden, particularly in low-transmission areas. The observed false positives by mRDT may be as a result of persistence of HRP-2 antigen after successful antimalarial treatment. The persistent HRP-2 positivity is more common when treatment is done with artemisinin combination therapy and may last for 2-20 days post-treatment (Oulton et al., 2022).

There have been previous reports of relatively high gametocyte prevalence in school-age children in Bagamoyo district than what we observed; one study in Kiwangwa village reported a gametocyte prevalence of 14% (Sumari et al., 2017), and another study in Buma and Yombo reported a prevalence of 18% (Hofer et al., 2023).Given the difference in study

villages, a direct comparison between studies may not be appropriate. Nonetheless, the difference between the gametocyte prevalence in our study and previous reports could be reflective of heterogeneity in malaria transmission and carriage rates in the population. A large proportion of gametocyte carriers in our study were adults, indicating a shift in the age-distribution of gametocyte carriage. This shift could be attributed to intensive efforts to reduce and prevent malaria in children than in adults, in recent years (Kamazima & Makemba; Odufuwa et al., 2020). Furthermore, the gametocyte prevalence in male participants for in this study was higher compared to their female counterparts, which could be due to fact the common social-economic activities in the study area, such as fishing at night, and animal grazing, are dominated by men and boys, making them more exposed and vulnerable to malaria infection than women and girls.

The gametocyte sex ratio, which is the proportion of male gametocytes, is a useful predictor of the malaria infectiousness of human host and their potential to transmit malaria parasites (Santolamazza et al., 2017). Although the likelihood of mosquito infection largely depends on gametocyte density, parasite fertilization in the mosquito requires sufficient numbers of male and female gametocytes to be present in the mosquito blood meal (Tadesse et al., 2019). Quantifying both male and female gametocytes in human hosts allows a better prediction of infectiousness than measurement of the total gametocytes or the more abundant female gametocytes (Bradley et al., 2018). The overall gametocyte sex ratio in the current study is similar to what has been observed in other endemic areas (Bradley et al., 2018). The gametocyte sex ratio in adult gametocyte carriers of our study is an indication that adults could also be playing a significant role in malaria transmission. Additional prospective studies are needed to fully understand the age-specific contribution to mosquito infection, particularly in light of the similar gametocyte density and sex ratio observed among age groups in this study. Nevertheless, the current findings have important community-level implications for ongoing malaria control efforts. Adults, like school-age children, represent a large group of gametocyte carriers in the study area, suggesting that interventions which focus solely on children may be unlikely to interrupt malaria transmission.

ii. Malaria infection in field mosquitoes

Field mosquito surveillance has been used to estimate transmission of malaria (Rossi, Belli, Mancini, & Sabatinelli, 1986) as well as developing vector control interventions to reduce malaria transmission. Our field mosquito collections support previous reports that *An. funestus s.s*, *An.arabiensis* and *An.gambiae* s.s, are the predominant malaria vectors in Bagamoyo (Killeen & Sougoufara, 2023; Kisinza et al., 2017).The number of field indoor biting mosquitoes collected during our study was relatively low, which could be attributed to the mass rollout of vector control interventions such as LLINs in the area (Odufuwa et al., 2020). These interventions could have led to reductions in endophilic Anopheline mosquito populations. The reduction in indoor biting mosquitoes is particularly important for malaria control as exophilic mosquito populations, which may be newer species and not easy to control (Stevenson et al., 2012) may take up a significant role in maintaining transmission. In this case, an effective transmission-blocking vaccine will be an ideal tool to supplement the available malaria interventions.

Although a limited number of field mosquitoes were analyzed in our study, the observed malaria infection rate in these mosquitoes was higher compared to what has been reported elsewhere in Tanzania (Mwalimu et al., 2024). The observed malaria positivity in field mosquitoes highlights the need for new malaria control tools to supplement the current interventions. A transmission-blocking vaccine may be a great addition to the current local malaria control toolbox for the study area.

4.2.2. Natural transmission-blocking antibodies

Our antibody survey data showed evidence of naturally acquired antibody responses against *P. falciparum* gametocyte antigens; Pfs230 and Pfs48/45, as previously reported (J. Bousema et al., 2007; T. Bousema et al., 2010; Drakeley et al., 2006; Ouédraogo et al., 2011), as well as some evidence of stability and longevity of the detected anti-gametocyte responses. All the participants tested were seronegative for anti-Pfs25 IgG and this is likely due to Pfs25 transcripts being translated post blood ingestion in mosquito midgut. The seroprevalence for anti-Pfs230 and anti-Pfs48/45 increased with age, and our data showed that titres for anti-Pfs230 but not anti- Pfs48/45, also increased with age. This observation suggests that anti-gametocyte responses particularly anti-Pfs230 IgG, may be long-lived and stable, even when antigen exposure decreases with increasing age or low transmission intensity. The seroprevalence in children is most likely from the initial immune response to gametocytes, as

previously observed (J. Bousema et al., 2007; Drakeley et al., 2006). There was no significant difference in anti-Pfs48/45 titres detected in children, adolescents and adult but a significant difference was observed in seroprevalence between areas of low and relatively high transmission. This indicates a potential role for recent parasite exposure in the presence of Pfs48/45-specific immunity.

A large proportion of participants with anti-Pfs230 or anti- Pfs48/4 IgG in the absence of gametocytes or parasite infection was observed. The detected anti-gametocyte responses may have been as result of a potential remnant from previous gametocyte infection prior to sampling or levels of gametocytes that cannot be detected by even sensitive qPCR. This observation together with the absence of any association between anti-Pfs230 antibodies, and gametocyte presence suggest that antibodies may serve as markers of gametocyte exposure, rather than biomarkers of active gametocytemia. Concurrent gametocytes may reflect development of anti-gametocyte responses, but a direct association may not always be observed (J. Bousema et al., 2007; Drakeley et al., 2006). Anti-gametocyte antibodies induced before sample collection may persist for several weeks after gametocyte clearance. On the other hand, antibody induction /boosting to circulating gametocytes at the time of sampling may take longer, e.g., after their death/destruction and subsequent clearance(J. Bousema et al., 2007; T. Bousema et al., 2010).

The 10-months period over which anti-gametocyte responses were measured coincided with the end of one long rainy season and the start of the next rainy season. Data from this period indicated that both anti-Pfs230D1M and anti-Pfs48/45 IgG responses are long-lived, although anti-Pfs48/45 responses fluctuated more with the average rainfall compared anti-Pfs230D1M responses. More detailed longitudinal studies should be undertaken with a bigger sample size and larger age range to firmly establish the age-dependency of anti-gametocyte malaria immunity as well as providing more evidence on the longevity of these responses.

The observation of natural responses against recombinant Pfs230 and Pfs48/45 antigens and their association with common proxy-markers of malaria exposure (transmission intensity, age) in this study suggest that natural parasite exposure may boost vaccine induced immunity(Farrance, Rhee, et al., 2011; Theisen et al., 2014). A recent Phase 1 trial of Pfs48/45 and Pfs230 vaccine candidates in Burkina Faso found that vaccine-specific responses were boosted by pre-existing immunity against respective antigens (Tiono et al., 2024), which contrasts prior findings that exposure to *P. falciparum* might diminish

subsequent boosting by vaccination (Jepsen et al., 2013). These experiences indicate the need that transmission-blocking vaccine evaluation in endemic areas to take into account naturally acquired immune responses to the sexual-stage vaccine antigens.

4.2.3. Pfs25 antibodies induced by Pfs25-IMX313 in Matrix-M

Although the Pfs25-IMX313 antigen has previously been tested in naïve volunteers in a viral-vectored heterologous prime-boost regimen with the ChAd63/MVA Pfs25-IMX313 vaccines (De Graaf et al., 2021), the Pfs25 antibodies induced in our study were 10-fold higher and long-lasting compared to the levels observed in the earlier study. Our findings are in agreement with another clinical study where malaria-naïve healthy adults received up to four doses of Pfs25-EPA conjugates formulated with Alhydrogel (Talaat et al., 2016), suggesting that protein-in-adjuvant vaccine formulations may be superior over viral vectored vaccines in inducing antibody responses against mosquito stage malaria parasites. However, further studies may be required to determine whether the higher Pfs25 antibody titres induced in our study could compensate for the lack of natural boosting. A strength of our study is that two doses were evaluated in an endemic setting, with target population who are naturally exposed to malaria. In addition, the Pfs25-IMX313 in our study was administered with Matrix M adjuvant which is known to have significant immune stimulatory activity in humans(Singh, Plieskatt, Chourasia, Fabra-Garcia, et al., 2021; Singh, Plieskatt, Chourasia, Singh, et al., 2021; Singh et al., 2019). Recombinant vaccines generally require an adjuvant to enhance and prolong an adequate immunity. It is suggested that the Matrix-M adjuvant mobilizes immune cells at the vaccine inoculation site and that the adjuvant facilitates drainages of antigen and immune cells to the draining lymph nodes (Stertman et al., 2023).

An essential requirement for an antigen to be effective as a TBV is its ability to induce robust antibody production even at low vaccine doses. Our study demonstrated that the low dose of Pfs25-IMX313 is as good as the high dose level. The lack of a clear improvement between low and high vaccine dose levels in our study, implies that the low vaccine dose would be favorable, as it would minimize the risk of side effects in vaccinated individuals and the cost of vaccine production may be lower compared to the high dose of the vaccine. Altogether, our findings have informed decisions to start a trial in Burkina Faso, using 10μg of Pfs25-IMX313 head-to-head against 10μg of Pfs48/45 full length soluble protein.

The concept of a transmission-blocking vaccine (TBV) has gained prominence following the renewed focus on malaria elimination and the challenges facing current malaria interventions. The Malaria Eradication Research Agenda (malERA) has recommended TBVs as potential tool for malaria elimination but it can also be used as a component of a multi-stage malaria vaccine. In order to break the malaria transmission cycle, TBVs will require mass deployment at community level. Thus, developing a TBV with a satisfactory safety profile is mandatory. The data from the clinical trial (unpublished) in which this PhD study is nested, showed that the formulation of Pfs25-IMX313 in matrix-M® adjuvant demonstrated a favourable safety profile, being well tolerated at all dose levels, and no participants withdrew due to adverse events. The observed reactogenicity in our study was similar to what has been reported in previous transmission-blocking vaccine trials, which evaluated similar vaccine candidates (De Graaf et al., 2021; Tiono et al., 2024). In addition, no antibody cross-reactivity was detected against the oligomerisation domain of human C4bp, in the vaccinated groups. It was thus unlikely that vaccination with an antigen fused to IMX313, which has 21% homology to the human C4bp (HC4bp) sequence (Ogun et al., 2008), would generate an immune response against hC4bp. This study further supports the use of IMX313 as a carrier nanoparticle for transmission-blocking vaccine antigens.

4.2.4. TRA of Natural and Vaccine-induced antibodies

i. TRA of field sera reactive to Pfs230 and Pfs48/45 antigens

The lack of significant inhibition of oocyst intensity by individuals who were reactive to either Pfs230 or Pfs48/45 antigens only suggests that the antibodies could be binding to non-blocking epitopes or that the antibody levels were not sufficient to effect oocyst reduction in the mosquitoes. The observation of high transmission reducing activity in the two field samples reactive to both Pfs48/45 and Pfs230 antigens is similar to reports from previous field studies (T. Bousema et al., 2010). The observed TRA may be attributed to an additive or a synergistic effect of different TRA mechanisms by the two antibody types. Pfs230 antibody-related TRA involves complement-mediated lysis of gametes (Healer et al., 1997); Pfs48/45 antibodies can inhibit zygote development independent of complement (Richard Carter, Graves, Keister, & Quakyi, 1990). However, it is also possible that these two

individuals with a high TRA were reactive to an unknown transmission-blocking antigen, and further investigations such as antibody depletion assays, are required to fully understand the immune responses promoting the observed TRA in the two participants.

ii. TRA of Pfs25 antibodies induced by Pfs25-IMX313 in Matrix-M

Our SMFA data showed strong malaria transmission-reducing activity of Pfs25 antibodies in the serum of volunteers in the vaccinated groups, with only two of the twenty vaccinated volunteers failing to show reduction in parasite development in test mosquitoes. Although the TRA values of individual volunteers were slightly variable, they moderately correlated with antibody concentrations measured by ELISA. These findings partly congruent with prior extensive pre-clinical work and the available clinical data (Chichester et al., 2018; Sagara et al., 2018; Talaat et al., 2016). However, our findings show that high anti-Pfs25 antibody titres may not always result in high TRA, particularly in malaria-exposed populations. This observation may be attributed to induction of antibodies against non-blocking epitopes of the Pfs25 antigen (Shukla et al., 2023). The observed TRA in most D0 (pre-vaccination) sera samples supports the presence of natural transmission-blocking antibodies in semi-immune individuals as previously reported (J. Bousema et al., 2007; T. Bousema et al., 2010; Drakeley et al., 2006; Ouédraogo et al., 2011). Indeed, majority of these D0 samples were seropositive for ether anti-Pfs230 and/or anti-Pfs48/45, when tested alongside the field sera samples.

As expected, the average oocyst counts (2-31) in infected mosquitoes in our feeding assays were higher compared to average oocyst counts (2-10) in naturally infected field mosquitoes (Beier, Copeland, Mtalib, & Vaughan, 1992; Gouagna, Yao, Yameogo, Dabiré, & Ouédraogo, 2014). The colonized mosquitoes used in this study experience different selective pressures which result in loss of heterogeneity and natural immunity against malaria parasites (Kengne-Ouafo et al., 2019). Therefore, the observed ex-vivo levels of oocyst inhibition may be an underestimation of what the vaccine can achieve in the field.

In comparison with other Pfs25-based vaccine candidates, the anti-Pfs25 antibodies induced in this study achieved extremely effective transmission-reducing activity; a Phase I trial in healthy adults using either Pfs25 virus-like particles or Pfs25H-EPA conjugate vaccine with Alhydrogel®) suggested that a more immunogenic formulations were needed for significant TRA (Chichester et al., 2018; Talaat et al., 2016), and when Pfs25H-EPA in Alhydrogel®

was further tested in endemic settings, significant functional activity was induced but the TRA was only seen after four vaccine doses, and antibody titers rapidly waned (Sagara et al., 2018). Other studies testing Pfs25 and Pfs230 conjugated to EPA indicated that anti-Pfs230 antibodies promoted better functional activity than anti-Pfs25 antibodies (Healy et al., 2021). The highly effective TRA of Pfs25 antibodies induced by the Pfs25-IMX313 vaccine in our study merits further investigations of this vaccine candidate, particularly in children and in other endemic areas, with the aim of improving TBV immunization strategies. It will also be of importance to characterize the IgG subclasses, epitope specificity and cellular immune mechanisms mediating the observed TRA.

4.2.5. Genetic variation in field Pfs25 sequences

The observation of 100% identity between the Pfs25 field sequences and Pfs25 reference sequence, suggesting no or very limited frequency of polymorphs in the field Pfs25 gene sequences was unsurprising. This is due to lack of evidence of the protein within the human host despite the Pfs25 transcript being readily detectable in gametocytes, thus the antigen is believed to be under little or no immune pressure. Nonetheless, polymorphisms in Pfs25 that affect the structure of the protein could reduce the effectiveness of vaccine-induced antibodies. It is important to regularly look out for polymorphs in transmission-blocking candidate proteins in the field.

4.3. Limitations of the Study

i. It was not possible to evaluate the Pfs25-IMX313/Matrix-M vaccine candidate in children because the approval to vaccinate children with the candidate vaccine (IMP) was delayed by Tanzania Medicines and Medical Devices Authority (TMDA). Children are known to harbor the sexual parasites responsible for malaria transmission. Progress of this candidate vaccine to the next development stages will be very challenging without phase 1 evaluation in children.

ii. The SMFAs were conducted using the *Anopheles stephensi* mosquitoes, which are not common in the study area. Local strains like the Ifakara *Anopheles gambiae* mosquito strain, were not readily available at the laboratory were SMFA were conducted. Also, it was not feasible to determine the vaccine TRA in other vector species like; *Anopheles funestus* and *Anopheles arabiensis*, which are known to transmit malaria in the study area.

Nonetheless, the use of *Anopheles stephensi* mosquitoes does not have any impact/influence on the observed TRA results.

iii. The budget limitations made it impossible to perform antibody depletion assays to ascertain if the TRA observed in the two participants from the serosurvey, was promoted by either Pfs48/45 or Pfs230 IgG.

iv. The participants were recruited from only five locations, which were easily accessible, and this may have restricted the study population and sample size.

v. Another limitation is that all transmission blocking assays were done with the same strain of parasites NF54 which has been in culture for decades and may not be representative of wild-strain parasites.

CHAPTER 5: CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

The observed malaria prevalence in humans and malaria positivity in the limited number of analysed field mosquitoes suggest transmission in Bagamoyo is still substantial, and the available interventions alone may not be able to bring down malaria transmission to levels that support elimination.

The observed gametocyte counts in adults warrants the need for transmission-blocking vaccine strategies to target children as well as adults, particularly in low transmission settings. The Pfs48/45 and Pfs230 seroprevalence in this study suggest that both Pfs48/45 and Pfs230 antigens could be ideal vaccine targets in endemic areas, as there is potential for natural boosting with these two antigens.

The Pfs25-IMX313 in Matrix-M vaccine candidate in this study induced long-lasting transmission-blocking antibodies which significantly reduced malaria parasite development in mosquitoes. These findings support further development of Pfs25-IMX313 in Matrix-M, as a promising transmission-blocking vaccine candidate and warrants further analysis of the study samples to characterise the cellular immune responses as well as investigate the antibody isotypes and epitopes which are involved in mediating the highly effective TRA in this study.

5.2. Recommendations

The following recommendations are made in the context of the findings of this study:

i. The Pfs25-IMX313 in Matrix-M should as well be evaluated in children as they bear the highest malaria burden in most malaria transmission settings. Therefore, to be effective, malaria transmission-blocking strategies must be able to significantly decrease transmission to and from the young segments of the population.

ii. Transmission-reducing activities should be directly measured in the human host in order to overcome challenges associated with the cumbersome mosquito feeding assays, which are at times problematic to standardize.

iii. To address challenges associated with the requirement of maintaining high levels of potent vaccine-induced antibodies that preclude mosquito infection, further studies are

recommended to focus on combining two transmission-blocking vaccines with different TRA mechanisms.

iv. In recent decades, the research capacity and infrastructure across many research institutions like IHI, in the global south, have remarkably improved. These institutions should be given more opportunities to conduct first-in human trials to stimulate innovative research and reduce the overdependence on developed countries for research and local interventions against many diseases of public health importance. In this case, malaria transmission occurs largely in Low- and Middle-Income Countries, where the disease epidemiology, and the genetics of the endemic population will have significant impact on use of the vaccine and the desired outcomes. Relying on results from first-in human evaluations conducted in naïve settings may not yield relevant information on the safety and immunogenicity for a vaccine that will eventually be deployed in endemic settings.

v. The evaluation of transmission-blocking vaccines candidates in endemic settings should also involve regular monitoring of transmission-blocking target proteins for genetic polymorphism. These polymorphisms may affect the structure of the transmission-blocking proteins, which could reduce the effectiveness of vaccine induced antibodies.

vi. The National Malaria Control Program should consider control strategies targeting children as well as adults, particularly in low transmission areas of Tanzania. With more interventions focusing in children, adults may become more important as reservoir of parasites. In addition, the National Malaria Control Program should consider developing engagement strategies to ensure high adherence to these vaccines, as protection is only as good as coverage.

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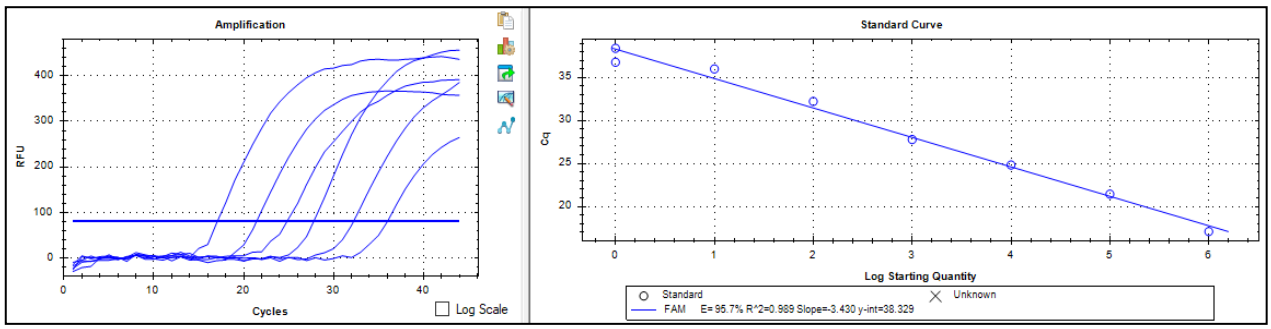
APPENDICES

Appendix 1: Primers and Probes used for gametocyte detection and quantification

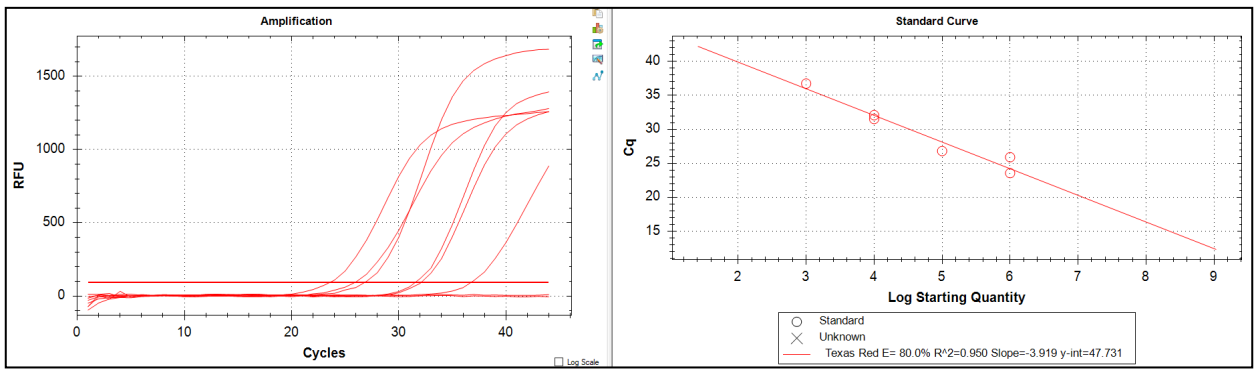
Adapted from Meerstein-Kessel *et al*, 2018

Gene ID	Gene name	Oligo sequence	Oligo modification [5'-3']	Concentration
		Gametocyte detection and quantification		**5x Oligo Mix**
PF3D7_0903800	*CCp4 fwd*	CAC ATG AAT ATG AGA ATA AAA TTG	-	4.5 µM
	CCp4 rev	TAG GCG AAC ATG TGG AAA G	-	4.5 µM
	CCp4 probe	AGC AAC AAC GGT ATG TGC CTT AAA ACG	TexasRed-BHQ2	0.625 µM
PF3D7_1469900	*PfMGET fwd*	CGG TCC AAA TAT AAA ATC CTG	-	1.125 µM
	PfMGET rev	TGT GTA ACG TAT GAT TCA TTT TC	-	1.125 µM
	PfMGET probe	CAG CTC CAG CAT TAA AAA CAC	FAM – BHQ2	0.625 µM

Appendix 2: qRT-PCR reaction efficiency for PfMGET marker used for the detection and quantification of male gametocytes in the malaria survey participants



Appendix 3: qRT-PCR reaction efficiency for CCp4 marker used for the detection and quantification of female gametocytes in the malaria survey participants



Appendix 4: Data Collection Tools

DATA COLLECTION FORMS- TBVB STUDY

Participant ID: ..... **Date...../...../.....**

Informed consent obtained: **Written: .....**

Oral: .....

1. Age in Years .....
2. Gender .....
3. Village .....
4. Weight .....
5. Taken anti malaria for the past two month 1) Yes 2) No

6. Result for: mRDT 1) Yes 2) No
7. Result for: Microscopy 1) Yes 2) No

Gametocytes density: (per 500 WBCs) |_____|/500 WBCs

ELISA Test Results

1. Date of ELISA tests |___|___|___|___|
2. Proteins screened:

	Anti Pfs25 concentration (pg/mL)	**Anti Pfs230 concentration (pg/mL)**	**Anti Pfs48/45 concentration (pg/mL)**	**Anti Pfs28 concentration (pg/mL)**
Standard1				
Starndard2				
Participant sample1				
Participant sample2				

Comments.....
.....

Supervised by initial..... Time.....

TBVB study, data collection form, v1.0, 2021.04.01 Page 1 of 5

MFA of Lab-reared *An. gambiae* mosquitoes fed with a mixture of field donor gametocyte isolates and Pfs25-immunised serum

1. Date of feeding |___|___|___|___|
2. Feeding number |___|___|___|
3. Gametocyte density (per 500 WBCs) |_____|/500 WBCs
4. MFA time

   Start time..... End time..... Total duration.....

Comments.....
.....

Supervised by initial..... Time.....

Cup number	Mosquitoes fed with gametocytes in Naïve serum (Positive control)	Mosquitoes fed with gametocytes in Pfs25-immunised serum	Number of blood-fed mosquitoes (max 50)
e.g. Cupx1 TBVB01 0104	√		**Xn1**
e.g. Cupx2 TBVB01 0104		√	**Xn2**

- **Cups to be labeled with study code, Participants ID number, date, source of gametocyte - isolate (field or cultured), (type of serum: test/control) and number of blood fed mosquitoes**

MFA with Lab-reared *An. gambiae* mosquitoes fed with a mixture of Lab-cultured NF54 gametocytes and Pfs25-immunised serum

1. Date of feeding |___|___|___|___|
2. Isolates-No. (culture flasks) used |_____||_____| |_____||_____|
3. Gametocyte density (per 200 RBCs) |_____|/200 RBCs
4. MFA time

Start time..... End time..... Total duration.....

Comments.....
.....

Supervised by initial..... Time.....

Cup number	Mosquitoes fed with gametocytes in Naïve serum (Positive control)	Mosquitoes fed with gametocytes in Pfs25-immunised serum	Number of blood-fed mosquitoes (max 50)
e.g. Cupx1 TBVB01 0104	√		**Xn1**
e.g. Cupx2 TBVB01 0104		√	**Xn2**

- **Cups to be labeled with study code, Participants ID number, date, source of gametocyte - isolate (field or cultured), (type of serum: test/control) and number of blood fed mosquitoes**

Mosquitoes dissection form

MEMBRANE FEEDING ASSAY FORM	
Protocol Code: TBVB	**Study Title Abbreviated**: TBVB
Participant's Initials: |___|___|___|	Participant's ID number: |___|___|-|___|___|

Date of performing mosquitoes dissection (7 days after direct feeding assay)	|___|___|-|___|___|___|
Number of mosquitoes alive 7 days after direct feeding assay	|___|___|

NUMBER OF OOCYSTS PER MOSQUITO

Mosquito	Number of oocysts	Mosquito	Number of oocysts
1st	|___|___|	19th	|___|___|
2nd	|___|___|	20th	|___|___|
3rd	|___|___|	21st	|___|___|
4th	|___|___|	22nd	|___|___|
5th	|___|___|	23rd	|___|___|
6th	|___|___|	24th	|___|___|
7th	|___|___|	25th	|___|___|
8th	|___|___|	26th	|___|___|
9th	|___|___|	27th	|___|___|
10th	|___|___|	28th	|___|___|
11th	|___|___|	29th	|___|___|
12th	|___|___|	30th	|___|___|
13th	|___|___|	31st	|___|___|
14th	|___|___|	32nd	|___|___|
15th	|___|___|	33rd	|___|___|
16th	|___|___|	34th	|___|___|
17th	|___|___|		
18th	|___|___|		

Optional Comments:

Field mosquito collections

	Site X1	Site X2	Site X3	Mosquito Spiecies1	Mosquito Spiecies1	Totals
Number of houses from which mosquitoes were caught.						
Average number of mosquitoes collected per house						
Number of live mosquitoes positive of Pf						
Total number of mosquitoes collected per site						

Appendix 5: Research Permit from IHI

F120-ILH-v20.0

Plot 463, Kiko Avenue, Mikocheni | P.o. Box 78,373 Dar es Salaam. Tanzania | Phone: +255222774756 Email: irb@ihi.or.tz

INSTITUTIONAL REVIEW BOARD

ISO 9001:2015 certified

IFAKARA HEALTH INSTITUTE
research | training | services

May 31, 2021

National Institute for Medical Research
P O Box 9653
Dar Es Salaam
Email; headquarters@nimr.or.tz

Charles Mulamba
Ifakara Health Institute
P O Box 74
Bagamoyo

IHI/IRB/No: 29-2021

INSTITUTIONAL CLEARANCE CERTIFICATE FOR CONDUCTING HEALTH RESEARCH

On 28 May 2021, the Ifakara Health Institute Review Board (IHI-IRB) reviewed from study titled: ***"Evaluation of transmission blocking activity of vaccine induced antibodies to Pfs25 and seroprevalence of natural antibodies to Malaria gametocyte antigens in Tanzania volunteers (TBVB)"*** submitted by the Principal Investigator Charles Mulamba.

The following documents were reviewed and approved:

1. Protocol
2. Informed Consent Forms (English & Swahili)
3. Budget
4. Tools
5. CVs

The study has been approved for implementation after IRB consensus. This certificate thus indicates that; the above-mentioned study has been granted an Institutional Ethics Clearance to conduct this study in Bagamoyo District.

The Principal Investigator of the study must ensure that, the following conditions are fulfilled during or after the implementation of the study:

1. PI should submit a six-month progress report and the final report at the end of the project
2. Any amendment, which will be done after the approval of the protocol, must be communicated as soon as possible to the IRB for another approval
3. All research must stop after the project expiration date, unless there is prior information and justification to the IRB.
4. There should be plans to give feedback to the community on the findings
5. The PI should seek permission to publish findings from NIMR
6. The approval is valid until 28th May 2022

The IRB reserves the right to undertake field inspections to check on the protocol compliance

Chairperson
Prof. Esther Mwaikambo

IRB Secretary
Dr Mwifadhi Mrisho

0

Appendix 6: Research Permit from NIMR

THE UNITED REPUBLIC OF TANZANIA

National Institute for Medical Research
3 Barack Obama Drive
P.O. Box 9653
11101 Dar es Salaam
Tel: 255 22 2121400
Fax: 255 22 2121360
E-mail: nimrethics@gmail.com

Permanent Secretary (Health)
Ministry of Health, Community
Development, Gender, Elderly & Children
Government City Mtumba, Health Road
P.O. Box 743
40478 Dodoma

NIMR/HQ/R.8a/Vol. IX/3716

01st July 2021

Charles Mulamba
Ifakara Health Institute
Box 74
Bagamoyo

RE: ETHICAL CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: **Evaluation of transmission-blocking activity of vaccine-induced antibodies to Pfs25 and seroprevalence of natural antibodies to malaria gametocyte antigens in Tanzanian volunteers (TBVB) (Mulamba C. et al.),** has been granted ethical clearance to be conducted in Tanzania.

The Principal Investigator of the study must ensure that the following conditions are fulfilled:

1. Progress report is submitted to the Ministry of Health, Community Development, Gender, Elderly & Children and the National Institute for Medical Research, Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from National Institute for Medical Research.
3. Copies of final publications are made available to the Ministry of Health, Community Development, Gender, Elderly & Children and the National Institute for Medical Research.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine as per NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Sites: Ifakara Health Institute's Clinical Trials Centre.

Approval is valid for one year: 01st July 2021 to 30th June 2022.

Name: Prof. Yunus Daud Mgaya

Signature
CHAIR PERSON
MEDICAL RESEARCH
COORDINATING COMMITTEE

Name: Dr. Aifello Wedson Sichalwe

Signature
CHIEF MEDICAL OFFICER
MINISTRY OF HEALTH, COMMUNITY DEVELOPMENT, GENDER, ELDERLY & CHILDREN

CC: Director, Health Services-TAMISEMI, Dodoma.
RMO of Pwani region.
DMO/DED of Bagamoyo district.

RESEARCH OUTPUTS

Mulamba, C., Williams, C., Kreppel, K., Ouedraogo, J. B., & Olotu, A. I. (2022). Evaluation of the Pfs25-IMX313/Matrix-M malaria transmission-blocking candidate vaccine in endemic settings. *Malaria Journal*, 21(1), 159.

Mulamba, C.,Kreppel, K., Olukayode, O. G., Kweyamba, P. A., Lazaro, L. O., Chabo, M. S., Kamage, J. J., & Olotu, A. I., Williams, C., (2025) *Plasmodium falciparum* gametocyte burden in a Tanzanian heterogenous transmission setting. *DOI: 10.1186/s12936-025-05270-4*

Mulamba, C., Olotu, A. I., Kreppel, K., Olukayode, O. G., Kweyamba, P. A., Lazaro, L. O., Mtaka, M. I., Kalinga, W. F., & Williams, C. (2025) Seroprevalence of antibodies to *Plasmodium falciparum* transmission-blocking target proteins Pfs230D1M and Pfs48/45 in Bagamoyo, Tanzania. *Frontiers*.

Seminars, Conferences and Trainings

Name of Event (Training/Conference/Seminar)	Dates	Organizer/Place	Participation (Attendance/Oral Presentation/Poster Presentation)	Mode of attendance
Institutional Scientific Seminar	19th February, /2024	Ifakara Health Institute (IHI)/Bagamoyo, Tanzania.	Oral presentation	In-person
International Pandemic Sciences Conference	10th -11thJuly, 2023	Pandemic Sciences Institute (PSI), University of Oxford/UK	Poster presentation	In-person
Training on Good Clinical and Laboratory Practice	3rd -5th March, 2023	IHI/ Bagamoyo, Tanzania	Attendance	In-person
Annual Conference for the Multi-Stage Malaria Vaccine Consortium	22nd -23rd November, 2022	Jenner Institute, University of Oxford/Gambia	Oral presentation	Via Zoom
Institutional Scientific Seminar Series	16th August,2022	Ifakara Health Institute (IHI)/Bagamoyo, Tanzania.	Oral presentation	In-person
Annual Conference for the Multi-Stage Malaria Vaccine Consortium	26th -27th October, 2021	Jenner Institute, University of Oxford	Oral presentation	In-person
Virtual lecture Séries on Cutting-edge Immunology: from Chemistry to Metabolism	19th – 23rd October 2020	Leiden University Medical Center/ Leiden, Netherlands.	Attendance	Via Zoom
AfriBop Training program on the Immunology of Malaria and other parasitic infections.	23rd – 30th October 2020	University of Cape Town, Cape Town, South Africa	Attendance	Via Zoom
Annual Conference for the Multi-Stage Malaria Vaccine Consortium	10th -12th March, 2020	Jenner Institute, University of Oxford/ Dakar, Senegal.	Oral presentation	In-person
Symposium on Transmission-Blocking Vaccine Trials	6th -8th November, 2019	IHI and Jenner Institute, University of Oxford/Bagamoyo, Tanzania.	Attendance	In-person

Poster Presentation

Malaria transmission-blocking vaccine development: Assessing the gametocyte burden and gametocyte-antigen immunity in low transmission settings

Charles Mulamba[1], Prisca Kweyamba[1], Wilmina Kalinga[1], Ivanny Mtaka [1], Muhamed Chabo[1], Linda Lazaro[1], Sarah Mswata[1], Ally Olotu[1], David Mekhaiel[2] and Chris Williams[2]

[1]Ifakara Health Institute, Bagamoyo, Tanzania

[2]Transmission-blocking malaria vaccine group, Jenner Institute, University of Oxford

Background

Malaria transmission-blocking vaccines (TBVs) are designed to prevent the development of parasites in mosquitoes, consequently blocking onward transmission, from mainly human carriers of the infectious forms of the parasites.

Malaria gametocytes-carriers, are usually asymptomatic (1), and often not targeted by convetional malaria interventions. TBVs could accelerate malaria elimination by targeting these malaria reserviours .

A malaria survey was conducted in Tanzania, to assess the *P. falciparum* gametocyte burden, as well as investigating natural transmission-blocking antibody responses against leading TBV parasite target antigens; Pfs25, Pfs230, and Pfs48/45.

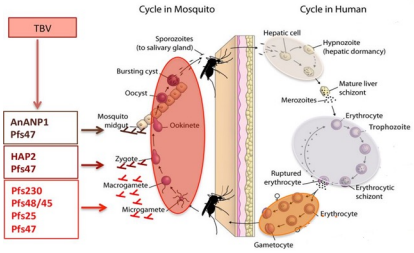
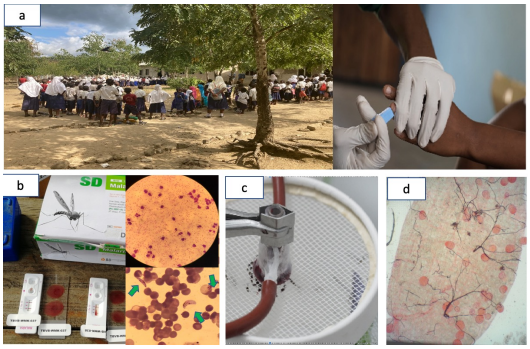


Figure 1: Potential targets for TBVs in the Malaria Transmission Cycle (2).

Methods

The malaria survey was conducted between 2022 and 2023, in volunteers between 5-45 years.

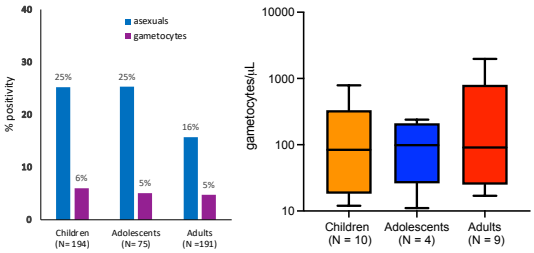


a) Participant recruitment & sample collection; **b)** Malaria testing and quantification; **c)** Mosquito feeding assays of gametocytes in immunized test serum; **d)** Oocyst count in mosquito midguts.

Gametocyte Burden

It is thought that children between 5-14 years harbour a greater number of gametocytes and more infectious (3,4).

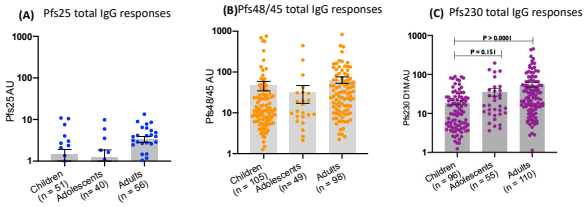
Here, a similar gametocyte positivity was observed in children (5-12 years) & adults (18-45 years) , gametocyte density was highest in adults .



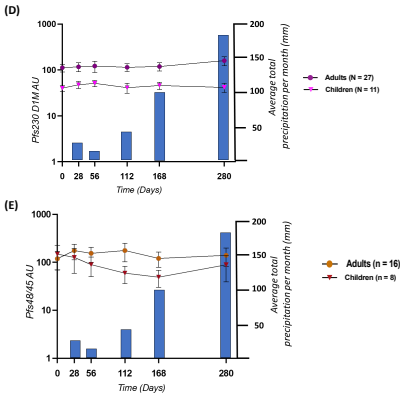
RT-qPCR detection **(a)**, and quantification **(b)** of *P. falciparum* gametocytes , using previously established method (5).

Natural Antibody responses against Pfs25, Pfs230D1M, & Pfs48/45

Total IgG responses were measured by ELISA, based on a previously established method, individuals with antibody unit readout of 10 and below for each response were considered negative. Overall, **52% of volunteers (n =262)** tested positive for anti–Pfs230D1M total IgG, **48% of volunteers (n = 252)** tested positive for anti–Pfs48/45 total IgG. All volunteers (n = 157) tested for anti-Pfs25 IgG were negative.

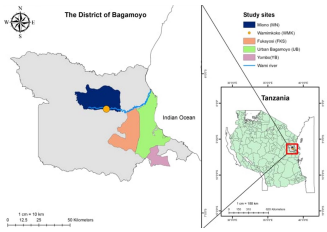


Antibody units for Pfs25 total IgG(A), Pfs230 D1M total IgG (B), and Pfs48/45 total IgG. Antibodies were measured following a single time point. Each dot corresponds to a different individual's response.



Mean anti–Pfs230D1M total IgG responses **(D)**, and Mean anti–Pfs48/45 total IgG responses**(E)**, shown for children and adults over time. Average precipitation per month of sampling indicated by blue bars.

Sequencing TB genes in the field



As part of the malaria survey conducted across the Bagamoyo district (left). PCR was performed on blood samples from infected individuals to amplify and sequence the genes encoding the three antigens of interest.

To date, all the Pfs25 genes sequenced from across the district, show no SNPs and have a 100% sequence homology to the reference. This is likely due to the fact the Pfs25 protein is not translated in humans, so is not under immune pressure.

Pfs48/45 and Pfs230 proteins however are present in the human stages. Work is currently underway to fully sequence these genes.

Conclusion

- Immune response to Pfs230 seems to develop over a long period of time , while Pfs48/45 responses peak in children
- Contrary to the current thinking, this work indicates that adolescents as well as adults are just as important reservoirs for transmission as children.
- TBVs should target adults as well as children
- Pfs48/45 and Pfs230 could be ideal vaccine targets with potential for natural boosting

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