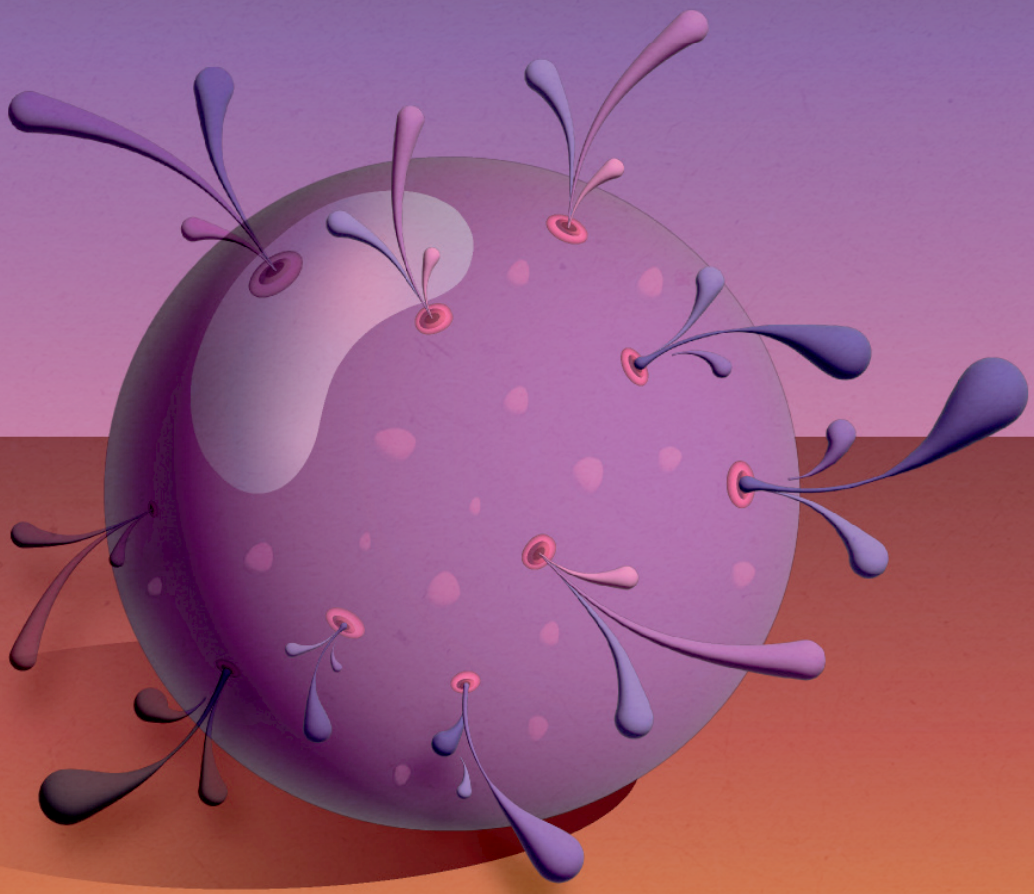
# Complementing the fight against malaria



Maartje Robin Inklaar

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# Complementing the fight against malaria

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# Complementing the fight against malaria

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# Complementing the fight against malaria

Dissertation to obtain the degree of doctor

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according to the decision of the Doctorate Board

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by

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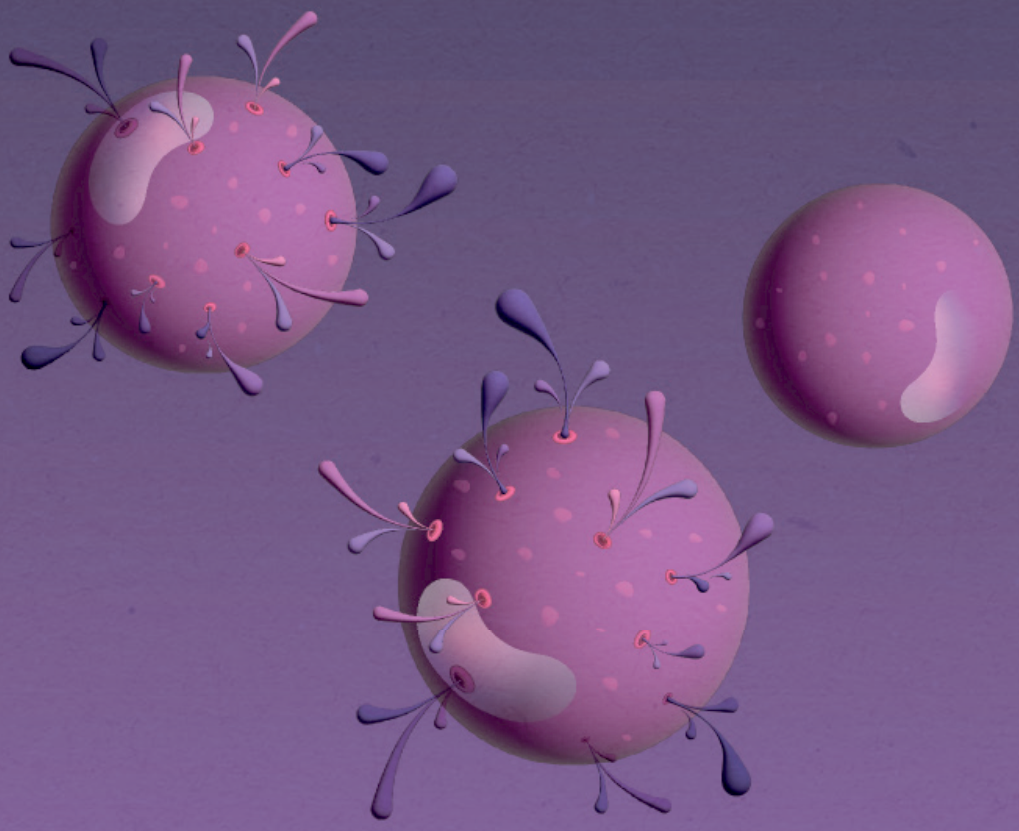
# Table of Contents



Chapter 1

# Introduction

## Small parasite, big impact

Malaria has had a firm grip on public health for thousands of years. Nowadays, the disease caused by parasitic infection still prevails in many regions, primarily on the African continent. Many regions are struggling with the consequences of this devastating parasite. In the year 2023 alone, there were an estimated 263 million malaria cases and 597,000 malaria deaths globally[1]. A large proportion of malaria cases and deaths, 94% and 95% respectively, occurred on the African Continent. Children under the age of five are most often the victim of the malaria parasite, depending on the force of infection and acquisition of immunity, and 76% of deaths on the African continent are assigned to children that age. The parasite species *Plasmodium falciparum* is the most prevalent on the African continent, though it is only one of the five species that can cause malaria in humans. Together with *Plasmodium vivax*[2], which is dominant in the Asia-pacific region and across Central and South America, *P. falciparum* is responsible for the greatest malaria threat to human health. The remaining species include *Plasmodium malariae* and *Plasmodium ovale*, which can sporadically cause severe disease but generally cause mild symptoms. Last, *Plasmodium knowlesi* is associated with severe morbidity and mortality though only in the South-East Asia region. These three *Plasmodium* species are thus comparatively less important for the global malaria burden. Essential for the spread of the malaria parasite, is the *Anopheles* mosquito, with 41 out of 465 formally recognised species that have the capacity to transmit human malaria parasites at a level that is a concern for public health[3].

## The Plasmodium lifecycle

The biology and complex lifecycle of the *P. falciparum* parasite (**Fig. 1**) form the basis of the multifaceted problem described above. The infectious stage of the parasite, the sporozoite, resides in the salivary glands of the *Anopheles* mosquito waiting for the mosquito to find the parasite's future human host. The mosquito uses its proboscis to probe (hence the term) the skin in search for a blood vessel, which can take up to several minutes[4], whilst sporozoites are inoculated. The number of sporozoites injected is highly variable, from very small numbers to hundreds or even thousands of sporozoites by a single probing mosquito[5]. The sporozoite is capable of actively migrating by traversal of cells to glide towards its first stop, a hepatocyte[6]. A sporozoite will often traverse several hepatocytes[7], but once invaded, the sporozoite transforms and multiplies within the hepatocyte, forming tens of thousands of parasites. As merosomes, still covered in host plasma membrane, they emerge from

the infected liver cell and once arrived in the lungs they egress as merozoites[8]. These parasites are released into the bloodstream and will invade erythrocytes to remodel the host cell from the inside out to exploit its nutrients for development[9]. Merozoite invasion commences the asexual replication cycle that will continue through ring, trophozoite and schizont stages forming 16-32 new merozoites. Consequentially, the exponential amplification of infected erythrocytes causes the disease symptoms of malaria such as rigors, headache, nausea, and muscle pains.

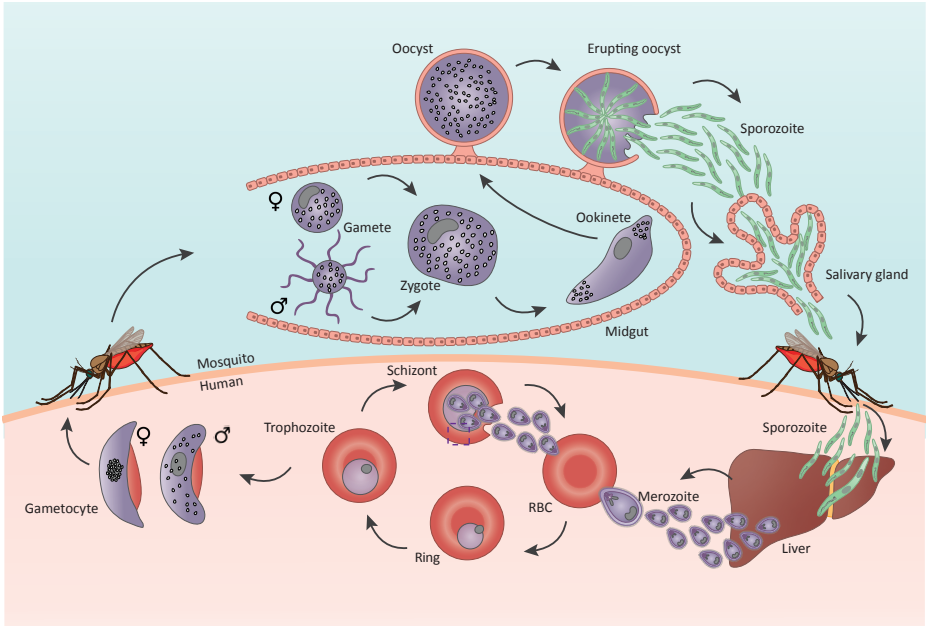



**Figure 1. The *P. falciparum* lifecycle.**

The Plasmodium parasite lifecycle is divided between two hosts, one being the Anopheles mosquito, the other human. The parasite enters the human host as a sporozoite that is deposited into the dermis by a mosquito. Sporozoites travel through the bloodstream to invade a hepatocyte and use this shelter for transformation into merozoites. An outburst of merozoites seeks to invade erythrocytes and commence the asexual replication cycle, including ring, trophozoite and schizont stages. Sexual commitment leads to development into male and female gametocytes that once matured are transferred to the mosquito midgut via a blood meal. An activated male microgamete fertilizes a female gamete to form a zygote, that will further develop into an ookinete capable of crossing the epithelial layer. The parasite settles underneath the midgut basal lamina, forming an oocyst in which sporozoites are formed that after migration to the salivary gland mature and can continue the cycle.

A proportion of parasites will exit the asexual replication cycle and undergo sexual commitment as dictated by both a transcriptional switch[10] and environmental stimuli and will develop into either a female or male gametocyte. A gametocyte

requires circa 10 days to mature and during that time it remains sequestered in the bone marrow, to thereafter emerge into the peripheral circulation as an infectious gametocyte[11]. At this point it is a waiting game; mature gametocytes circulate until a mosquito takes a bloodmeal, the parasite is then transported to its new surroundings, the mosquito midgut. Upon the gametocyte's arrival in the lumen of the mosquito midgut the shift in temperature and presence of the mosquito-derived molecule xanthurenic acid will cause the gametocyte to egress from its erythrocyte exterior[12]. The female and male parasites are now activated and rapidly transform into macrogametes and microgametes with a flagella, respectively. Male microgametes fuse with and fertilize female macrogametes, leading to the formation of zygotes. Several key parasite surface proteins are involved in the fertilization process and will be described below in the section on transmission blocking vaccines. Zygotes develop into elongated ookinetes capable of traversing the midgut epithelial layer and forming oocysts underneath the basal lamina of the midgut. Within the oocyst more than 10,000 sporozoites will form[5]. Rupture of the oocyst releases the sporozoites, allowing the parasite to access the haemolymph, the circulatory system of the mosquito. The sporozoites employ their gliding motility to enter and accumulate in the salivary gland. This malignant parasite is now able to continue its lifecycle when the mosquito bites another human.

## Malaria diagnosis, treatment and prevention

Malaria is traditionally diagnosed by microscopy that can detect all malaria-causing parasites by analysing blood films with Giemsa staining, though this method has limitations in terms of low sensitivity and throughput. The dependence on skilled microscopists was one of the factors leading to the development of rapid diagnostic tests. Rapid (immuno-chromatographic) diagnostic tests detect circulating parasite antigens specific for one or multiple *Plasmodium* species and are responsible for more than 75% of diagnoses[13]. Antigen HRP2 (histidine-rich protein) is most widely used for *P. falciparum* diagnosis, other options include pLDH (parasite lactate dehydrogenase) and aldolase[13].

When a mosquito infected with the malaria parasite bites a human without previous exposure, malaria can develop and cause the first symptoms, such as fever, headache and chills, in general within 8-15 days. Prompt malaria treatment is essential to prevent mild symptoms from deteriorating to severe life-threatening disease. Treatment can also stop the parasite from spreading to mosquitoes and thus other people. Resistance patterns are an important factor in choice of

treatment, as for example resistant parasites to antimalarials including chloroquine and sulfadoxine-pyrimethamine have been observed for decades. The most widely used and highly efficacious antimalarial combination is Artemether-Lumefantrine, consisting of the fast-acting Artemether and the partner drug Lumefantrine for clearing remaining parasites. In the case of severe malaria, antimalarials quinine and artesunate are the drugs of choice[13,14]. The WHO recommends Artemisinin-based combination therapies (ACT) as first- and second-line treatment for uncomplicated *P. falciparum* malaria. The ACT approach combines Artemisinin with its derivatives, including artesunate, dihydro-artemisinin, and artemether[15]. It is important to note that parasites with partial artemisinin resistance have emerged in Africa[16]. Although ACTs currently retain efficacy in African settings, artemisinin resistance forms a threat to anti-malaria progress.

Preventing infection would of course be preferred over curing the disease. This could be achieved by effective chemoprophylaxis that clears parasites as soon as these appear in the bloodstream and thereby prevents blood-stage infection. Chemoprophylaxis is most commonly available for people travelling to malaria endemic regions[17,18]. Chemoprophylaxis is not commonly used in populations living in malaria-endemic regions, as sustained use of chemoprophylaxis is expensive, impractical and has adverse health effects. However, some populations who live in areas of seasonal malaria and are at high risk of malaria receive chemoprophylaxis during the peak of malaria season, which is called seasonal chemoprevention[19,20]. Nevertheless, inhabitants of these endemic regions mostly rely on two core malaria interventions that target mosquitoes, insecticide-treated nets (ITNs) and indoor residual spraying (IRS)[21,22].

Several disruptions have allowed malaria to increase its grip on public health, leading to increased mortality and incidence rates. The COVID-19 pandemic is one example that caused disruption to (health-) services worldwide, and as a result also affected the services to prevent, detect and treat malaria, mainly due to delivery issues. Other challenges for malaria control over the past years include emergence of drug and insecticide resistance, lack of resources, humanitarian crises, problems with program implementation and climate change. These issues are especially detrimental to countries already experiencing a high disease burden. For example, Venezuela experienced a massive rise in malaria cases due to the collapse of their health system and Ethiopia also saw their malaria cases and deaths increase primarily due to conflict. When striving for progress against malaria, it is important to take climate change and its considerable risk into account. However, the impact of climate change on malaria transmission and incidence are difficult to

assess primarily due to sparsity of data, with heated discussions between scientists as a result[23]. Regardless, climate change is widely accepted as an important factor in driving malaria outbreaks in epidemic transmission areas. Large changes in temperature, humidity and rainfall all influence the behaviour and survival of the *Anopheles* mosquito transmitting the malaria parasite. Also, heatwaves, flooding or other extreme weather events can affect the transmission and malaria burden directly by contributing to climates more suitable for mosquito propagation. For example, stagnant bodies of water and disruption of infrastructure (making healthcare inaccessible) due to floods in Pakistan in 2022 led to a five-fold increase in malaria cases in that region[1]. Such catastrophic consequences should only increase the already existing need to act on climate change and it calls for sustainable and durable approaches against malaria.

On a more positive note, there are reasons to be optimistic about the future of the battle against malaria. Already, there are many countries that have a low burden of disease that are now moving towards malaria elimination. In fact, 26 nations reported zero cases of malaria for 3 consecutive years in 2024, when in 2000 these were all endemic. In the last two years, Azerbaijan, Belize, Tajikistan, Cabo Verde and Egypt were declared malaria-free by the World Health Organisation (WHO)[1].

Overall, the fight against malaria continues and there are several challenges, including drug resistance and climate change, that need to be faced to establish progress. Large-scale investment could fuel the development of new efficacious vaccines. The malaria burden in Africa posed by *P. falciparum* infection prevalence has been halved between 2000 and 2015 due to interventions, most primarily insecticide-treated nets[24]. Thus, the addition of other interventions with a leading role for vaccines will most likely be essential to further reduce the grip that malaria has on public health.

## Vaccines

Considering the extensive and intricate malaria lifecycle, it should not come as a surprise that there are a variety of parasite stages, each with different surface proteins expressed, that can be targeted by a malaria vaccine. Vaccines targeting pre-erythrocytic stages aim to prevent infection, while targeting asexual blood stages can prevent disease symptoms and vaccines targeted at the sexual stages aim to prevent spreading of the parasite.

Pre-erythrocytic vaccines are designed to prevent infection by targeting the sporozoite and liver stages of the parasite. This is achieved by the induction of T-cells to target infected hepatocytes or induction of an antibody response to target the sporozoite surface[25]. In 1973 the first report of a protective pre-erythrocytic vaccine was published, showing that radiation attenuated whole sporozoites administered to a volunteer by hundreds of mosquito bites conferred protection to subsequent challenge with *P. falciparum* sporozoites[26,27]. Now, 50 years later, two vaccines are recommended by the WHO and thus marked as safe and effective[28,29]. The pre-erythrocytic vaccine RTS,S/AS01 is based on the most abundant sporozoite surface antigen, the circumsporozoite protein (CSP), and was approved in October 2021 for broad use among children residing in regions with moderate to high *P. falciparum* transmission. RTS,S/AS01 first entered a large phase 3 clinical trial in 2009 in 11 countries in sub-Saharan Africa, with results showing that severe malaria cases were reduced by one-third after three doses and over a 3–4-year period[30]. The WHO decided to recommend a large pilot rollout to obtain better understanding of the vaccine's real-world impact. This pilot was conducted in the form of a Malaria Vaccine Implementation Program in Ghana, Malawi and Kenya in 2019, and RTS,S/AS01 was shown to provide 30% reduction in severe malaria and 20% reduction in hospitalization rates for children aged 5-17 months, as measured after 2 years[31,32]. RTS,S/AS01 has a moderate efficacy rate and is cost-effective[33], but it is questionable whether the vaccine can fill the gap between supply and demand, with only 18 million initial doses available for allocation from 2023 to 2025[34]. Another vaccine, R21/Matrix-M, is based on the same antigen as RTS,S/AS01 and designed to increase anti-CSP antibodies. After a 3-dose regimen and 12-month follow-up R21/matrix-M was found to have circa 75% efficacy against clinical malaria at four different sites, ranging from low to high (seasonal and perennial) transmission and across sub-Saharan Africa[35]. In October 2023, WHO recommended the widespread use of R21/Matrix-M and claimed that the availability of two malaria vaccines makes broad-scale deployment across Africa possible. It is however stressed by experts that the vaccines should be combined with the use of established tools, including rapid diagnostic tests, antimalarial drugs, ITNs and IRS. Although RTS,S/AS01 and R21/Matrix-M may substantially contribute to a reduction in the number of malaria cases and prevention of (severe) morbidity, elimination is not within reach and thus the need for more efficacious vaccines remains.

An important question to ask is: how can the current vaccines or vaccine strategies be improved? Is it useful to add another component to the existing pre-erythrocytic vaccines and could that be a vaccine targeted at a different stage of the parasite? A possibly attractive target for vaccine development is the asexual *Plasmodium*

parasite, the blood stage that causes clinical symptoms. There are several challenges concerning blood stage vaccine development, including the polymorphisms of target antigens, the short timeframe (within minutes) in which the antibodies must bind merozoites passing between erythrocytes, the large number of parasites that need to be targeted and low immunogenicity in humans[25,36]. In the period between 2000-2015 there were 30 blood-stage vaccine trials registered and completed, with results showing little protection against controlled human malaria infection or naturally occurring infection[25]. The most advanced blood-stage vaccine candidate is the conserved *P. falciparum* reticulocyte-binding protein homologue 5 (RH5), which has a critical function during erythrocyte invasion by the merozoite[37]. In a Phase 1b clinical trial the RH5.1/Matrix-M™ vaccine was administered in delayed 3-dose regimen to healthy Tanzanian children, who produced antibodies with growth inhibitory activity of <60%, comparable to levels associated with protection in non-human primates[38]. The first results of an ongoing Phase 2b clinical trial in Burkina Faso (ClinicalTrials.gov: NCT05790889) involving RH5.1/Matrix-M™ were recently published [39]. A comparison was made between a 0,1 and 2 month vaccine regimen and delayed third-dose (0,1 and 5 month) regimen, with efficacies of 40% and 55% respectively[40]. Generally, RH5.1/Matrix-M™ is reported as safe and well-tolerated, with only mild adverse events, which supports its further development as a target for malaria vaccines.

Another category of vaccines, next to pre-erythrocytic and asexual blood stage vaccines, does not target parasite stages residing in the human host, but is directed at the sexual stages that develop in the mosquito midgut, shortly after these parasites have arrived within the mosquito bloodmeal. The next paragraph will elaborate on these so-called transmission blocking vaccines (TBVs) and their potential as vaccination strategy.

## Targeting transmission: making use of the parasite's weak spot

Transmission to mosquitoes occurs via sexual stage parasites, mature male and female gametocytes present in the peripheral circulation, that are transported to the mosquito midgut in a blood meal. During development in the mosquito midgut, *Plasmodium* parasites suffer dramatic losses[41]. One study found that an estimated number of circa 450 *Plasmodium falciparum* gametocytes were detected in patient blood and this number drops to ~13 zygotes and as little as ~2 oocysts in the mosquito[42]. Thus, transmission can be considered a bottleneck within the

*Plasmodium* lifecycle, with very low parasite numbers. It might seem contradicting, but transmission of the *Plasmodium* parasite is also extremely efficient, with an $R_0$ value reaching as high as 3000 for certain endemic settings[43]. The median $R_0$ value is calculated at 115, indicating that every infected individual can transmit the parasite to over 100 other individuals in a naïve population[43]. This illustrates how impactful a transmission intervention could be when striving for reduction of malaria in high transmission areas.

In the mosquito midgut the gametocyte sheds its red blood cell exterior, and the emerged gamete lacks protection by a host cell for roughly 24 hours before zygote formation is finalised[44]. This provides a relatively large timeframe for antibodies to attack compared to merozoites that roam around in a vulnerable state for only several minutes before erythrocyte invasion[45]. Furthermore, the antigens of the sexual stages have lower sequence diversity compared to pre-erythrocytic and blood stages[46]. The sexual stages thus form a major bottleneck in the *Plasmodium* lifecycle (Fig. 1) and are an excellent target for an intervention strategy.

Transmission blocking vaccines (TBVs) are designed to induce human antibodies against the sexual stages of *Plasmodium* that will arrest sporogenic development in the mosquito. The induced antibodies, together with sexual stage parasites, are transferred via the blood meal to the mosquito midgut where they bind their antigen target once the parasite has emerged from the host cell. The human antibodies cause parasite lysis through complement activation or neutralize fertilization and development, and as such prevent oocyst development. By blocking parasite development in the mosquito, the number of infected mosquitoes is reduced, which in turn lowers the number of human malaria infections. Thus, a TBV does not protect the vaccinated individual directly, though the community would reap the benefits of the decreased number of infectious mosquitoes.

TBVs consist of antigens expressed on the surface of gametocytes and the mosquito developmental stages, including gametes, zygotes and ookinetes. There are only a few TBV candidates discovered to date[47]. The first candidates that were discovered (Pfs230, Pfs48/45, Pfs25 and Pfs28) were named after their originally determined molecular weight[48]. Pfs25 and Pfs28 are expressed after activation of gametocytes and fertilization in the mosquito midgut, on the zygote and ookinete surface. Therefore, no antibodies against Pfs25 and Pfs28 are elicited during natural human infection. On the contrary, Pfs230 and Pfs48/45 are both already expressed on gametocytes circulating in the human bloodstream and later in the lifecycle abundantly present on the gamete surface; hence the induced

and naturally acquired antibodies can act after erythrocyte egress and before gamete fertilization[49,50]. Naturally induced antibodies against Pfs230 and Pfs48/45 can only develop when the human immune system has successfully targeted the gametocytes, causing these otherwise hidden antigens to be exposed. Importantly, naturally acquired anti-Pfs230 and Pfs48/45 antibodies have repeatedly been associated with high titres, transmission reducing (TR) immunity[44] and after purification are shown to block transmission[51].

The gold standard in measuring transmission reducing activity (TRA) is the standard membrane feeding assay (SMFA). In an SMFA set-up *in vitro* cultured gametocytes and whole serum or purified antibodies are fed to mosquitoes in a controlled laboratory setting[52]. Mosquitoes are dissected after one week to count the developed oocysts and compare this to a negative control to calculate the reduction in mean oocyst count per mosquito (transmission reducing activity, TRA). When the prevalence of infected mosquitoes is used to measure the percentage of inhibition, the outcome is called transmission-blocking activity (TBA). A similar approach to SMFA, the direct membrane feeding assay (DMFA), uses blood of naturally infected individuals containing gametocytes instead of cultured gametocytes to feed a laboratory colony of local mosquitoes. The blood is fed directly to the mosquitoes, or alternatively, plasma can be used. This plasma can be replaced by naïve plasma, and it is possible to add specific antibodies to assess their transmission reducing activity. In field settings the DMFA offers an important tool to investigate functionality against naturally circulating and genetically diverse *Plasmodium* strains at representative parasite densities in locally relevant mosquito species [53].

Out of the four above mentioned TBV candidates, only Pfs48/45, Pfs230 and Pfs25 managed to enter clinical trials. The latter, Pfs25, is extensively studied and pre-clinical results showed promising transmission-blocking activity [reviewed in[48]. However, subsequent human trials failed to induce a potent and durable TR antibody response[54-56]. The development of the vaccine candidate based on the GPI-anchored Pfs48/45, which forms a complex with Pfs230[57], was hampered due to issues with preparing a properly folded recombinant protein[58]. Progress was made when only one Pfs48/45 domain (6C) was included in a construct for vaccination[59]. Though, a vaccine formulation with the Pfs48/45 6C-domain did not induce sufficiently high levels of antibodies to result in transmission reduction[60,61]. The ProC6C-AlOH/Matrix-M vaccine was shown to induce antibodies with TRA, which are attributed to ProC6C, though only 7/20 samples remained active at day 180 [61]. Finally, the TBV candidate Pfs230 has shown most promising results in humans and is currently tested in a Phase 2 clinical trial (ClinicalTrials.gov: NCT03917654).

The target population for TBVs extends from infancy to adulthood, same as the infectious reservoir for transmission. School-aged children tend to have the highest per person infectivity, though adolescents and adults remain infectious and are thus a contributor to transmission from humans to mosquitoes[62]. Furthermore, individuals with asymptomatic infections are responsible for the largest portion of the human infectious reservoir, and thus important drivers for transmission, as reported by a longitudinal study performed in Uganda[63]. Importantly, TBVs require high vaccine coverage, which can be determined for a specific region by mathematical modelling, to be effective[62]. The fact that a TBV does not confer protection against malaria to the vaccinee itself is one of its disadvantages, since such altruistic vaccination is associated with ethical issues that could decrease the willingness of individuals to receive the vaccine[64]. A TBV depends on an adequate humoral response of the vaccinee's immune system, which could potentially obstruct the TBV effectiveness. If sufficient level of antibodies can be maintained for a sufficient period (preferably more than one transmission season), vaccinees may be protected due to herd immunity in the long term. Herd immunity has been shown to have an important role in preventing the spread of disease[65] and TBVs have been predicted to have an impact on public health[66]. Though, establishing the clinical benefits of TBV herd immunity requires cluster-randomized trials, that are estimated to require an estimated 32 clusters with around 1000 participants per cluster, making this an intricate and expensive endeavour[67].

## Human attractiveness to mosquitoes

It is important to realise there are many factors influencing transmission potential, including the duration of human infectiousness, variation in vector competence among different mosquito populations and the human attractiveness to mosquitoes[68]. The actual mosquito bite and the processes leading up to this event should therefore not be ignored when attempting to block malaria transmission.

Prior to taking the actual blood meal from a human host, a mosquito has completed a complex behavioural trajectory. While resting, a hungry female mosquito becomes activated when she senses the elevated $CO_2$ levels associated with exhaled breath[69]. Upon activation, the mosquito will take flight and navigate a variety of olfactory (smell), visual, and temperature cues to finally arrive on exposed skin. Upon contact, a mosquito may evaluate the skin surface to select an appropriate bite site, to engorge her meal and in the process (potentially) deposit parasites. Several members of the *Anopheles* genus are highly anthropophilic and have an exquisitely developed sense

of smell, enabling them to distinguish humans from other animals[70]. In addition to the human versus non-human distinction, mosquitoes also seem to display a preference for certain humans. While this aspect is familiar to many, the biological basis for differences in mosquito attraction among humans is poorly understood. In regions where mosquito bites are merely a nuisance, differences in mosquito attraction are not a matter of life and death, yet in malaria endemic regions, the amount of mosquito bites a person receives is related to a higher risk of malaria infection.

The factors that drive mosquito attraction among humans have been a topic of scientific and public debate, with a wild variety of explanatory hypotheses ranging from impact of blood type[71-73], to dietary choices such as eating garlic[74] or consuming beer[75] and environmental variables like the phase of the moon[76]. Scientific discourse on the matter also has a rough edge, with many underpowered studies voicing strong claims[71-73]. Recent studies that characterized individuals that consistently and strongly attract mosquitoes identified elevated levels of certain carboxylic acids on the skin of high attractors[77,78]. Furthermore, strong mosquito attraction has been associated with reduced diversity of skin microbes[79]. Yet despite these recent advances, a complete picture of the personal characteristics and behaviours that co-determine the degree to which individuals attract mosquitoes is still lacking.

In Chapter 5, we address the factors that drive how strongly humans attract mosquitoes through an unconventional study design. In the summer of 2023, we set up a field lab at the Netherlands' largest music festival, Lowlands, to include hundreds of participants in the “Mosquito Magnet” study. In this study a custom-built mosquito behaviour assay determined the participants' 'Mosquito Magnet Score' - a metric describing how strongly participants attracted *Anopheles stephensi* mosquitoes. All participants completed a questionnaire on personal characteristics, had their temperature and blood alcohol levels measured, and supplied a skin swab for microbial characterization. Interestingly, study participants showed a wide variety of attraction levels, with some participants not attracting a single mosquito, whereas others elicited sustained attraction of all mosquitoes in the cage. A total of 524 participants volunteered, resulting in, to the best of our knowledge, the largest cohort study of mosquito attraction to date.

In addition to its scientific goals, project Mosquito Magnet had science outreach and dissemination as an equally important goal. The project drew over 500 participants that each were personally guided through the experiment, its motivations and implications, and in-depth discussions regarding the impact mosquitoes and malaria has on societies around the globe.

By providing participants with a 'scientific experience' on a tangible topic, we increased scientific literacy and raised public awareness for the fact that mosquitoes at a festival may be annoying, yet their presence has devastating consequences in other contexts.

It should be noted that the study population at Lowlands festival deviates strongly from the general population of the Netherlands and is even further removed from local populations in endemic malaria settings. Despite these connotations, we robustly identified a variety of personal characteristics and behavioural attributes that correlated with the observed attractiveness of participants, described in more detail in Chapter 5. These correlations provide a lead for further research, which could potentially contribute to strategies aiming to block transmission of malaria parasite. However, TBVs are arguably much closer to implementation, with Pfs230D1 currently involved in Phase 2 clinical trials. Nonetheless, the prospect of in the future implementing multiple anti-transmission strategies is a favourable one.

## Pfs230 at the front of the race

A publication in 1983 by Rener *et al*. for the first time mentions the *Plasmodium falciparum* gamete surface protein we now know as Pfs230[80]. This protein in its native form could be precipitated and measured as 255 kDa due to recognition by two monoclonal antibodies, later characterised as synergistically transmission blocking. Two years later it was Vermeulen *et al*. who published a gamete surface protein with a slightly different molecular weight of 230 kDa[81]. Ironically, the publication mentions that the 230 kDa protein, which coprecipitates with 48 and 45 kDa proteins, likely has no role in transmission blocking, though experiments were performed without active complement. In 1987, Richard Carter, Isabella Quakyi and others working at the National Institute of Allergy and Infectious Diseases (Bethesda, Maryland) showed that Pfs230 was a target of complement-dependent transmission blocking antibodies[82]. It took several years before Williamson and Kaslow reported the sequence of Pfs230 after cloning the Pfs230 gene in *E. coli* [83]. The antigen was immuno-affinity purified with a functional mAb and digested with trypsin to obtain purified peptides. This analysis allowed the design of degenerate nucleotide probes, with which complementary DNA fragments containing an ORF (Open Reading Frame) could be isolated and sequenced. As a result, the authors concluded that Pfs230 is approximately 300 kDa after cleavage of the 360 kDa precursor, though the Pfs230 name remained unchanged.

For the identification of regions that could induce transmission-blocking activity and could therefore be vaccine candidates, Pfs230 was divided into 6 subsections that were

expressed as recombinant proteins fused to Maltose Binding Protein, expressed in *E. coli* and used for mice immunizations[84]. The authors of this publication were the first to induce functional antibodies against recombinant Pfs230, though only against the 76 kDa N-terminal fragment, and showed significantly reduced oocyst development in SMFA. This outcome shifted the focus of TBV research towards the N-terminal domains of Pfs230 in search of functional epitopes. Several studies put forward the first domain of Pfs230 as the main target[85] and in 2016 the first clinical Pfs230 product, the *Pichia Pastoris*-expressed Pfs230D1M, was developed and characterized by Macdonald *et al.*[86]. Fusing Pfs230D1 to ExoProtein A (EPA) was shown to enhance immunogenicity[87]. Phase 1 clinical trial data report 88.9% TRA after the third dose of the Pfs230D1-EPA vaccine in malaria-experienced Malians[88]. Recent results show that within 6 weeks of receiving Pfs230D1-EPA/AS01 there is no reduction of transmission reduction, though over two years the proportion of infected mosquitoes as tested by direct skin feeding (DSF) was 77,8% lower[89]. The Pfs230 research field is currently awaiting results from a completed Phase 2 clinical trial with Pfs230D1M-EPA/AS01B (ClinicalTrials.gov: NCT03917654) and a Phase 1 trial involving Pfs230D1-CRM-197/ Matrix-M (ClinicalTrials.gov: NCT06507605) is still ongoing.

Over the past years there have been several studies investigating recombinant Pfs230 fragments, all concluding that only the Pro domain (upstream of D1) and D1 can induce functional antibodies[90-92]. This led to the hypothesis that only these two N-terminal Pfs230 domains contained functional epitopes. However, an extremely potent anti-Pfs230 monoclonal antibody raised against gametocyte extract did not target the Pro domain or D1[93]. Presumably, the target of this mAb is Pfs230 domain 4, as indirectly shown by studying the impact of non-synonymous SNPs in Pfs230 of clinical isolates on transmission-blocking activity. We did not manage to recombinantly produce Pfs230 domain 4, therefore it is currently not possible to assess Pfs230D4 as vaccine candidate. This interesting finding did direct our research towards a monoclonal antibody that targets a domain of Pfs230 outside of the ProD1 region. We determined the target of this non-functional mAb raised against native Pfs230 as Domain 7, as described in Chapter 3. This chapter highlights the importance of complement for functional antibodies against Pfs230 and shows how this non-functional mAb was converted to be potently transmission blocking. Inspired by this direct evidence that Pfs230 might comprise more functional epitopes, we produced recombinant fragments of Pfs230 in an expression system not used previously for this antigen and tested the fragments in a mice immunization study. In Chapter 4, we provide the results of this study, which puts forward Pfs230 domain 12 as a new TBV candidate. Together, our research shows that regardless of large efforts searching for Pfs230 epitopes, it is important

to follow scientific leads, even if small, since they might result in important new assets for TBV research.

The Pfs230 structure is vital for the subunit design for Pfs230 immunogens, and it was the above-mentioned Richard Carter who made an important contribution to the Pfs230 domain architecture. Carter *et al*. presumed that Pfs230 consisted of 14 domains with an even number of cysteines (2, 4 or 6) forming disulfide bonds[94], which was ultimately confirmed by cryogenic electron microscopy of the Pfs230-Pfs48/45 complex[95,96]. Pfs230 disruptant male parasites can successfully emerge from the erythrocyte exterior, exflaggelate and become microgametes, though they are incapable of forming foci of fertilization with adjacent red blood cells[82,97]. The reduction in fertilization was however not complete; oocyst formation was 92–96% inhibited, causing researchers to question the essentiality of Pfs230 for transmission. So, is disrupting the function of Pfs230 important for functionality of anti-Pfs230 antibodies? Interestingly, almost all functional antibodies against Pfs230 act dependent on human complement activity[86,98-102], indicating an important role for complement in a Pfs230 based TBV.

## Contemplating complement

The complement system is part of the human innate immune system, the fast line of defence, which protects the human body from invaders in case they managed to cross the skin and epithelial layers[103]. The complement system consists of precursor serum proteins that can be activated upon recognition of infectious organisms, damaged tissue or surfaces identified as foreign. The initiation of the complement cascade occurs through three different pathways: the alternative pathway, the lectin pathway and the classical pathway (Chapter 2, Figure 2). Each of these pathways culminates in the formation of convertases on the pathogen surface that, as the name suggests, convert other complement proteins that are then deposited on the surface. This cascade of proteolytic events generates the major effector mechanisms of the complement system; opsonization (and consequently phagocytosis), pathogen lysis by the membrane attack complex (MAC) and the production of pro-inflammatory anaphylatoxins. The MAC is a terminal assembly of complement components forming ring-structured pores that insert into the membrane of gram-negative bacteria, enveloped viruses, and parasites. This causes the influx of calcium ions and activates lytic signals, leading to cell death[104].

While the end of the pathways is thus identical, the initiation of the pathways by recognizing invading pathogens differs substantially. Activation of the lectin pathway occurs via pattern recognition proteins that encounter conserved pathogenic carbohydrate motifs. The classical pathway is kicked-off through a pentameric or hexameric antigen-antibody complex that is formed on the pathogen surface, facilitating the C1q protein to latch on to the antibody complex[105]. IgM can readily activate the classical pathway due to its multimeric structure, while IgG molecules can form hexamers to recruit C1q and activate complement. The alternative pathway commences through spontaneous hydrolysis of complement proteins that results in convertase formation. This process can occur on all surfaces, including host cells if not for the protection provided by complement regulators present on host cells that negatively impact the alternative pathway. Complement can effectively clear pathogens, including parasites, from the human body but most pathogens have evolved to escape complement attack[106,107]. The *Plasmodium* parasite is also confronted with complement upon its encounters with human blood throughout the *Plasmodium* lifecycle. Therefore, cunning as it is, *P. falciparum* has evolved to escape complement attack. Chapter 2 dives deeper into this topic of how *Plasmodium falciparum* can complete its lifecycle through the human and mosquito host without being halted by complement and how complement could be used to develop more efficacious vaccines and antibodies against malaria.

## Objectives and outline of this thesis

There is a long road ahead of us before reaching the goal of malaria eradication. While interrupting *Plasmodium* transmission with a TBV could be an important tool for elimination and eradication, TBVs are still far removed from large scale implementation. There are only a handful of TBV candidates identified so far, and even the most advanced candidate (Pfs230D1) has not yet successfully passed all phases of clinical trials. More efficacious TBV candidates are thus needed. With the content of this thesis, we add new Pfs230 TBV candidates to the current list, which we hope will help TBV research and development move forward. Furthermore, in this thesis we highlight the intricate relationship between complement and the *Plasmodium* parasite, which could provide valuable insights for development of anti-malaria vaccines and therapeutics.

Throughout the *Plasmodium* lifecycle the parasite encounters the human and mosquito complement systems. These complement systems have the potential to pose a serious threat to the *Plasmodium* parasite. However, several recent studies revealed the evasion mechanisms the *P. falciparum* employs for its survival. In

**Chapter 2: Deceiving and escaping complement – the evasive journey of the malaria parasite,** we give an in-depth overview of the evasion mechanisms known and speculate on the existence of even more undiscovered mechanisms. Furthermore, the efficacy of the RTS,S vaccine might rely (partly) on complement activation as an effector mechanism, on which we elaborate in this comprehensive review.

Human complement activation is essential for the activity of anti-Pfs230 antibodies. This is clearly illustrated in **Chapter 3: Pfs230 Domain 7 is targeted by a potent malaria transmission-blocking monoclonal antibody.** We generated a subclass-switched complement-fixing variant of an antibody targeting Domain 7 of Pfs230. The antibody showed no functionality before the switch, but the subclass-switched monoclonal antibody potently reduced *P. falciparum* infection in *Anopheles* mosquitoes. We demonstrate the importance of complement for anti-Pfs230 transmission-blocking vaccines and identify Pfs230 Domain 7 as a new target for transmission-blocking antibodies.

Finding new targets for transmission-blocking antibodies is crucial for increasing the list of TBV candidates. We therefore produced eight single Pfs230 domains in *Drosophila melanogaster* to assess their immunogenicity in mice. In **Chapter 4: Pfs230 Domain 12 is a potent malaria transmission-blocking vaccine candidate**, we describe that mice antibodies induced by Pfs230 Domain 12 show strong TRA. The D12-specific antibodies reduced mosquito transmission of cultured parasites as well as parasites acquired from naturally infected gametocyte carriers from Burkina Faso. Sera from individuals naturally exposed to *P. falciparum* recognized Pfs230 Domain 12 and showed increased antibody levels with age. The results put Pfs230 Domain 12 forward as a potent new TBV candidate.

When attempting to block malaria parasite transmission, it essential to consider the role of the human attractiveness to the mosquito. In **Chapter 5: Blood, sweat, and beers: investigating mosquito biting preferences amidst noise and intoxication in a cross-sectional cohort study at a large music festival** we discuss the results of the large-scale Mosquito Magnet Trial, where we researched the factors influencing attractiveness of festivalgoers to *Anopheles stephensi*. We show that hedonist traits such as drinking beer and sharing your sleeping accommodation can attract more mosquitoes, whilst using sunscreen keeps mosquitoes at a distance.

Finally, all the above findings, including implications and future prospectives, are extensively discussed in **Chapter 6: General discussion – Complementing the fight against malaria**.

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Chapter 2

# Deceiving and Escaping Complement - The Evasive Journey of the Malaria Parasite

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## Abstract

During its lifecycle, the malaria parasite is exposed to the human and mosquito complement systems. Early experiments demonstrated that activation of complement can pose a serious threat to parasites, but recent studies revealed complement evasion mechanisms by malaria parasites that are important for parasite survival. Blood stage parasites and gametes recruit regulators to neutralize human complement activation, while ookinetes inhibit mosquito complement activation by disrupting midgut epithelial nitration in response to invasion. Here we provide an in-depth overview of the evasion mechanisms currently known and speculate on the existence of yet unidentified ones. Finally, we discuss how these mechanisms could provide novel targets for urgently needed malaria vaccines and therapeutics.

# Malaria parasites know their way around complement

Malaria continues to pose a major global health burden, with an estimated 241 million cases and 627 thousand deaths in 2020 alone [1]. Despite extensive malaria control and elimination efforts, the decline in disease incidence has recently stalled, calling for novel control tools and strategies. Human malarias are caused by *Plasmodium* parasites that are transmitted by female *Anopheles* mosquitoes. *Plasmodium falciparum* infection results in the most virulent form of malaria in humans and is responsible for most of the mortality, mainly in young African children. *Plasmodium* parasites encounter the human (**Box 1**) and the mosquito (**Box 2**) complement systems as they complete their developmental cycle (**Figure 1**).

The human complement system consists of serum proteins that can recognize pathogens and initiate a proteolytic cascade that leads to pathogen elimination (**Box 1**). Several *Plasmodium* life stages are exposed to human serum and are therefore vulnerable to the complement system (**Figure 1**). Early research has suggested that neutralizing complement is important for survival, as parasites can be killed when exposed to serum from animal species that are not susceptible to infection [2,3]. More recently, several **complement evasion** (see **Glossary**) mechanisms employed by malaria parasites have been discovered and demonstrated to be important for parasite survival. Furthermore, malaria parasites also encounter the mosquito complement system as they develop in the vector and evasion of this defence system appears to be essential for parasite survival and **transmission** [4,5].

In recent years, we have gained new insight into the fascinating interactions of the malaria parasite with both the human and the mosquito complement systems. It has become clear that evasion of these innate immune responses is important for parasite survival, and neutralization mechanisms may therefore provide urgently needed novel targets for malaria vaccines and therapeutics. Recent advances in the field fueled the need for a review. Here, we provide a comprehensive overview of the parasite's complement evasion mechanisms, discuss current knowledge gaps and outline opportunities for vaccine and therapeutics development.

## Human complement activation pathways and evasion by pathogens (Box 1)

The human complement system consists of more than sixty proteins and activation products, and forms a first line of defence against invading pathogenic bacteria, viruses and parasites [87-89]. Complement activation is generally initiated by recognition of antibody complexes (Classical pathway/CP), carbohydrates (Lectin pathway/LP) or spontaneous tick-over (Alternative pathway/AP) (Figure I). The three pathways converge at the formation of C3 convertases. C3 convertases cleave additional C3, producing membrane-bound C3b and soluble C3a. When high concentrations of C3b are reached, C5 convertases are formed that cleave C5 into C5b and C5a. C5b initiates the assembly of the membrane attack complex (MAC), also referred to as C5b-9.

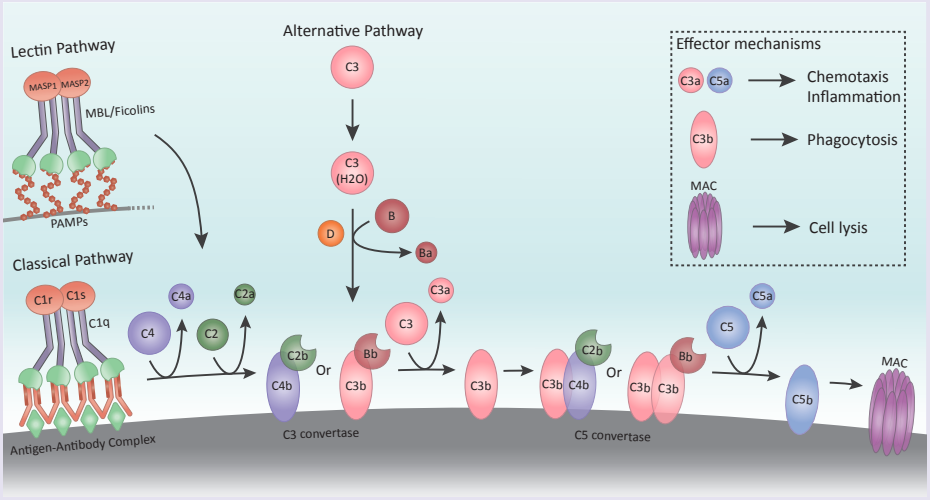


**Figure I. Activation of human complement pathways.**

Initial activation of complement by recognition of antibody complexes (Classical pathway, CP), carbohydrates (Lectin pathway, LP) or spontaneous tick-over (Alternative pathway, AP) leads to the formation of the C3 convertases C4bC2b (formerly known as C4b2a) and C3bBb respectively. C3 convertases cleave C3, causing deposition of covalently bound C3b. The accumulation of surface-bound C3b leads to phagocytosis and also initiates the assembly of the C5 convertase, which in turn leads to the proteolysis of C5. The anaphylatoxins C5a and, to a lesser extent, C3a cause chemotaxis and phagocytosis. C5b attaches to the surface and initiates the formation of the C5b, C6, C7, C8 and C9 containing membrane-attachment-complex (MAC) responsible for cell lysis.

Activated complement proteins lead to different effector mechanisms; C3a and C5a induce chemotaxis of immune cells and inflammation, deposited C3b mediates phagocytosis and MAC formation leads to pathogen lysis. To prevent detrimental effects on human cells, complement activation is tightly regulated by specific

membrane proteins and recruitment of soluble serum proteins [87]. Soluble regulators include factor H which negatively regulates the cleavage of C3 via AP, C4 binding protein (C4BP) which accelerates decay of C3 convertases formed via CP and LP, C1-inhibitor (C1-INH) which inactivates C1r, C1s, MASP1 and MASP2, and vitronectin which prevents MAC formation. Properdin is the only positive regulator described to date, and stabilizes the C3 convertase of the AP.

To prevent damage by complement, human pathogens have developed four general mechanisms of complement evasion [8,90]. These include (I) recruitment of complement regulators, (II) inhibition of complement activation by direct interactions with complement proteins, (III) inactivation of complement by enzymatic degradation and (iv) passive evasion mechanisms such as complement-insensitive cell walls of gram-positive bacteria.

**Mosquito complement activation (Box 2)**

In vertebrates, thioester-containing proteins (TEPs) include α2-macroglobulins and the complement proteins C3/C4/C5, which bind to the surface of pathogens and trigger phagocytosis or cell lysis [91]. A family of 19 TEPs have been identified in the genome of *Anopheles gambiae*, a major mosquito vector of malaria in Africa [92]. *A. gambiae* TEP1 shares some sequence homology and a similar structural organization with the mammalian complement factor C3 [93,94]. It binds to the surface of bacteria, labelling them for phagocytosis by mosquito hemocytes [95], and also binds to the surface of ookinetes as they emerge from the mosquito midgut, forming a complex that ultimately lyses the parasite [36,96].

TEP1 is present in two forms in the mosquito hemolymph, a long full-length inactive form and a shorter, proteolytically processed form ($TEP1_{cut}$) that is stabilized by forming a complex with two leucine-rich repeat (LRR) proteins, LRIM1 (leucine-rich repeat immune protein 1) and APL1 (Anopheles *Plasmodium*-responsive leucine-rich repeat 1) [93,97]. Silencing either LRIM1 or APL1 results in loss of TEP1 function by premature activation and TEP1 deposition in mosquito tissues (Figure II) [93,97].

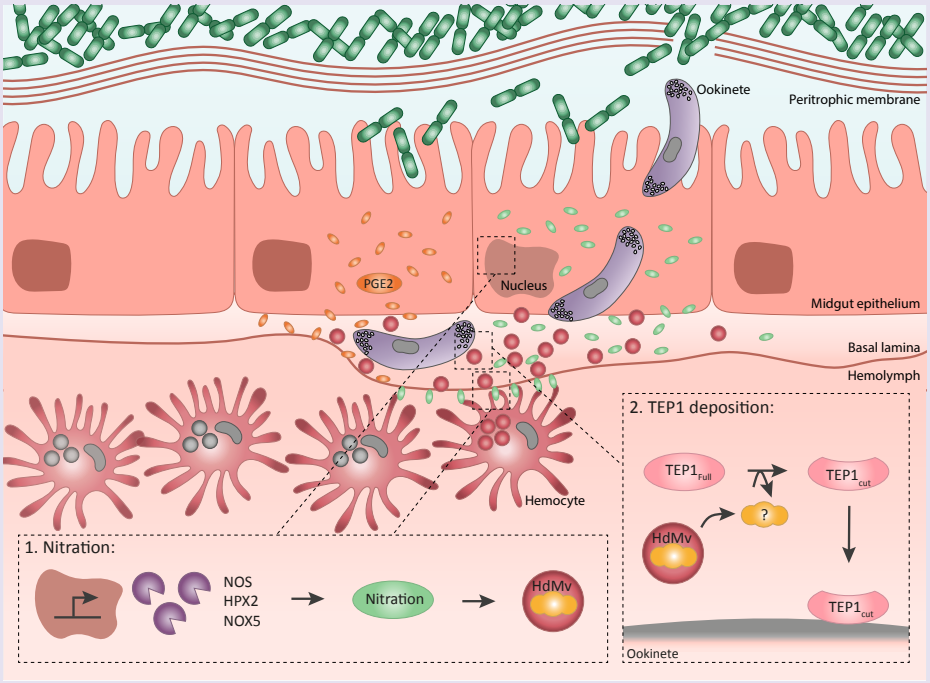


**Figure II. Current working model of mosquito complement activation in response to *Plasmodium* midgut invasion.**

Ookinetes disrupt the peritrophic matrix as they traverse the mosquito midgut, allowing direct contact of the microbiota with midgut epithelial cells. Ookinetes also cause irreversible damage to the invaded cells and induce expression of nitric oxide synthase (NOS), followed by JNK-mediated induction of heme peroxidase 2 (HPX2) and nicotinamide adenine dinucleotide phosphate oxidase 5 (NOX5) expression (inset 1). These enzymes nitrate the damaged cells and the basal lamina as the cell undergoes apoptosis. When midgut cells detect bacterial immune elicitors, they release prostaglandin E2 (PGE2), which attracts hemocytes to the invasion site. Hemocytes patrol the basal surface of the midgut and undergo apoptosis if they come in contact with a nitrated midgut basal lamina, releasing hemocyte-derived microvesicles (HdMv). HdMv release is necessary for propagation of TEP1 binding and activation on the ookinete surface [35]. Presumably, HdMv release promotes activation of the mosquito complement system by delivering a factor(s), not yet known, required for effective local activation of TEP1 (inset 2).

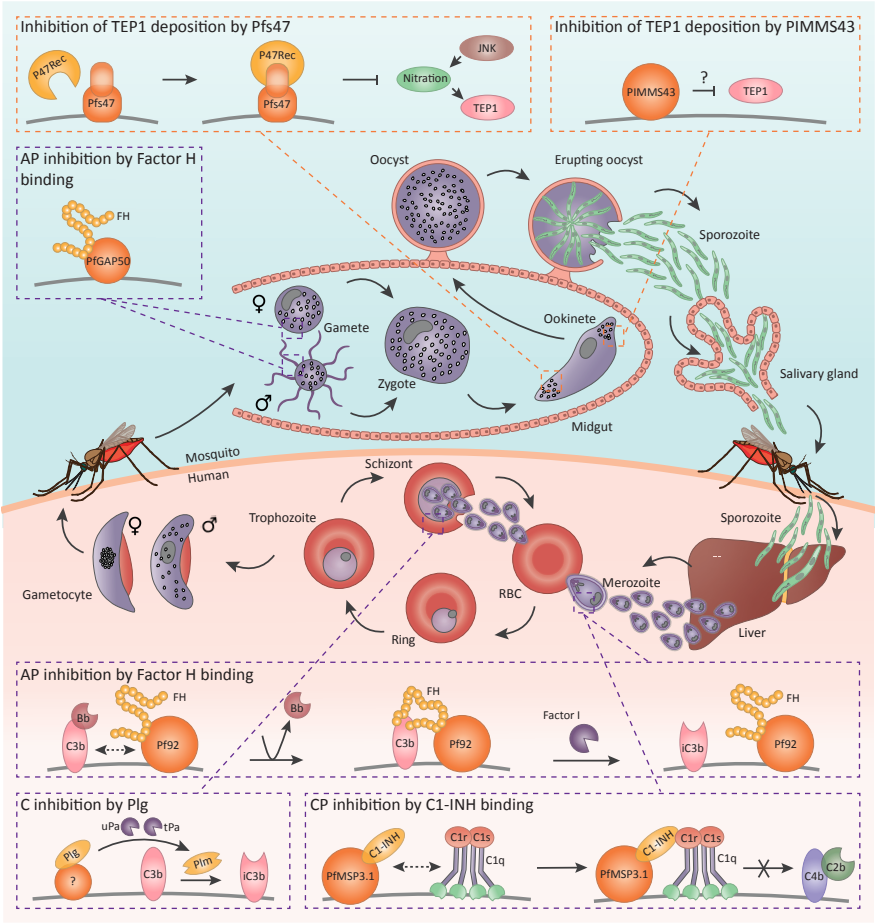


**Figure 1. Human and mosquito complement evasion by *Plasmodium* parasites and Anopheline mosquitoes.**

The *P. falciparum* lifecycle is depicted in the centre. Human and mosquito complement evasion mechanisms are shown in purple and orange dashed line boxes, respectively. Merozoites inhibit complement by recruiting Factor H (FH) via surface protein Pf92 and C1-Inhibitor (C1-INH) via PfMSP3.1; FH negatively regulates C3 convertase formation by promoting Factor I-mediated cleavage of C3b to inactive C3b (iC3b) and C1-INH recruitment prevents C4bC2b convertase formation by inhibiting proteolytic activity of serine protease C1r. Infected RBCs of the schizont stage inhibit complement by recruiting plasminogen (Plg); the urokinase-type and tissue-type plasminogen activators (uPA and tPA, respectively) mediate the conversion of Plg to plasmin (Plm), thereby promoting C3b inactivation. Gametes recruit FH to inhibit the AP of human complement inside the mosquito midgut. The *P. falciparum* surface protein Pfs47 interacts with a mosquito midgut receptor (P47Rec) and inhibits JNK-mediated epithelial nitration in ookinete-invaded cells, rendering mosquito complement ineffective. Ookinetes also employ PIMMS43 to evade mosquito complement when traversing the midgut.

# Human complement evasion by different stages of malaria parasites

While malaria parasites spend most of their life inside host cells, three extracellular forms are exposed to human complement: **sporozoites**, **merozoites** and **gametes**. Sporozoites are deposited in the skin by a mosquito bite, reach the blood stream through gliding and cell traversal and are transported to the liver where they invade hepatocytes, a journey can take several hours [6]. After transformation and replication inside hepatocytes, merozoites egress and start asexual replication inside RBCs. At the end of each replication cycle, merozoites are released from infected RBCs (iRBCs), and take several minutes to invade new RBCs [7]. During asexual replication, some parasites commit to become gametocytes, the sexual stage of the parasite. Gametocytes are the transmissible form of the parasite that, when taken up by a female mosquito via a blood meal, activate into gametes. Gametes egress from RBCs inside the lumen of the mosquito midgut and are exposed to human complement that is also present in the blood meal [8,9]. Research has shown that sporozoites, merozoites and gametes, as well as iRBCs are prone to complement-mediated damage and/or have adopted evasion mechanisms described below (**Figure 1**).

## Sporozoites

Recent research indicates that the antibody-mediated **classical pathway (CP)** of complement activation can initiate an attack on *P. falciparum* sporozoites. Kurtovic *et al*. showed that naturally acquired antibodies against circumsporozoite protein (CSP) can fix C1q on the sporozoite surface and induce sporozoite death [10]. A follow-up study underlined those findings by showing that immunizations with whole sporozoites elicit antibodies that can damage sporozoites by CP activation and thus, inhibit sporozoite invasion of hepatocytes [11]. Furthermore, this study showed that C3 deposition and sporozoite death also occur with pre-immunization sera, pointing to activation of the **alternative pathway (AP)** of complement on the sporozoite surface [11]. Interestingly, inactivated C3b (iC3b) was also detected by western blot [10], suggesting that sporozoites actively neutralize AP activation. More evidence for complement evasion by sporozoites comes from a study with the murine malaria parasite *P. berghei*. Kawamoto *et al*. showed that the AP of human complement is activated on the surface of *P. berghei* sporozoites, resulting in C3 deposition and a decrease in hepatocyte infectivity [3]. Interestingly, C3 deposition was not observed on *P. berghei* sporozoites incubated with mouse serum, but was observed on *P. berghei* sporozoites that had been treated with trypsin [3]. Altogether, these studies provide indirect evidence that sporozoites may have evolved strategies to evade the fast-acting vertebrate complement system, especially the AP, but the molecular mechanisms underlying

evasion remain unidentified. One such mechanism could be the inactivation of C3b by Plasmin (Plm) as a recent study found that *P. falciparum* and *P. berghei* sporozoites recruit Plasminogen (Plg), and its activators urokinase-type (uPA) and tissue-type (tPA) that convert Plg into Plasmin (Plm) [12]. While this study found that Plm formation enhanced sporozoite motility in the skin and sporozoite liver infection, likely through Plm-mediated degradation of extracellular matrices, it did not assess whether Plm impacted complement activation on sporozoite surfaces [12].

## Asexual blood stages

One of the most common AP evasion mechanisms utilized by pathogens, the recruitment of **complement regulator** Factor H (FH) [13], has also been observed for *P. falciparum* blood stages [14,15]. FH and its splice variant Factor H like protein 1 (FHL-1) are known to play an important role in complement inhibition by downregulating the AP [16]. The human FH protein family also contains five FH-related proteins (FHR1-5) that lack complement regulatory domains and are increasingly considered as deregulators, i.e. enhancers, of complement activation on pathogen surfaces by competing with FH and FHL-1 recruitment [16]. Both schizont infected red blood cells (iRBCs) and merozoites bind FH to their surface [14,15,17]. Merozoites bind the complement control protein (CCP) domains 5-6 of FH via the glycosylphosphatidylinositol (GPI)-anchored merozoite surface protein Pf92 [15], but it is unclear how iRBCs bind FH [14]. Importantly, merozoite surface bound FH retains its ability to serve as cofactor for factor I, mediating C3b cleavage into iC3b [15]. *Pf92* deletion causes increased complement-mediated destruction of merozoites, but *in vitro* parasite growth is only marginally affected, suggesting that other evasion mechanisms may exist [15]. FH binding to iRBCs mediates C3b inactivation, increases *in vitro* growth and reduces lysis [14]. Further research found that FHR-1 also binds to the surface of merozoites and iRBCs, and promotes complement activation *in vitro* by competing with FH binding [18].

Recruitment of human C1-inhibitor (C1-INH) is another well-known pathogen evasion mechanism [19] used by merozoites [20]. C1-INH binds to the complement C1s protease and arrests CP activation before the C4bC2b convertase is formed. First, it was reported that purified C1-INH binds the surface of merozoites via the glycan group of *P. falciparum* glycosylphosphatidylinositol (GPI) molecules that mediate membrane anchoring of certain proteins [21]. This study showed that C1-INH binding to merozoites reduced RBC invasion but did not assess whether C1-INH recruitment impacted complement activation on the merozoite surface [21]. A later study showed that C1-INH binds to the merozoite surface protein *Pf*MSP3.1, which does not contain a GPI anchor, *in vitro*. *Pf*MSP3.1 knockout merozoites fail to recruit C1-INH from normal

human serum, suggesting that C1-INH recruitment mainly occurs through *Pf*MSP3.1 rather than GPI molecules [20]. Importantly, surface bound C1-INH retained its ability to form protease inhibitor complexes that prevent C4bC2b convertase formation [20]. Concurrently, the *Pf*MSP3.1 knockout strain showed increased **complement deposition** and, somewhat surprisingly, enhanced erythrocyte invasion [20].

The mechanism of complement evasion by merozoites and how this affects their ability to successfully invade erythrocytes has been controversial. One working hypothesis stems from a study showing that naturally acquired human antibodies against merozoite surface proteins MSP1 and MSP2 enhance complement deposition on the merozoite surface by fixing C1q, and this results in reduced RBC invasion [22]. An alternative hypothesis proposes that deposition of the complement molecules C3 and iC3b on the merozoite surface enhances binding to complement receptor 1 on RBCs, explaining the increase in RBC invasion that is for instance observed for the *Pf*MSP3.1 knockout strain [20,23-25]. The discrepancy may be due to differences in the experimental set-ups used, and it is unclear whether C3 deposition is beneficial or detrimental for *in vivo* merozoite RBC invasion and proliferation [26].

Intracellular parasites can also be damaged by complement activation on the iRBC membrane. Recent studies revealed that *Plasmodium*-infected RBCs evade complement not only by recruiting FH [14,15], but also Plg [27] to the surface of iRBCs. iRBCs showed significantly higher levels of surface-bound Plg compared to non-infected RBCs, and it remains to be determined whether this increased binding is mediated by *Plasmodium* proteins exported to the surface of iRBCs or whether proteins from the RBC itself are also involved. Plm formation on iRBCs occurred in the absence of its typical activators tPA and uPA, but the addition of tPA greatly enhanced this conversion. Importantly, Plg recruitment leads to reduced MAC formation on schizont-infected RBC membranes, and promotes parasite growth [27].

*Plasmodium* parasites have also developed another method to evade complement attack by the CP, illustrated by a study with antibodies against the well-characterized erythrocyte membrane protein 1 (PfEMP1) [28]. Antibodies against PfEMP1 could activate complement in an ELISA assay with recombinant PfEMP1, but failed to fix C1q on the iRBC surface. The authors speculated that this could be due to the focal display of PfEMP1 in knobs, preventing antibody hexamerization that is required for C1q fixation [28]. Whether complement evasion by spatial organization of cell surface proteins is unique to PfEMP1 or also applies to other *Plasmodium* proteins requires further investigation.

Human complement is not only primed to kill *Plasmodium* parasites directly through lysis, but it can also target them for destruction through other mechanisms. *Plasmodium* parasites avoid splenic clearance by cytoadhesion of iRBCs to the endothelial cells in the microvasculature, an interaction that is mediated by PfEMP1 [29]. Interestingly, the complement protease C1s prevents cytoadherence of iRBCs *in vitro* by cleavage of PfEMP1, and it was hypothesized that this would promote iRBCs clearance by the spleen [30]. However, only non-adhered iRBCs are affected by C1s cleavage, as cells that have already adhered cannot be dislodged [30], illustrating the intricate interactions between the parasite, endothelial cells and the complement system. It is not clear to what extent this mechanism affects survival of *P. falciparum in vivo*. Altogether, the effect of the different complement evasion strategies on RBC invasion and parasite growth *in vivo* remains to be established.

### Gametes

The first evidence that (extracellular) sexual stage parasites are prone to complement attack came from studies with avian *P. gallinaceum* zygotes. These were lysed by AP complement from non-avian species, while zygote surface proteins prevented lysis by chicken complement and enabled transmission to mosquitoes [31,32]. Currently, there is one evasion mechanism known to be employed by female gametes; the recruitment of FH (and FHL-1) on the gamete's surface [9]. During gametocyte activation, the glideosome-associated protein PfGAP50 relocates from the inner membrane complex to the surface of the activated gamete, where it binds FH via its CCP5-7 domains [9]. A monoclonal antibody against FH fully blocked development of *P. falciparum* oocysts in mosquitoes. Polyclonal antibodies against PfGAP50 reduced FH binding to gametes by approximately 60%, and concurrently reduced the formation of oocysts, but not completely, suggesting that other surface proteins may also recruit FH [9]. Further research is required to identify these protein(s). A recent study discovered that gametes, as well as zygotes and ookinetes, bind Plg and its activator(s) to generate Plm and that inhibition of Plm led to reduced ookinete formation [12]. The authors attributed this finding to Plm's ability to degrade fibrinogen and fibrin present in the blood meal, but the effect on complement activation on parasite surfaces was not assessed.

## Mosquito complement evasion by ookinetes

*Plasmodium* parasites develop into motile ookinetes in the gut lumen that traverse the mosquito midgut and develop into oocysts [33]. Mosquito defence responses that target the ookinete stage require sequential activation of midgut epithelial **nitration** [34], local release of hemocyte-derived microvesicles [35], and activation

of the mosquito complement system [36] (**Box 2**). Midgut traversal by *P. berghei* ookinetes causes irreversible damage to the invaded cells that triggers a strong nitration response [37-39]. If patrolling hemocytes come in contact with a nitrated midgut basal lamina (**Box 2**), they undergo apoptosis and release microvesicles that promote activation of the thioester-containing protein 1 (TEP1), a key effector of mosquito complement [34-36,40]. It is estimated that about 80% of *P. berghei* ookinetes are eliminated by TEP1 as they come in contact with mosquito hemolymph [36].

Given the high efficiency of TEP1-mediated *A. gambiae* elimination of *P. berghei* ookinetes, it came as a surprise that this response was not effective when this African vector was infected with several *P. falciparum* parasite lines of African origin [41-43]. Classic quantitative trait locus (QTL) analysis and linkage group selection identified *Pfs47* as the gene that allows some *P. falciparum* strains to evade the mosquito complement system [44]. Pfs47 is a GPI-anchored protein present on the surface of *Plasmodium* gametes, zygotes and ookinetes that belongs to the 6-Cysteine protein family [45,46]. Disruption of the *Pfs47* gene results in TEP1-mediated *P. falciparum* elimination, and this phenotype can be rescued by genetic complementation or by silencing TEP1 expression [44]. *P. falciparum* parasites that lack Pfs47 trigger an epithelial nitration response very similar to that of *P. berghei* parasites [37,44].

Pfs47 has several haplotypes, with strong geographic structure at the continental level [47-49]. Previous studies revealed a molecular language of compatibility, and based on these observations, the "lock-and-key theory" was proposed [47]. According to this model, Pfs47 serves as a "key" that interacts with a specific mosquito receptor, i.e. "the lock", to escape mosquito complement. There are different haplotypes of Pfs47, and local vectors constantly select for parasites with a compatible version of Pfs47 that enables them to become "invisible" to the mosquito immune system and hence survive and be transmitted [47]. The putative Pfs47 mosquito receptor (Pfs47Rec) has been identified as a novel mosquito midgut protein of unknown function with clear orthologs in all anopheline species [5]. How the interaction of *Plasmodium* Pfs47 with the mosquito Pfs47Rec disrupts epithelial apoptosis and prevents nitration is currently under investigation.

Disruption of a second surface protein, *Plasmodium* Infection of the Mosquito Midgut Screen 43 (PIMMS43), expressed in ookinetes and sporozoites, results in complete elimination by the mosquito immune system [4]. Silencing key effectors of mosquito complement rescues oocyst formation of the PIMMS43 knockout, but the oocysts that form are small and contain only a few non-viable sporozoites [4]. This indicates that, besides immune evasion, PIMMS43 is also required for normal

oocysts and sporozoite development. This is in contrast with Pfs47, which only seems to play a role in immune evasion, as disruption of mosquito complement rescues Pfs47 knockout parasites and results in large oocysts with sporozoites of normal appearance [42]. Furthermore, although both Pfs47 and PfPIMMS43 exhibit significant geographic structure between different regions in Africa, their fixation index (FST) profiles do not fully overlap [4,47], suggesting that different selection pressures are exerted on these two genes. Taken together, these observations indicate that these two proteins serve different functions.

*Plasmodium* traversal of the mosquito midgut is fast, as it takes *P. berghei* ookinetes about 90 seconds to cross a cell once invasion is initiated, while maximal epithelial nitration takes a few hours [50]. Thus, effective activation of TEP1-mediated responses requires a coordinated response that involves cooperation between epithelial, cellular and humoral components of the mosquito immune system [48]. *P. falciparum* transmission appears to be highly effective, at least in part, because the parasite has evolved a strategy to avoid detection by the mosquito defense system. These observations beg the question of whether epithelial nitration and local microvesicle release by immune cells may also be important local mediators of vertebrate complement activation in response to epithelial damage. Interestingly, a recent study showed that extracellular vesicles activate the classical complement pathway and damage retinal endothelial cells by MAC deposition [51].

## Do *Plasmodium* parasites benefit from human complement evasion by anopheline mosquitoes?

As human complement in the blood meal is still active in the mosquito midgut [9], it is not surprising that mosquitoes have evolved strategies to inhibit complement activation. *Anopheles stephensi* and *Anopheles gambiae* recruit FH to the midgut wall [8] while saliva of some other anopheline species contains a small protein called Albicin that inhibits the AP [52,53]. It is clear that the ability of *Anopheles* mosquitoes to block human complement enhances mosquito fitness, and thus benefits malaria parasite transmission. However, it is unclear whether parasites, in particular sporozoites and gametes, also benefit directly from these anopheline complement evasion mechanisms, for instance through Albicin recruitment. Hijacking of vector proteins has been described for *Borrelia burgdorferi*, the agent of Lyme disease that is transmitted by ticks, which escapes complement-mediated killing by recruiting a tick salivary protein to its surface [54,55]. Further research should determine the importance of mosquito complement inhibitors for malaria parasite survival and malaria transmission.

# Employing complement to target malaria parasites

## Vaccines

Complement activation/evasion is highly relevant when developing malaria vaccines because the efficacy may depend either on eliciting antibodies that fix complement or on targeting proteins that mediate complement evasion. The RTS,S vaccine, which targets the abundant CSP on the surface of sporozoites, is the only malaria vaccine approved to date, but only confers partial protection [56]. The efficacy of this vaccine has been demonstrated through many clinical trials, and recent studies revealed that complement fixing antibodies elicited by this vaccine may play an important role in protection. Naturally acquired complement-fixing antibodies against CSP were associated with protective immunity in longitudinal cohort studies with children from malaria-exposed populations [10]. More recently, it was shown that antibodies induced by RTS,S are able to fix complement proteins, including C1q, and can mediate opsonization [57,58]. Participants in the phase 1/2a challenge trial that reached higher levels of C1q-fixing antibodies against CSP seemed to have a lower probability of developing parasitemia after controlled human malaria infection, although the observed difference was not significant [58]. Both naturally acquired and RTS,S-induced anti-CSP antibodies are mainly of the complement fixing subclasses IgG1, IgG3 and IgM [10,57,59]. RTS,S-induced IgG levels correlate with **complement fixation**, but for IgM-levels there are contradicting results [58,60]. The RTS,S vaccine only includes the central repeat region of CSP, consisting of the tandem NANP repeats, and the C-terminal domain. RTS,S-induced antibodies are directed at both the central repeat region and the C-terminal domain, but the NANP-directed antibodies are more strongly correlated with C1q fixation [57]. In a different study, human antibodies induced against full length PfCSP, presented on *P. berghei* sporozoites, mediate human complement activation and *P. falciparum* sporozoite lysis [61]. Altogether these data suggest that complement fixation may contribute to the efficacy of both naturally acquired and vaccine-induced CSP antibodies.

For the malaria transmission blocking vaccine candidate Pfs230, which targets gametes and is currently being tested in a Phase II clinical trial (ClinicalTrials.gov: NCT03917654), it has been well established that efficacy depends on complement activation [62-64]. Extensive *in vitro* and *in vivo* research over the years shows that monoclonal antibodies with complement-fixing isotypes induced by Pfs230 can target macrogametes and zygotes to block transmission through complement-mediated lysis [65-69]. Pfs230-induced polyclonal antibodies showed strong efficacy, but even in the absence of complement some activity remained, challenging the

assumption that Pfs230 antibodies are completely dependent on complement [70]. Nevertheless, it is clear that the efficacy of Pfs230-based vaccines is greatly enhanced by complement, and therefore Pfs230 vaccines should contain adjuvants that induce complement fixing immunoglobin subtypes such as IgG1 and IgG3.

Next to the relatively straightforward skewing of antibody responses to complement fixing subtypes, targeting the pathogen's complement evasion proteins is also an attractive approach to develop novel vaccines. The concept of neutralizing complement evasion was first demonstrated for *Neisseria meningitidis*. *N. meningitidis* recruits FH to evade complement, and targeting the protein responsible for FH recruitment was very effective for eliminating this pathogen. This formed the basis for the development of the MenB-FHbp vaccine, which has recently been licensed in the European Union for active immunization to prevent invasive meningococcal infection [71,72]. Analogously, recently identified complement evasion proteins from *P. falciparum*, such as PfMSP3.1, could be considered as vaccine candidates. Naturally acquired antibodies against a C-terminal fragment of PfMSP3.1 have previously been associated with growth inhibition of parasites co-cultured with monocytes *in vitro* [73]. This PfMSP3.1 fragment has advanced into clinical trials, but only showed moderate efficacy [74,75]. PfGAP50 also has reasonable potential for vaccine development, since polyclonal rabbit anti-PfGAP50 antibodies moderately reduce parasite transmission to mosquitoes [9]. PfGAP50 expressed in plants induced rabbit antibodies that showed moderate reduction of zygote formation *in vitro* [76]. It remains unclear whether PfGAP50 is a promising vaccine candidate.

The potential of the surface protein Pfs47, which allows *P. falciparum* to escape the mosquito immune system (**Figure 1**, **Box 2**) [48], as a transmission blocking vaccine (TBV) target has been evaluated. Full length Pfs47, including all three domains, did not induce antibodies with significant transmission reducing activity (TRA) [77]. However, a shorter version consisting of 58 amino acids induced anti-Pfs47 antibodies in mice that potently blocked *P. falciparum* transmission to mosquitoes. Curiously, the TRA did not depend on the activity of the mosquito or the human complement system, but instead greatly reduced ookinete formation, suggesting a possible role in *Plasmodium* fertilization [77]. Further preclinical studies found that coupling of the Pfs47 fragment to a virus-like particle carrier significantly improved TRA potency of antibodies induced in mice [78].

Together, these promising early studies illustrate the potential of complement evasion proteins as targets for vaccine development. Other *P. falciparum* complement evasion proteins, such as Pf92 on the merozoite surface and PIMMS43 on the ookinete and

oocyst surface, are potential targets that could be explored [4,15]. Furthermore, it is possible that a combination of strategies could result in more effective malaria vaccines; for instance, a vaccine that would neutralize CP inhibition by the parasite may potentiate a vaccine that relies on the induction of antibodies that activate CP.

### Biomolecules

An alternative to vaccination is the elimination of pathogens by passive immunization with recombinant antibodies and derivatives thereof. To achieve complement activation by CP, the Fc tail of an antibody needs to associate with C1q. Structural studies revealed that (antigen-bound) IgG1 antibodies can form hexamers through Fc-Fc interactions and that hexamerization increases the avidity for (hexameric) C1q, thus enhancing complement activation [79]. Further studies identified amino acid mutations that enhance hexamerization and thus complement activation, giving rise to the proprietary HexaBody® platform [79,80]. This platform has been successfully used to increase complement-dependent cytotoxicity on cancer and bacterial cells [80-82]. Importantly, HexaBody antibodies are currently under evaluation in a clinical trial (ClinicalTrials.gov: NCT03576131), confirming their potential as a promising future therapeutic strategy [83]. It would be interesting to test whether this innovative principle could also be applied to malaria antibodies to increase their potency, in particular for mAbs that are complement-dependent, such as antibodies directed at Pfs230 [66].

A different approach, although still in the experimental phase, is the development of biomolecules that consist of FH domains that are fused to Fc tails. These molecules could compete with FH binding on a pathogen, activating the AP and the CP through the Fc tail. This strategy is currently being explored for an array of different pathogens that recruit FH and has shown to reduce bacterial loads *in vivo* [84,85]. This concept could also be explored to target *P. falciparum* merozoites and gametes as these bind FH to their surface.

## Concluding remarks

In this review, we highlight the recently discovered complement evasion mechanisms employed by different developmental stages of *Plasmodium* parasites and by their *Anopheles* vectors. Most evasion mechanisms described to date rely on recruitment of complement regulators. In particular, FH recruitment seems to be a common strategy, as both *Plasmodium* merozoites and gametes, as well as the mosquito midgut, recruit FH to evade human complement, a strategy also shared with other pathogens [13].

The discovery of novel evasion strategies and the elucidation of the molecular mechanisms that *Plasmodium* parasites use to avoid damage by the complement system opens the possibility of developing novel vaccines and therapeutics. The sporozoite stage is an attractive vaccine target since it represents a bottleneck in the *Plasmodium* lifecycle and successful targeting would result in prevention of infection and disease. Indeed, the only licensed malaria vaccine targets this stage, but with limited efficacy [86]. This warrants further research to determine whether sporozoites also employ evasion mechanisms to avoid damage by the human complement system (see **Outstanding Questions**), since these could provide leads for more efficacious anti-sporozoite vaccines. Other intriguing questions are whether *Plasmodium* triggers the **Lectin Pathway** of complement activation, and which complement evasion mechanisms are essential for survival. Furthermore, neutralization of *Plasmodium* mosquito immune evasion strategies in genetically engineered mosquitoes may provide an interesting avenue to explore. Altogether complement evasion by *Plasmodium* parasites is an exciting and emerging field of research that may provide novel strategies to combat malaria.

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## Glossary

**Alternative Pathway (AP):** This cascade of complement protein activation starts by spontaneous hydrolysis and deposition of C3 on any surface. Host surfaces are protected by complement regulators.

**Classical Pathway (CP):** This complement pathway is typically initiated by the binding of antibodies to a surface.

**Complement deposition**: Complement proteins that are covalently attached to the surface of a pathogen.

**Complement evasion**: A mechanism by which a pathogen escapes from attack by the complement system.

**Complement fixation**: Antibodies bind surfaces, form multimers and subsequently activate the CP by C1q binding.

**Complement regulator:** These regulators positively or negatively control the complement cascade.

**Gamete:** Sexual stage parasite that has egressed from the red blood cell in the mosquito midgut and undergoes fertilization.

**JNK signalling pathway:** This mitogen-activated protein kinase pathway is important for the signalling that leads to nitration.

**Lectin Pathway (LP):** Binding of mannose-binding lectin, ficolin and collectins to the pathogen surface activate this complement pathway.

**Merozoite:** Parasite form that invades red blood cells.

**Nitration:** The exchange of $NO_2^+$ for $H^+$ on proteins is a mosquito immune response which can be triggered by the invasion of the ookinete.

**Ookinete:** Motile form of the parasite that is formed after fertilization and traverses the mosquito midgut wall.

**Sporozoite:** The infectious form of the malaria parasite that is injected by mosquitoes and migrates to the human liver to proliferate.

**Transmission**: The transfer of an infectious agent from one host to another.

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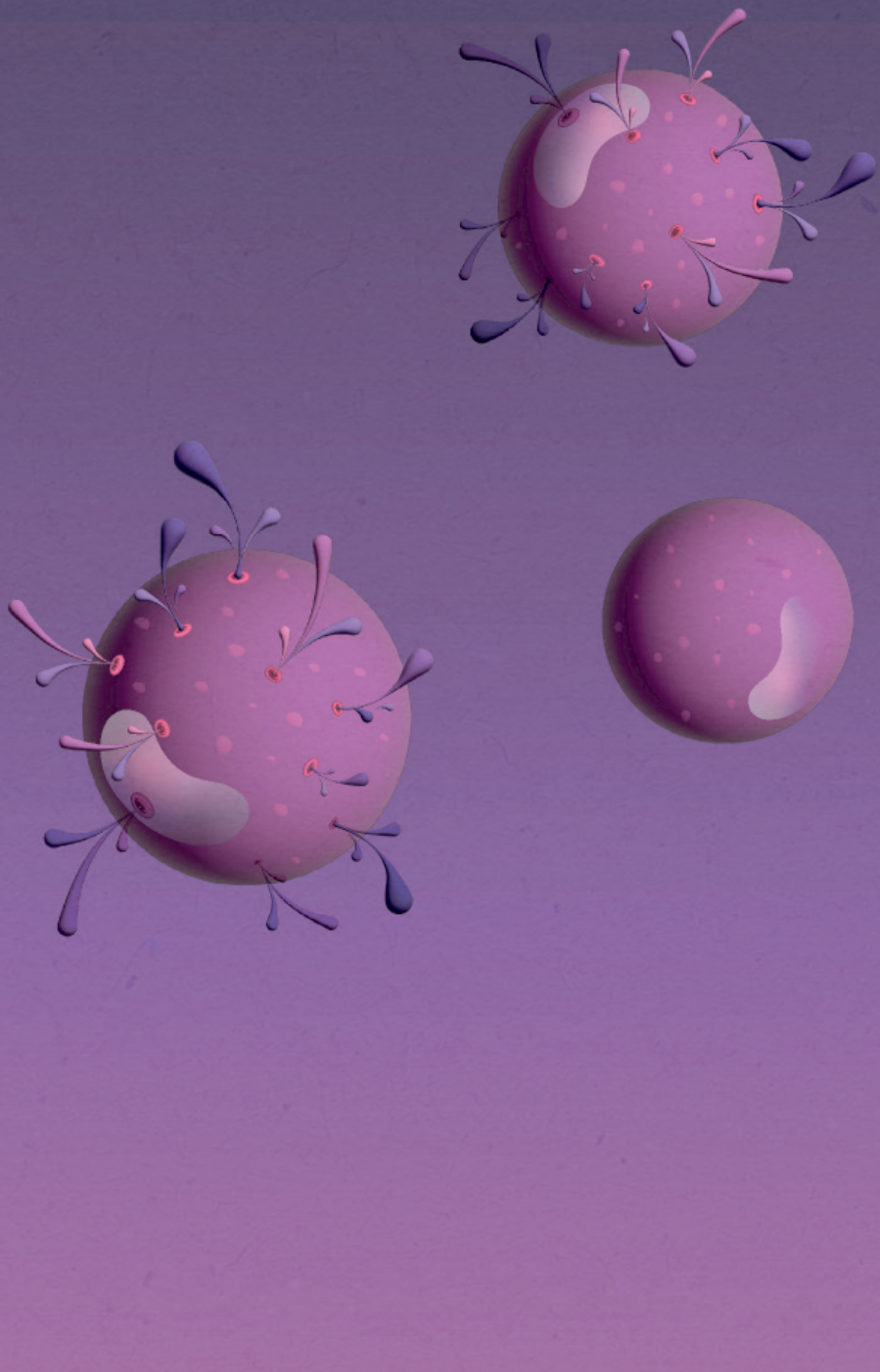

Chapter 3

# Pfs230 Domain 7 is targeted by a potent malaria transmission-blocking monoclonal antibody

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## Abstract

Malaria transmission-blocking vaccines (TBVs) aim to induce antibodies that block *Plasmodium* parasite development in the mosquito midgut, thus preventing mosquitoes from becoming infectious. While the Pro-domain and first of fourteen 6-Cysteine domains (Pro-D1) of the *Plasmodium* gamete surface protein Pfs230 are known targets of transmission-blocking antibodies, no studies to date have discovered other Pfs230 domains that are functional targets. Here, we show that a murine monoclonal antibody (mAb), 18F25.1, targets Pfs230 Domain 7. We generated a subclass-switched complement-fixing variant, mAb 18F25.2a, using a CRISPR/Cas9-based hybridoma engineering method. This subclass-switched mAb 18F25.2a induced lysis of female gametes *in vitro*. Importantly, mAb 18F25.2a potently reduced *P. falciparum* infection of *Anopheles stephensi* mosquitoes in a complement-dependent manner, as assessed by standard membrane feeding assays. Together, our data identify Pfs230 Domain 7 as target for transmission-blocking antibodies and provide a strong incentive to study domains outside Pfs230Pro-D1 as TBV candidates.

## Introduction

After a decade of considerable gains, the decline in malaria cases and deaths has completely stalled in recent years[6], underscoring the need for novel interventions including highly effective vaccines. *Plasmodium* parasites are the causative agents of malaria, with *Plasmodium falciparum* being responsible for the vast majority of deaths. Transmission of *Plasmodium* parasites through the human population occurs via *Anopheles* mosquitoes and commences with the uptake of gametocyte-infected erythrocytes from an infected human during a mosquito blood meal. In the mosquito midgut, female and male gametocytes activate and develop into gametes that egress from the erythrocyte. Male gametes fertilize female gametes, resulting in zygotes that differentiate into ookinetes capable of traversing the mosquito midgut epithelial layer. After traversal, oocysts are formed, in which sporozoites develop that migrate to the salivary glands and render a mosquito infectious to humans upon a next bite.

Malaria transmission-blocking vaccines (TBVs) are designed to induce antibodies against antigens on the parasite or mosquito midgut surface. When taken up together with gametocytes, these antibodies can interrupt sexual development in the mosquito midgut and reduce parasite transmission. As a consequence of this vaccine-induced transmission reducing activity (TRA), TBVs can limit the number of infectious mosquitoes and play an important role in efforts to eliminate malaria[7].

Pfs230D1-EPA is the most clinically advanced TBV candidate and is currently studied in a phase 2 clinical trial in Mali (ClinicalTrials.gov: NCT03917654). Pfs230D1-EPA contains an N-terminal fragment of the gamete surface protein Pfs230, including a part of the Pro-domain and the first of fourteen 6-cysteine (6-Cys, also known as cysteine motif) domains (Fig. 1A)[1,8]. Preclinical studies with a range of Pfs230 fragments showed that only vaccines containing the Pfs230 Pro-domain and/or Domain 1 (Pfs230Pro-D1) were able to induce functional antibodies with transmission-reducing activity (TRA)[9-13]. Importantly, the vast majority of Pfs230-specific antibodies are dependent on human complement that is also present in the mosquito blood meal[8,14-17], while some antibodies retain strong TRA in the absence of complement[18,19]. The observation that recombinant fragments covering Domains 2 up to 14 (Pfs230D2-D14) did not induce functional antibodies[9,11,12] led to the current hypothesis that these domains do not contain functional epitopes. We recently challenged this hypothesis by showing that the functional murine monoclonal antibody (mAb) 2A2.2a, elicited against full-length Pfs230 in *P. falciparum* gametocyte extract, does not target Pfs230Pro-D1[20]; thus far, the

targeted domain remains unknown. In line with this observation, another recent study identified murine mAbs that have TRA but do not recognize Pfs230Pro-D1[17].

Here, we aimed to identify functionally relevant Pfs230 epitopes outside Pfs230Pro-D1. We selected the high affinity murine mAb 18F25 IgG1 (18F25.1), elicited against female gametes[21,22] and showed that it binds to Pfs230-D7. Since mAb 18F25.1 is a non-complement-fixing subtype (IgG1) antibody, whereas all of the described Pfs230 antibodies with functional activity are complement-dependent, we generated a complement-fixing subtype variant, 18F25 IgG2a (18F25.2a). This mAb 18F25.2a induced complement-mediated gamete lysis and strongly reduced *Plasmodium* transmission in standard membrane feeding assays (SMFA). Together, we show that Pfs230D7 is a target for a potent transmission-blocking mAb and provide new leads for the development of Pfs230-based vaccines.

## Results

### mAb 18F25 binds Pfs230 Domain 7

To identify mAbs that target Pfs230D2-D14 (Fig. 1A), we screened murine mAbs, elicited against whole parasites and parasite extract, for binding to full-length Pfs230 and absence of binding to Pro-D1. We observed that mAb 18F25.1[22] binds to a conformational epitope on native Pfs230 in gametocyte extract with recognition being lost after reduction of disulfide bonds (Fig. 1B). While mAb 18F25.1 binds full-length Pfs230, it failed to recognize recombinant Pfs230CMB (containing Pro-D1), thus indicating that it targets an epitope on Pfs230D2-D14 (Fig. 1B). Next, we mapped the target domain of mAb 18F25.1 using a panel of recombinant Pfs230 fragments expressed in the wheat germ cell-free system (Supplementary Fig. 1)[9]. We first examined binding to the Pro-domain and fragments containing four consecutive 6-Cys domains and found that mAb 18F25.1 only reacted with D5-D8 and to a lesser extent D7-D10 (Fig. 1C). This suggests that the majority of interactions are with residues in D7 and/or D8. In a western blot with double-domain fragments, mAb 18F25.1 exclusively bound to D7-D8 (Supplementary Fig. 1). Using single-domain constructs D7 and D8, we found that mAb 18F25.1 binds to D7 and not D8 (Fig. 1D). Thus, mAb 18F25.1 targets an epitope that is (primarily) located on Pfs230D7.

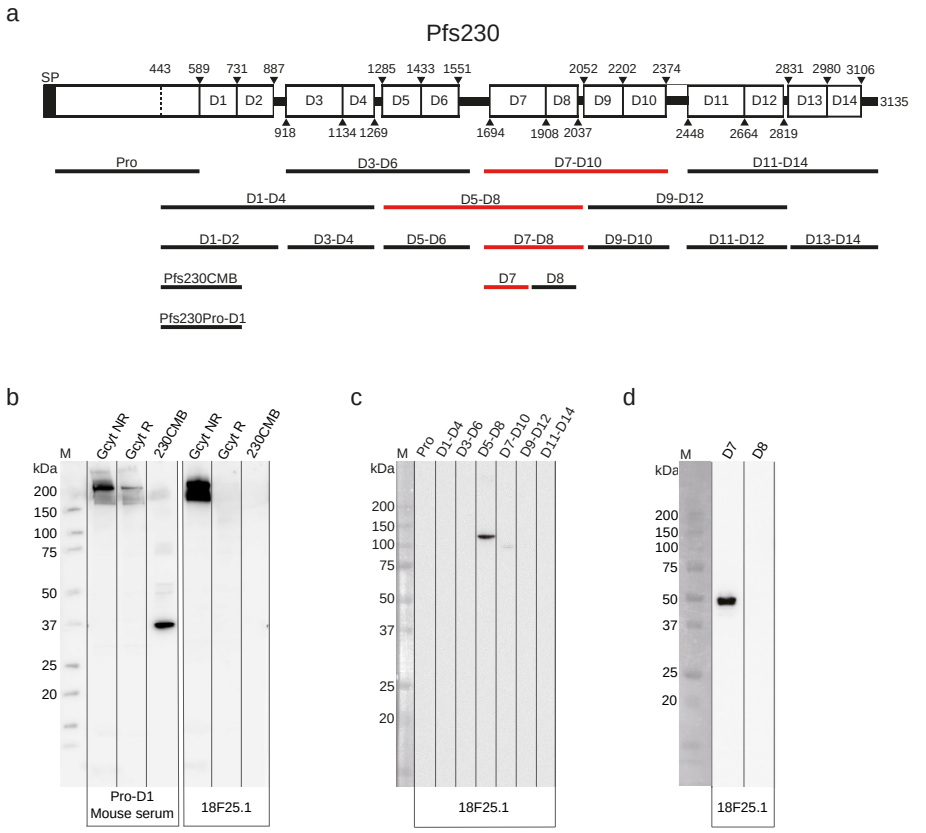


**Figure 1. mAb 18F25 targets Pfs230D7.**

(a) Schematic illustration of the Pfs230 domains and design of the constructs. Domain, linker segments and fragment boundaries are indicated with amino acid numbers (Supplementary Table S1)[1]. Pro is an abbreviation for the Pro-domain, which contains cleavage sites (mapped between 477-487 and 523-555 [2]) that are cleaved during gamete formation. SP is an abbreviation for signal peptide. The constructs in red are recognized by mAb 18F25.1 and all contain D7. (b) Western blot with *P. falciparum* NF54 gametocyte extract (Gcyt) under reduced (R) and non-reduced (NR) conditions and 20 ng of recombinant Pfs230CMB (aa 444-730) under NR conditions[3]. All samples were separated on one gel and transferred to a blot. The blot was cut in two parts that were incubated with mAb 18F25 IgG1 (18F25.1) and serum from mice immunized with Pro-D1 (aa 443-736)[4], respectively. Serum raised against Pfs230Pro-D1 was included as positive control and recognized both 230CMB and native Pfs230. Pro-D1 and 230CMB are abbreviations for Pfs230Pro-D1 and Pfs230CMB, respectively. (c,d) Western blots with recombinant Pfs230 fragments shown in (A) that were incubated with mAb 18F25.1. Note that the recombinant fragments were expressed with a glutathione S-transferase (GST) tag and therefore migrate as larger products.

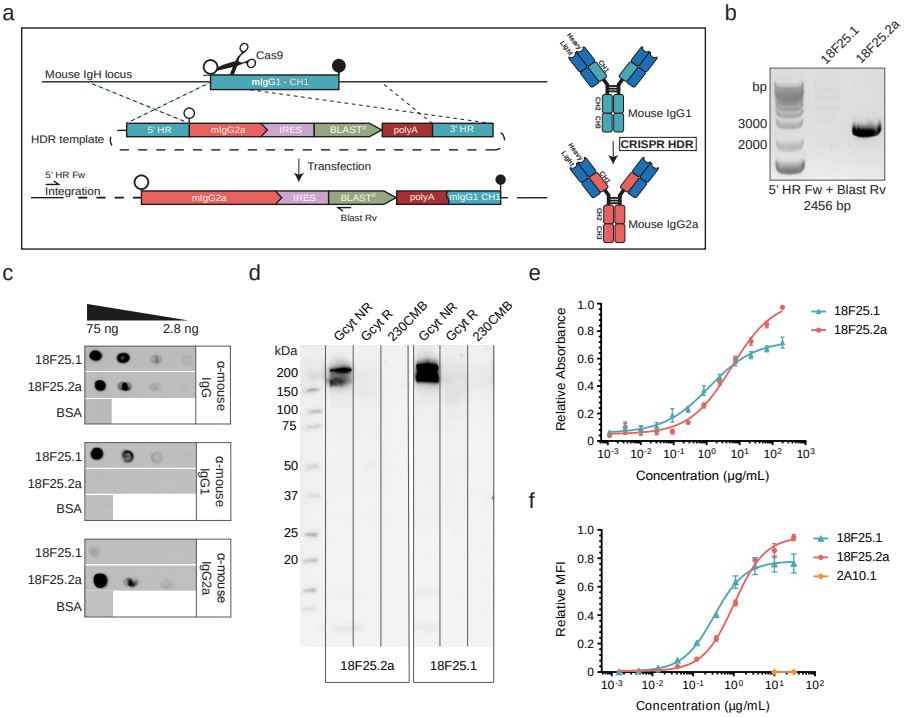


**Figure 2. Generation of a subclass-switched 18F25 mAb using CRISPR/Cas9-based hybridoma engineering.**

(a) Schematic overview of the general strategy used to switch the isotype of 18F25 from IgG1 to IgG2a. A guide RNA directed Cas9 to make a double strand break in the CH1 domain of Ighg1 (gene locus), which was repaired by integration of the HDR template. This HDR template contained the coding sequence for the CH1-CH3 domains of Ighg2c (mIgG2a), Internal Ribosome Entry Site (IRES), Blasticidin resistance gene (BLAST$^R$) and polyA signal sequence. The first 54 amino acids of CH1 from Ighg1 were removed after integration. HDR: Homology Directed Repair; HR: Homology Region. (b) Integration PCR on genomic DNA isolated from the 18F25.1 parental and the 18F25.2a-expressing hybridoma cell lines. The annealing sites of the primers are depicted in (A). (c) Dot blot with a titration (75, 25, 8.3 and 2.8 ng) of purified 18F25.1 and 18F25.2a antibody spotted on a nitrocellulose membrane. Three identical blots were subsequently incubated with α-mouse IgG, α-mouse IgG1 or α-mouse IgG2a secondary antibody. 75 ng bovine serum albumin (BSA) was included as negative control. (d) Western blots with non-reduced (NR) and reduced (R) *P. falciparum* NF54 gametocyte extract (Gcyt) and 20 ng of recombinant Pfs230CMB (230CMB). All samples were separated on one gel and transferred to a blot. The blot was cut in two parts that were incubated with mAb 18F25.1 or mAb 18F25.2a, as indicated. (e) mAb 18F25 recognition of native Pfs230 in *P. falciparum* NF54 gametocyte extract as assessed by ELISA. Values are means ± SEM from three independent experiments with three technical replicates each. Values from each experiment were normalized against an internal control to allow averaging across experiments. (f) mAb 18F25 binding to native Pfs230 on the surface of *P. falciparum* NF54 female gametes by flow cytometry analysis. Monoclonal antibody 2A10.1 (α-PfCSP) was included as negative control. Relative mean fluorescent intensities (MFI) are means ± SEM from two independent experiments with three technical replicates each. Values from each experiment were normalized against an internal control to allow averaging across experiments. The gating strategy is shown in Supplementary Figure 3A.

## Subclass switch of mAb 18F25 IgG1 to IgG2a

mAb 18F25.1 binds to Pfs230D7, but fails to reduce transmission of *P. falciparum* to mosquitoes in SMFA in which cultured *P. falciparum* NF54 gametocytes were fed to *Anopheles stephensi* mosquitoes and TRA was determined using oocyst counts as readout[22]. The murine mAb 18F25.1 is a non-complement-fixing subtype IgG1 and we therefore switched the subtype to complement-fixing IgG2a by engineering the 18F25.1 hybridoma cell line using CRISPR/Cas9 (Fig. 2A). Using a guide RNA, Cas9 was directed to generate a double-stranded break in the constant heavy region 1 (CH1) of the *mIgG1* locus in the 18F25.1 hybridoma cell line. By simultaneously introducing a homology directed repair (HDR) template, the coding sequence of *mIgG1* CH1-CH3 was replaced by *mIgG2a* CH1-CH3 to generate a *mIgG2a* switch variant. Transfected cells were selected using blasticidin and subsequently a clonal cell line was established by limiting dilution. Correct genomic integration of the *mIgG2a* CH1-CH3 fragment in this cell line was verified by polymerase chain reaction (Fig. 2B) and subsequent Sanger sequencing. The cell line was expanded and expressed antibodies were purified from supernatant. Dot blots with subtype-specific detection antibodies confirmed that the modified cell line produced mIgG2a instead of mIgG1 (Fig. 2C). Next, we tested whether the specificity and affinity of mAb 18F25.2a was affected compared to the parental mAb 18F25.1. As expected, mAb 18F25.2a remained specific for Pfs230 in gametocyte extract (Fig. 2D). Furthermore, the affinity of 18F25.1 and 18F25.2a for Pfs230 in gametocyte extract were comparable (Fig. 2E). Binding of 18F25.1 and 18F25.2a to Pfs230 on the surface of live female gametes was similar with $EC_{50}$ values of 0.32 µg/mL (95% CI [0.12, 0.52]) and 1.02 µg/mL (95% CI [0.7, 1.34]), respectively (Fig. 2F). Together these results demonstrate that we successfully generated a subclass switch of 18F25 from IgG1 to IgG2a while retaining its specificity and affinity.

## mAb 18F25.2a strongly reduces P. falciparum transmission to mosquitoes

Having generated a complement-fixing subclass variant of mAb 18F25, we next assessed its functional activity. We first tested the capacity of this mAb to lyse purified female gametes *in vitro*, in the presence of active human complement. 18F25.2a induced strong lysis of gametes; 10 µg/mL 18F25.2a was sufficient to lyse 91% of gametes, while the non-complement-fixing mAb 18F25.1 at the same concentration induced substantially less lysis (24%) (Fig. 3A). Next, we performed SMFAs to assess TRA in mosquitoes. Addition of mAb 18F25.1 to the infectious blood meal did not result in significant TRA ($p>0.23$) across the concentrations tested (Fig. 3B), in agreement with previous findings[22]. In contrast, 10 and 30 µg/mL mAb 18F25.2a resulted in 62% (95% % CI [43, 75]) and 93% TRA (95% CI [89, 96]),

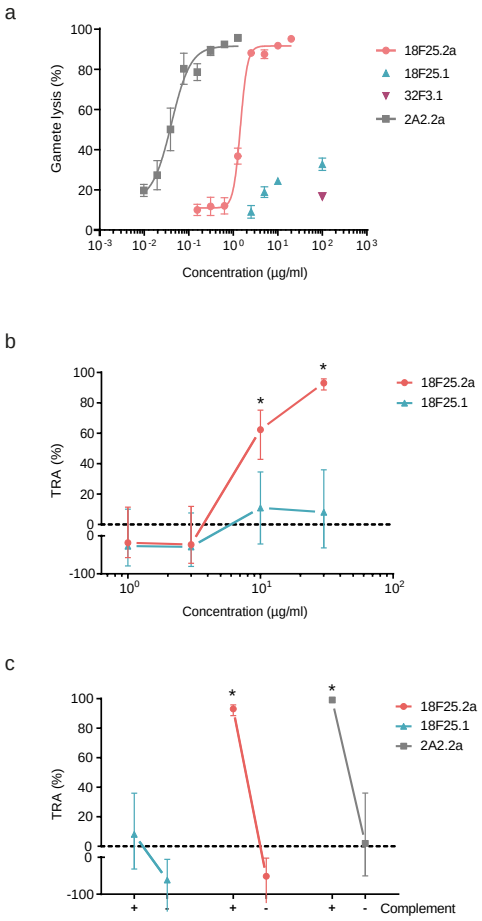


**Figure 3. mAb 18F25.2a reduces P. falciparum transmission in a complement-dependent manner.**
(a) Flow cytometry assay to determine the capacity of mAbs 18F25.1 and 18F25.2a to lyse isolated female gametes in the presence of active human complement. The gating strategy is shown in Supplementary Fig. 3B. Complement-dependent mAb 2A2.2a (α-Pfs230) was included as positive control and complement-independent mAb 32F3.1 (α-Pfs48/45) as a negative control. Lysis percentages are presented as means ± SEM from two independent experiments with three technical replicates each. Values from each experiment were normalized against an internal control to allow averaging across experiments. 0% lysis is defined as the number of live gametes present after incubation with normal human serum only. (b) mAbs 18F25.1 and 18F25.2a were tested in two independent SMFAs with *P. falciparum* NF54 gametocytes and *A. stephensi* mosquitoes, in the presence of active human complement. TRA estimates are shown with 95% confidence intervals and were determined using a mixed-effects negative binomial regression model[5]. (c) mAbs 18F25.1 and 18F25.2a were tested at 30 µg/mL in SMFA, in the presence (+) and absence (-) of active human complement. Complement-dependent mAb 2A2.2a (α-Pfs230) was included as positive control. TRA (%) estimates are shown with 95% confidence intervals and were determined using a mixed-effects negative binomial regression model[5]. Asterisks indicate significant TRA ($p < 0.05$). Oocyst counts from individual SMFA experiments can be found in Supplementary Figure 2.

respectively (Fig. 3B). These SMFA data strongly suggest that the capacity of the antibody to fix complement, determined by its subclass, is driving activity. To test whether the observed TRA is indeed complement-dependent, we also tested functional activity of mAbs in the presence of heat-inactivated complement. While 30 µg/mL 18F25.2a induced strong TRA in the presence of active complement, no significant TRA (p=0.98) was observed when complement had been inactivated (Fig. 3C). Together these data show that mAb 18F25.2a is a functional mAb that potently reduces transmission of *P. falciparum* parasites to *A. stephensi* mosquitoes in a complement-dependent manner.



## Discussion

In this study we have uncovered a new functionally relevant Pfs230 domain. We show that murine mAb 18F25, elicited by immunisation with whole female gametes, targets Pfs230D7. Furthermore, we demonstrate that the complement-fixing subtype mAb 18F25.2a blocks transmission to mosquitoes. After 30 years of research on this leading TBV candidate, these results identify Pfs230D7 as the first Pfs230 domain outside Pro-D1 that is targeted by a transmission-blocking mAb.

While TBVs hold promise as new tools for malaria elimination, very few candidates have been identified and tested (pre-)clinically[7]. Pfs25 was the first TBV candidate that was tested clinically, but despite promising results in rodents, it failed to induce potent and long-lasting TRA in humans[23]. Pfs230D1 and two Pfs48/45-based vaccines are currently being assessed in clinical studies (ClinicalTrials.gov: NCT05400746)[24,25], but it is not yet clear whether these can induce potent long-lasting TRA in humans. It is therefore important to continue discovery and development of novel TBV candidates. Here, we identify Pfs230D7 as a TBV candidate by showing that mAb 18F25 binds to a conformational epitope on Pfs230D7 and that it blocks transmission to mosquitoes in a complement-dependent manner.

Vaccines can ideally induce potent antibodies that target conserved epitopes. Although we characterized only one mAb against D7, thus providing limited insight in terms of potency, it is encouraging that mAb 18F25.2a is potent, showing 93% TRA at 30 µg/mL. We did not assess whether genetic variation in Pfs230D7 in circulating *P. falciparum* strains can affect the potency of mAb 18F25.2a. Interestingly, mAb 18F25 competed with murine mAb 11E3 which has TRA and recognizes *P. falciparum* isolates from geographically distinct areas[22]. This suggests that the functional target

epitope of 18F25 may be conserved. So far, Pfs230 fragments that included D7 did not manage to induce TRA in mouse immunization studies[9,11-13]. One possible explanation could be that non-functional epitopes within these fragments are immunodominant, preventing the induction of sufficient functional antibodies for TRA, as previously observed for the 6-Cys domain protein Pfs47[26]. Another explanation could be that fragments containing D7 failed to induce functional antibodies since these were (largely) incorrectly folded. 6-Cys domain containing proteins, with up to three disulfide bonds per domain, are notoriously difficult to express in their native conformation. This is exemplified by the struggles to produce correctly folded Pfs48/45, another 6-Cys family member and leading TBV candidate, where correct folding is essential for induction of functional antibodies[27]. It is striking that all fragments containing D7 that were produced in the wheat germ cell-free system and were used in the current study to identify D7 as target of 18F25, failed to induce TRA after mice immunization[9]; these were recognized by conformational mAb 18F25 (Fig. 1), suggesting that some properly folded protein was present. We hypothesize that the majority of protein in these preparations may have been incorrectly folded and prevented the induction of a functional response. In future studies, mAb 18F25 may in fact be utilized to purify correctly folded protein, an approach that was instrumental in the preclinical development of a Pfs48/45-based vaccine[27]. When correctly folded Pfs230D7 is obtained, its potency should be assessed, and compared to Pfs230D1, in mouse immunisation studies. In these studies, Pfs230D7 should also be combined with Pfs230D1 to explore whether including Pfs230D7 can enhance the efficacy of a Pfs230D1-based vaccine.

Almost all α-Pfs230 antibodies described to date are complement-dependent[8,14-17]. In agreement with this, our study shows that complement-fixing is the driving force behind the potency of 18F25.2a; this mAb does not reduce transmission in the absence of active complement, nor does the non-complement-fixing mAb 18F25.1 reduce transmission in the presence of complement (Fig. 3). The observed TRA is likely mediated by the lysis of gametes through the formation of membrane attack complex (MAC) as previously observed for Pfs230D1-specific antibodies[28,29].

We used a CRISPR/Cas9-based method to modify a murine hybridoma cell line to produce complement-fixing subtype 18F25.2a instead of non-complement-fixing subtype 18F25.1. Similar methods have previously been used to engineer the genomic immunoglobulin loci of rat and mouse hybridoma cell lines[30-33]. In our study, we generated HDR and guide RNA plasmids that enable replacement of the constant region of *mIgG1* by that of *mIgG2a,* and as such these plasmids can be utilized to switch the subclass of any mouse hybridoma cell line from IgG1 to

IgG2a. This method provides an attractive alternative approach to screening for spontaneous class switch mutants, a common method that has previously been used to generate the functional α-Pfs230 mAb 2A2.2a[34].

mAbs induced against sexual stage parasites, containing native full-length proteins, are valuable tools for antigen discovery. Indeed, two mAbs allowed the identification of Pfs230 as a TBV candidate, although the binding site on Pfs230 of these mAbs is unknown[35]. We recently found that another Pfs230-specific mAb induced against parasites, 2A2.2a, only recognized and blocked transmission of a subset of *Plasmodium* isolates. Analysis of polymorphisms in the Pfs230 protein of the different isolates suggested that Pfs230D4 may be the target of this mAb, but we could not express recombinant Pfs230D4 to confirm D4-specificity[20]. Here, we demonstrate that mAb 18F25, also induced against parasites, binds to a conformational epitope within Pfs230D7 and blocks transmission. Many more murine mAbs have been generated against native Pfs230 in parasites[17,22,36]; some of these block transmission but do not compete with mAbs 2A2 or 18F25. It is therefore tempting to speculate that yet more Pfs230 domains are targets for functional antibodies and that mAbs induced against whole parasites can be used to identify these.

Overall, the identification of Pfs230D7 as a target for the potent mAb 18F25.2a has important implications for the future of Pfs230-based TBV research. Our study provides new incentive to investigate Pfs230D7 as well as other non-Pro-D1 domains of Pfs230 as potential TBV candidates.

## Methods

### Hybridoma cell line and general culture conditions

The parental mouse hybridoma line expressing the anti-Pfs230 mAb 18F25.1 has been generated from immunized BALB/c mice[21] and characterized previously[22]. The cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 1× MEM Non-Essential Amino Acids Solution (Gibco), 10mM HEPES (Gibco), 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and Penicillin-Streptomycin (Gibco). The cells were propagated by splitting (1:1) with fresh medium daily.

### Western blot

*P. falciparum* NF54 gametocyte extract was prepared as described previously[37] and diluted to the equivalent of 500,000 gametocytes per well. For analysis under reducing conditions, a final concentration of 10mM dithiothreitol (DTT) was

added. Gametocyte extract and Pfs230CMB (aa 444-730)[3] samples were mixed with 4× NuPAGE™ LDS sample buffer and heated for 10 minutes at 70°C before loading on a 4-20% Bis-Tris gel (GenScript). 20 ng Pfs230CMB was loaded per well. Precision Plus Dual Color protein marker (Bio-Rad) was used as size standard. Proteins were transferred to a 0.45 µm nitrocellulose membrane using the Trans-Blot Turbo transfer system (Bio-Rad). The blots were blocked with 5% skimmed milk in PBS before incubation with 5 µg/mL 18F25.1, 5 µg/mL 18F25.2a or 1:5,000 polyclonal serum from mice immunized with Pfs230Pro-D1 (aa 443-736)[4]. After washing, the strips were incubated with 1:3,000 diluted polyclonal rabbit anti-Mouse IgG HRP (DAKO, Germany). Blots were developed with Clarity Max Western ECL substrate (Bio-Rad) and imaged on the ImageQuant™ LAS 4000 (GE Healthcare).

The gene sequences coding for the Pfs230 fragments were optimized for wheat codon usage, purchased from GenScript, and cloned into a pEU-E01-GST expression vector (CellFree Science, Matsuyama, Japan)[9]. The recombinant Pfs230 proteins were expressed as N-terminally GST-tagged proteins in wheat germ cell-free extract WEPRO7240G (CellFree Science) and purified by single-step GST-tag purification with a glutathione-Sepharose 4B column (Cytiva) using a robotic automated protein synthesizer Protemist DTII (CellFree Science) following manufacturer's instructions. The purified proteins were mixed with SDS-sample buffer and β-mercaptoethanol[38], and denatured at 37 °C for 30 min before loading on a 12.5% PAGE Tris gel (ATTO, Tokyo, Japan). 3.8 pmol of the proteins was loaded per well and Precision Plus Protein All blue standard (Bio-rad) was used as the size standard. Proteins were transferred to an Amersham Hybond P Low fluorescence 0.2 µm PVDF membrane (Cytiva) using the Trans-blot SD semidry transfer cell (Bio-rad). The blots were blocked with 5% skimmed milk in PBST before incubation with 1 µg/mL 18F25.1. After washing, the blots were incubated with 1:10,000 diluted polyclonal Sheep anti-Mouse IgG HRP (Cytiva). Blots were developed with Immobilon Western chemiluminescent HRP substrate (Millipore, MA) and imaged on the LAS-4000 (FUJIFILM, Tokyo, Japan). Unprocessed and uncropped scans of the western blots can be found in the Source Data File.

## Plasmid design and cloning

The genome of BALB/c mice was extracted from the BALB_cJ_V1 Ensembl Browser (accession number: GCA_ 001632525.1). The *Ighg2c* sequence coding for the mIgG2a immunoglobulin heavy constant region (accession number: MGP_BALBcJ_G0000005) was used for the HDR plasmid. The gene sequence was codon-optimized for mouse expression, flanked with BmsBI-v2 restriction sites and synthetized by GeneArt (Invitrogen) (Supplementary Table 2). For the generation of the HDR

plasmid, DNA was extracted from the mAb 18F25.1 producing hybridoma cell line using the Zymo Quick DNA Miniprep Plus Kit (D4068S). The 5' and 3' homology regions (HR) were amplified by polymerase chain reaction (PCR) using Platinum™ Taq High Fidelity (Invitrogen). The 5' HR was designed to contain the native splice acceptor region of the first exon of the *Ighg1* gene (MGP_BALBcJ_G0000007), and thereafter the *Ighg2c* coding sequence. The internal ribosome entry site (IRES), blasticidin resistance gene (Bsr) and PolyA sequence were PCR amplified from a donor plasmid kindly provided by M. Verdoes[33]. During PCR, BmsBI-v2 restriction sites and suitable overhangs were added to all amplicons. An overview of the used primers is provided in Supplementary Table 3. The different DNA fragments were incorporated in the pGGAselect entry vector using the NEB® Golden Gate Assembly Kit (BsmBI-v2) following the manufacturer's protocol (60 cycles).

To generate the guide RNA/Cas9, the pX330-U6-Chimeric_BB-CBh-hSpCas9 (gift from Feng Zhang; Addgene plasmid #42230)[39] was used as an entry vector. Suitable guide RNA sequences in the first exon of *Ighg1* were obtained using the CCTop Software[40]. The 5'-GGTCACCATGGAGTTAGTTT-3' guide was selected based on location, predicted off-targets and the CRISPRater efficacy score. The guide sequence was ordered as complementary single strand DNA oligos with the appropriate BbsI- overhangs. After phosphorylation using the T4 PNK enzyme (New England Biolabs) by incubation at 37 °C for 30 min, the oligonucleotides were annealed by incubating the mixture at 95 °C for 5 min followed by gradually cooling to 25 °C with an increment of -0.2 °C/s. Annealed oligos were inserted in the BbsI-digested entry vector by ligation using T4 ligase (New England Biolabs).The guide RNA/Cas9 and HDR plasmids were isolated from Stellar™ *E. coli* cells (TaKaRa Bio, Japan) using the HiPure filter kit (Invitrogen) according to manufacturer's protocol. The plasmids were verified by Sanger sequencing (Baseclear, Leiden).

## Hybridoma transfection

18F25.1 hybridoma cells, with a minimum viability of 90%, were used for nucleofection using the SF Cell Line 4D-Nucleofector X Kit L (V4XC-2020, Lonza). Briefly, cells were centrifuged, washed with PBS with 1% FBS and counted. One million cells were resuspended in the supplied SF medium containing 1 µg of HDR plasmid and 1 µg guide RNA/Cas9 plasmid, or 2 µg GFP plasmid (negative control). The cell suspensions were transferred to cuvettes and nucleofection was performed with the 4D-Nucleofection system (Lonza, CQ-104, Program SF). The cells were transferred to 6-wells plates with 3 mL of pre-warmed complete medium. Three days after transfection, the cells were transferred to 10 cm Petri dishes with 7 mL of complete medium supplemented with 7 µg/mL blasticidin (Invivogen, ant-bl-05).

Cultures were kept on blasticidin until GFP-transfected hybridoma cells were dead, and HDR-transfected cells were confluent with a viability above 80%. Antibiotic-resistant cells were seeded at 0.3 cells per well in five round bottom 96-well plates in 100 µL complete medium (without blasticidin) for clonal expansion.

## Clone selection

After 10 days of culture, supernatant from wells with high cell densities (110 clones) were analyzed for IgG1 and IgG2a expression by dot blot (described below). IgG2a-expressing clones were selected for further characterisation.

Genomic DNA from the parental hybridoma line and the selected hybridoma clones was isolated using the Zymo Quick DNA Miniprep Plus Kit (D4068S). To confirm integration, PCRs were performed using the polymerase kit PrimeSTAR GXL DNA (TaKaRa Bio, Japan) following manufacturer's instructions. PCR was performed with a forward primer (5' HR forward) annealing upstream of the 5' end of the HDR template and a reverse primer annealing on the blasticidin gene (Blasticidin reverse) (Supplementary Table 3). Another PCR was performed using a forward primer annealing on the blasticidin gene (Blasticidin forward) and a reverse downstream of the 3' homology region (3'HR reverse). There was overlap between the amplicons in order to cover the full integration site. The amplicons were visualized on a 1% agarose gel and the sequence was validated by Sanger sequencing (Baseclear, Leiden).

Finally, the clone that showed correct integration, high IgG2a expression levels and proper cell growth, was selected. The culture of this clone was expanded for antibody production.

## Antibody production

Hybridoma cells were left for 9 days without the addition of fresh medium for optimal mAb production. Supernatants from the parental hybridoma (18F25.1) and the engineered clone (18F25.2a) were centrifuged at 3,000 ×g for 10 minutes. Supernatants were loaded on 5 mL MabSelect™ Xtra columns (Cytiva) and antibodies were eluted using Glycine/HCL (pH2.5) buffer. The pH of peak fractions was immediately neutralized by adding Tris buffer (pH 8.8) to a final concentration of 0.1M. The fractions were pooled, buffer exchanged to PBS with 1 mg/mL trehalose and subsequently freeze-dried (Martin Christ GmbH, Germany). Antibody concentration was determined at 280nm on a NanoDrop™ 2000 spectrophotometer (ThermoScientific) assuming an extinction coefficient of 1.34.

Briefly, anti-Pfs230 mAb 2A2.2a has been derived from mice immunized with *Plasmodium falciparum* NF54 gametocyte extract and underwent spontaneous subclass switched as described previously[34,41]. Anti-CSP mAb 2A10.1 was produced by culturing the mice hybridoma cell line, as described above[42].

## Dot blot

Dot blots were used to determine the mAb subclass. Two µL of each dilution of mAb was spotted on a 0.45 µm nitrocellulose membrane (Bio-Rad). The blots were dried for 30 minutes and subsequently blocked for one hour with 5% skimmed milk in PBS. The blots were washed three times with PBS with 0.05% Tween (PBST) before incubation with 1:2,000 (in PBST) polyclonal rabbit Anti-Mouse IgGs HRP (Dako, P0260), Anti-Mouse IgG1 HRPO (Sigma, SAB3701171) or Anti-Mouse IgG2a HRPO (Sigma, SAB3701178) (in PBST). The blots were washed three times with PBST and once with PBS before incubation with Clarity™ Western ECL substrate (Bio-Rad). Imaging was performed on the ImageQuant™ LAS 4000 (GE Healthcare). The images of the blots were cropped and aligned. Unprocessed and uncropped scans of the dot blots can be found in the Source Data File.

## Gametocyte ELISA

*P. falciparum* NF54 gametocyte extracts were prepared as described previously[37]. Nunc MaxiSorp™ 96-wells plates (ThermoFisher) were coated overnight at 4$^0$C with 100 µL lysate per well, equivalent to 75,000 gametocytes. Plates were blocked with 5% skimmed milk in PBS, incubated with a 3-fold dilution series of primary antibody starting at 50 µg/mL (in PBS) and detected with 1:3,000 (in PBS) dilution polyclonal rabbit anti-Mouse IgG HRP (DAKO, P0260). The ELISA was developed by adding 100 µL tetramethylbenzidine (TMB). The color reaction was stopped by adding 50 µL 0.2M $H_2SO_4$ and the optical density was read at 450nm on an iMark™ microplate absorbance reader (Bio-Rad).

## Gamete purification

N-acetyl glucosamine treated 16-day old *P. falciparum* NF54 gametocyte cultures were centrifuged for 10 minutes at 2,000g and resuspended in FBS using a volume that equals half the culture volume. Gametocytes were activated on a roller bank for 45 minutes at room temperature and thereafter centrifuged for 10 minutes at 2,000×g at 4°C. The pellet was resuspended in 1 mL PBS, loaded onto a 7 mL layer of 11% w/v Accudenz (Accurate Chemical) and centrifuged for 30 minutes at 7,000g at 4°C without brake (Sorvall RC-5B Superspeed Centrifuge with HB-4 swing-out rotor). The top layer containing female gametes was collected, transferred to a 50 mL tube and filled up to 50 mL with PBS. Gametes were pelleted by centrifugation for

5 minutes at 2,000×g at 4°C. They were resuspended in 1 mL PBS and counted using a Bürker-Turk counting chamber.

## Gamete binding and lysis assays

Incubations were carried out in PBS supplemented with 2% FBS and 0.02% sodium azide. 50,000 purified gametes per well in a V-bottom non-treated 96-well plate (Costar) were incubated for 1 hour at room temperature with mAbs. In the case of a lysis assay, there is an addition of 20% normal human serum and incubation for 30 minutes at room temperature. Plates were centrifuged at 2,000×g for 3 minutes at 4°C and washed three times with PBS. Gametes were then incubated with either 1:200 Alexa Fluor™ 488 Chicken anti-mouse IgG (H+L) (Invitrogen) (binding assay) or 1:200 anti-Pfs47 (rat mAb 47.1)[43] labelled with DyLight™ 650 NHS ester (Thermo Scientific, Cat. No. 62266) (lysis assay). 1:1,000 eBioscience™ Fixable Viability Dye eFluor™ 780 (Invitrogen, Cat. No. 65-0865-14) was added and gametes were incubated for another 30 minutes at room temperature. After three rounds of washing with PBS, samples were resuspended in PBS. mAb binding to gametes and gamete lysis were assessed by flow cytometry by analysing a minimum of 2,000 gametes with the Gallios™ 10-color system (Beckman Coulter) and analyzed with FlowJo (BD, version 10.7.1).

## SMFA

18F25.1 and 18F25.2a antibodies were diluted in FBS and mixed with mature *P. falciparum* NF54 gametocytes and human serum that contains active complement. In conditions with inactive complement, the human serum was heated for 30 minutes at 56°C prior to mixing with the gametocytes. The blood meals were fed to *Anopheles stephensi* mosquitoes from a colony maintained at Radboudumc (Nijmegen, the Netherlands), as described previously[44]. Unfed and partially fed mosquitoes were removed. Mosquito midguts from 20 mosquitoes per condition were dissected 6-8 days after the blood meal. Midguts were stained with mercurochrome and oocysts were counted. TRA was defined as the reduction in oocyst intensity (oocysts per mosquito midgut) in a test condition compared to a negative control in which no antibody (FBS control) was added. We fitted a mixed-effects negative binomial regression model to the data and used this to estimate the TRA as previously described[5]. All samples were tested in two independent SMFA experiments for which the data are shown in Supplementary Fig. 2.

## Statistical analysis

Transmission reducing activity (TRA) was calculated as the reduction in oocysts compared to a negative control, using a negative binomial regression model as previously described[5]. SMFA data analyses were done in R (version 4.1.2).

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# Supplementary Information

**Supplementary Table 1.** Overview of recombinant Pfs230 fragments used or mentioned in this study. All constructs were produced in the wheat-germ cell free system[1,2] with the exception of the plant-produced Pfs230CMB[3] and the *Lactococcus lactis*-produced Pfs230Pro-D1 (previously called Pfs230Pro+I)[4].

| Name fragment | Amino acid boundaries |
|---|---|
| Pro | 22-588 |
| D1-D4 | 443-1274 |
| D3-D6 | 910-1560 |
| D5-D8 | 1280-2051 |
| D7-D10 | 1690-2393 |
| D9-D12 | 2052-2830 |
| D11-D14 | 2448-3135 |
| D1-D2 | 443-904 |
| D3-D4 | 910-1274 |
| D5-D6 | 1280-1560 |
| D7-D8 | 1690-2051 |
| D9-D10 | 2052-2393 |
| D11-D12 | 2448-2830 |
| D13-D14 | 2831-3135 |
| D7 | 1690-1907 |
| D8 | 1908-2051 |
| Pfs230CMB | 444-730 |
| Pfs230Pro-D1 | 444-736 |
| Pfs230C | 443-1132 |

**Supplementary Table 2.** Overview of sequences used to generate the homology directed repair plasmid for CRISPR/Cas9-based engineering of the mouse hybridoma cell line. Red nucleotides correspond to the original 50 nucleotides preceding the *Ighg2c* gene in the BALB/c genome. Nucleotides that are underlined and bold indicate the first complete codon of *Ighg2c*. The two preceding cytosines form a codon with a guanine originating from the VDJ domains after RNA splicing.

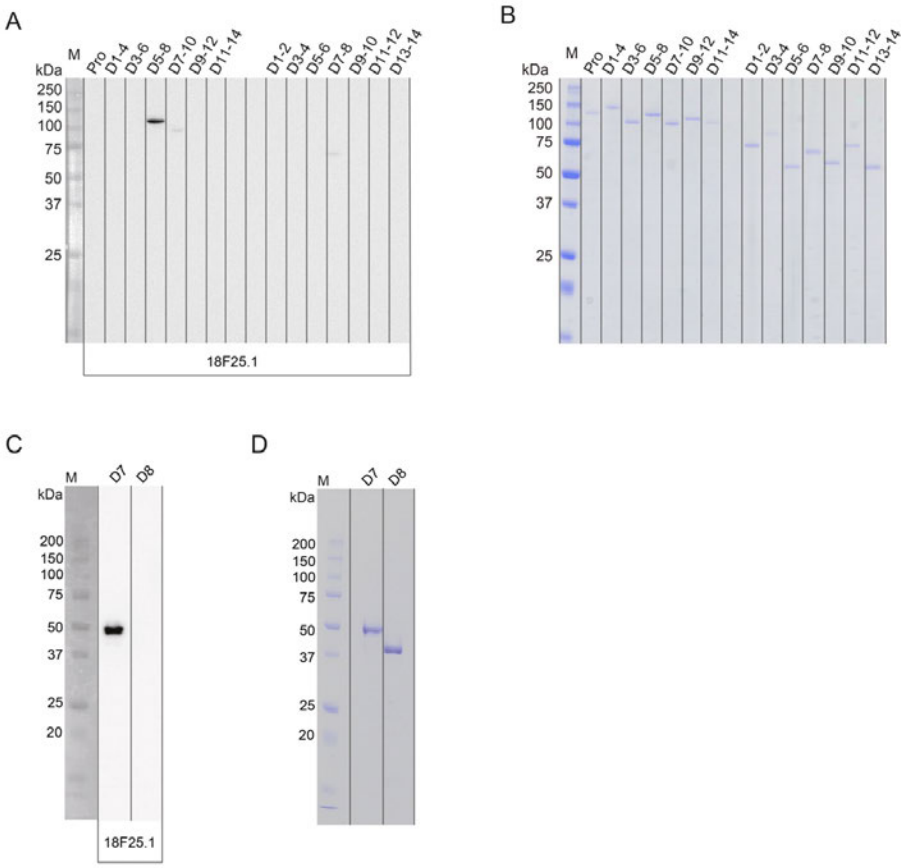
| | |
|---|---|
| mIgG2a synthetic gene | cagtcgtctcagctagcATCTGAGGCCACAGATAACAGAAAAGCTCACA-<br>CATCCTCCTCTCTTGCAGCCAAGACCACCGCTCCTAGCGTGTACCCTCTGGCTCCTGTGT-<br>GTGGCGACACAACAGGCAGCTCTGTGACACTGGGCTGTCTGGTCAAGGGCTACTTC-<br>CCCGAACCAGTGACACTGACCTGGAACAGCGGCTCTCTGTCTAGCGGCGTGCACA-<br>CATTTCCAGCCGTGCTGCAGAGCGACCTGTACACACTGTCCAGCAGCGTGACCGTGAC-<br>CAGCAGCACATGGCCTAGCCAGAGCATCACCTGTAACGTGGCCCATCCTGCCAGCTC-<br>CACCAAGGTGGACAAGAAGATCGAGCCTAGAGGCCCCACCATCAAGCCCTGTCCTC-<br>CATGCAAATGCCCCGCTCCTAATCTGCTCGGCGGACCCAGCGTGTTCATCTTCCCACCTA-<br>AGATCAAGGACGTGCTGATGATCTCTCTGAGCCCCATCGTGACCTGCGTGGTGGTGGAT-<br>GTGTCTGAGGACGACCCTGACGTGCAGATCAGTTGGTTCGTGAACAACGTGGAAGTGCA-<br>CACAGCCCAGACACAGACCCACAGAGAGGACTACAACAGCACCCTGAGAGTGGTGTCT-<br>GCCCTGCCTATCCAGCACCAGGATTGGATGAGCGGCAAAGAATTCAAGTGCAAAGT-<br>GAACAACAAGGACCTGCCTGCTCCTATCGAGAGAACCATCAGCAAGCCCAAGGGCTCT-<br>GTCAGGGCTCCTCAGGTGTACGTTCTGCCACCTCCTGAGGAAGAGATGACCAAGAAA-<br>CAAGTGACCCTCACCTGTATGGTCACCGACTTCATGCCCGAGGACATCTACGTGGAATG-<br>GACCAACAACGGCAAGACCGAGCTGAACTACAAGAACACCGAGCCTGTGCTGGACTC-<br>CGACGGCAGCTACTTCATGTACAGCAAGCTGCGCGTCGAGAAGAAGAACTGGGTCGA-<br>GAGAAACAGCTACAGCTGCAGCGTGGTGCACGAGGGACTGCACAACCACCACACCAC-<br>CAAGAGCTTCAGCAGAACCCCTGGCAAATGAgccggcatgagacggcat |
| 5'HR | GTTTGTGTATAGGCAAGAAGTGAATCCTGACCCAAGAATAGAGAGTGCTAAACG-<br>GACTTAGCTCAAAGACAACTGAAAAAGACAATGCCTGCAAAACAAAGCTAAGGCCA-<br>GAGCTCTTGGACTATGAAGAGTTCAGGGAACCTAAGAACAGGGACCATCTGTGTA-<br>CAGGCCAAGGCCGGTAGAAGCAGCCTAGGAAATGTCAAGAGCCAACGTGGCTGGGT-<br>GGGCAAAGACAGGAAGGGACTGTTAGGCTGCAGGGATGTGCCGACTTCAATTTGT-<br>GCTTCAGTGTTGTCCAGATTGTGTGCAGCCATATGGCCCAGGTATAAGAAGTTTAACAGT-<br>GGAACACAGATGCCCACATCAGACAGCTGGGGGGTGGGGGGGGGTGAACACAGA-<br>TACCCATACTGGAAAGCAGGTGGGGCATTTTCCTAGGAACGGGACTGGGCTCAATG-<br>GCCTCAGGTCTCATCTGGTCTGGTGATCCTGACATTGACAGGCCCAAATGTTGGATAT-<br>CACCTACTCCATGTAGAGAGTCGGGGACATGGGAAGGGTGCAAAAGAGCGGCCTTCTA-<br>GAAGGTTTGGTCCTGTCCTGTCCTGTCTGACAGTGTAATCACATATACTTTTTCTTGTAGCC |
| mIgG2a | AAGACCACCGCTCCTAGCGTGTACCCTCTGGCTCCTGTGTGTGGCGACACAACAGG-<br>CAGCTCTGTGACACTGGGCTGTCTGGTCAAGGGCTACTTCCCCGAACCAGTGACACT-<br>GACCTGGAACAGCGGCTCTCTGTCTAGCGGCGTGCACACATTTCCAGCCGTGCTG-<br>CAGAGCGACCTGTACACACTGTCCAGCAGCGTGACCGTGACCAGCAGCACATGG-<br>CCTAGCCAGAGCATCACCTGTAACGTGGCCCATCCTGCCAGCTCCACCAAGGTGG-<br>ACAAGAAGATCGAGCCTAGAGGCCCCACCATCAAGCCCTGTCCTCCATGCAAATGC-<br>CCCGCTCCTAATCTGCTCGGCGGACCCAGCGTGTTCATCTTCCCACCTAAGATCAAG-<br>GACGTGCTGATGATCTCTCTGAGCCCCATCGTGACCTGCGTGGTGGTGGATGTGTCT-<br>GAGGACGACCCTGACGTGCAGATCAGTTGGTTCGTGAACAACGTGGAAGTGCACACAG-<br>CCCAGACACAGACCCACAGAGAGGACTACAACAGCACCCTGAGAGTGGTGTCTGCCCT-<br>GCCTATCCAGCACCAGGATTGGATGAGCGGCAAAGAATTCAAGTGCAAAGTGAACAA-<br>CAAGGACCTGCCTGCTCCTATCGAGAGAACCATCAGCAAGCCCAAGGGCTCTGTCAG-<br>GGCTCCTCAGGTGTACGTTCTGCCACCTCCTGAGGAAGAGATGACCAAGAAACAAGT-<br>GACCCTCACCTGTATGGTCACCGACTTCATGCCCGAGGACATCTACGTGGAATGGACCAA-<br>CAACGGCAAGACCGAGCTGAACTACAAGAACACCGAGCCTGTGCTGGACTCCGACGG-<br>CAGCTACTTCATGTACAGCAAGCTGCGCGTCGAGAAGAAGAACTGGGTCGAGAGAAA-<br>CAGCTACAGCTGCAGCGTGGTGCACGAGGGACTGCACAACCACCACACCACCAA-<br>GAGCTTCAGCAGAACCCCTGGCAAATGA |

**Supplementary Table 2.** Continued

| | |
|---|---|
| IRES Bsr polyA | GTCGAGGCCCCTCTCCCTCCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAG-GCCGGTGTGCGTTTGTCTATATGTTATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAG-GGCCCGGAAACCTGGCCCTGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGC-CAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTT-GAAGACAAACAACGTCTGTAGCGACCCTTTGCAGGCAGCGGAACCCCCCACCTGGCGAC-AGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAAC-CCCAGTGCCACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAG-CGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTATGGGATCTGATCT-GGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAGGTTAAAAAAACGTCTAGG-CCCCCCGAACCACGGGGACGTGGTTTTCCTTTGAAAAACACGATGATAATATGGCCA-CAGAATTCGCCACCATGGCCAAGCCTTTGTCTCAAGAAGAATCCACCCTCATTGAAA-GAGCAACGGCTACAATCAACAGCATCCCCATCTCTGAAGACTACAGCGTCGCCAGCG-CAGCTCTCTCTAGCGACGGCCGCATCTTCACTGGTGTCAATGTATATCATTTTACTGGGG-GACCTTGTGCAGAACTCGTGGTGCTGGGCACTGCTGCTGCTGCGGCAGCTGGCAACCT-GACTTGTATCGTCGCGATCGGAAATGAGAACAGGGGCATCTTGAGCCCCTGCGGACG-GTGCCGACAGGTGCTTCTCGATCTGCATCCTGGGATCAAAGCCATAGTGAAGGACAGT-GATGGACAGCCGACGGCAGTTGGGATTCGTGAATTGCTGCCCTCTGGTTATGTGTGG-GAGGGCTAAGTACTAGTCGAGTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTC-CCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAAT-GAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGG-GCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGT-GGGCTCTATGGAGATCTTGTACA |
| 3'HR | CTAACTCCATGGTGACCCTGGGATGCCTGGTCAAGGGCTATTTCCCTGAGCCAGTGA-CAGTGACCTGGAACTCTGGATCCCTGTCCAGCGGTGTGCACACCTTCCCAGCTGTCCT-GCAGTCTGACCTCTACACTCTGAGCAGCTCAGTGACTGTCCCCTCCAGCACCTGGC-CCAGCGAGACCGTCACCTGCAACGTTGCCCACCCGGCCAGCAGCACCAAGGTGGA-CAAGAAAATTGGTGAGAGGACATATAGGGAGGAGGGGTTCACTAGAAGTGAGGCT-CAAGCCATTAGCCTGCCTAAACCAACCAGGCTGGACAGCCATCACCAGGAAATGGATCT-CAGCCCAGAAGATCAAAAGTTGTTCTTCTCCCTTCTGGAGATTTCTATGTCCTTTACACT-CATTGGTTAATATCCTGGGTTGGATTCCCACACATCTTGACAAACAGAGACAAATTT-GAGTATCACCAGCCAAAAGTCATACCCAAAAACAGCCTGGCATGACCTCACAC-CAGACTCAAACTTACCCTACCTTTATCCTGGTGGCTTCTCATCTCCAGACCCCAGTAACA-CATAGCTTTCTCTCCACAGTGCCCAGGGATTGTGGTTGTAAGCCTTGCATATGTACAGG-TAAGTCAGTAGGCCTTTCACCCTGACCCCAGATGCAACAAGTGGCCATGTTGGAGGGT-GGCCCAGGTATTGACCTATTTCCACCTTTCTTCTTCATCCTTAGTCCCAGAAGTATCATCTG-TCTTCATCTTCCCCCCAAAGCCCAAGGATGTGCTCACCATTACTCTGACTCCTAAGGTCAC-GTGTGTTGTGGTAGACATCAGC |

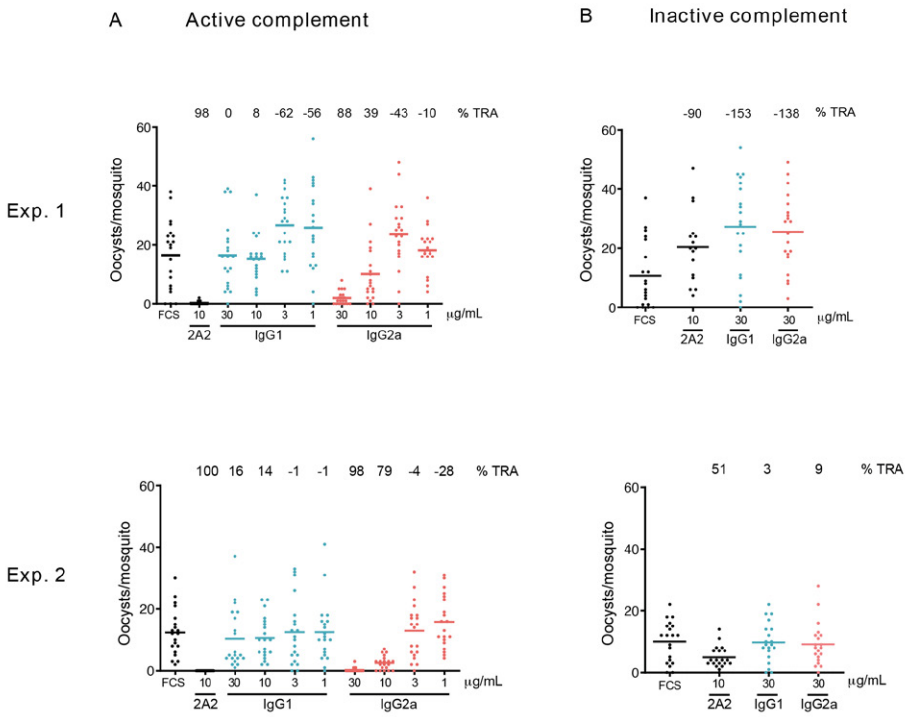
**Supplementary Table 3. Overview of primers used in this study.**

| **Plasmid Design** | |
|---|---|
| 5'HR Forward | cagtcgtctcaggagtcgacGTTTGTGTATAGGCAAGAAGTGAATCCTGAC |
| 5'HR Reverse | ctagcgtctcaTCTTGGCTACAAGAAAAAGTATATGTGATTACACTG |
| 3'HR Forward | cagtcgtctctacccgggCTAACTCCATGGTGACCCTGGGATG |
| 3'HR Reverse | ctagcgtctctatggaccggtGCTGATGTCTACCACAACACACGTGAC |
| IRES Bsr Poly A Forward | cagtcgtctcaggcaGTCGAGGCCCCTCTCCCTCC |
| IRES Bsr Poly A Reverse | ctagcgtctcagggtTGTACAAGATCTCCATAGAGCCCACC |
| mIgG2a Forward | cagtcgtctctAAGACCACCGCTCCTAGCGTG |
| mIgG2a Reverse | ATGCcgtctcATGCCGGCTC |
| **Integration PCR** | |
| 5'HR Forward | GAGAACCAAGCTAAAAAGTTATGTCAAACCAC |
| Blasticidin Reverse | ATACATTGACACCAGTGAAGATGC |
| Blasticidin Forward | CAGCAGAACCCCTGGCAAATG |
| 3'HR Reverse | CATCTACAAACCAGCTGAACTGGACC |



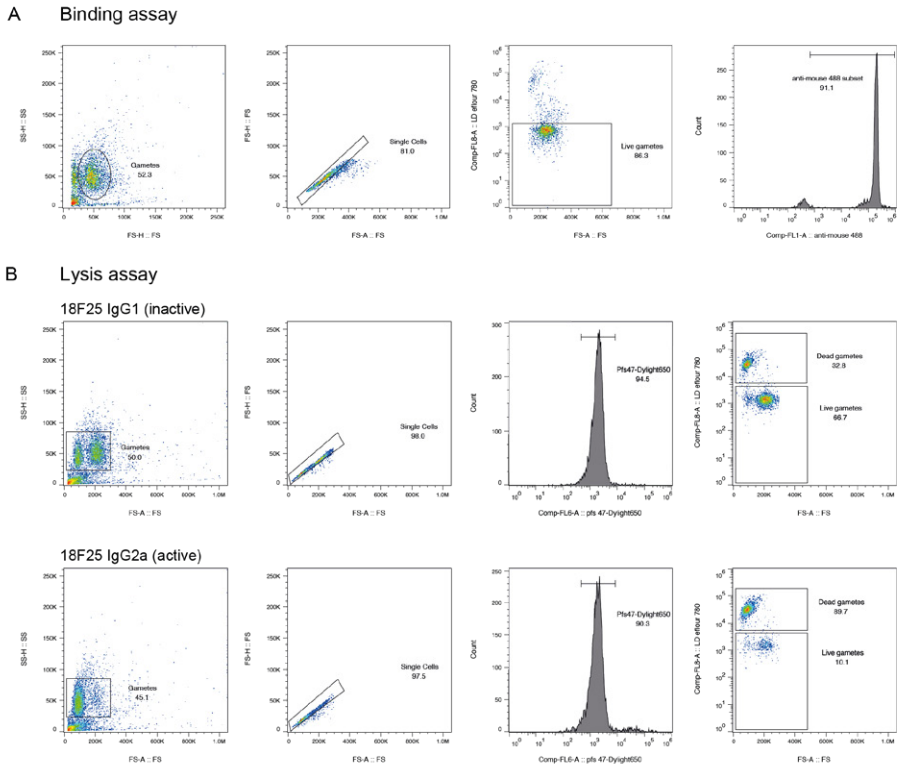

**Supplementary Figure 1. 18F25 detection of Pfs230 recombinant fragments.**

(A) Western blot with mAb 18F25.1 and recombinant multi-domain Pfs230 fragments. The recombinant fragments were expressed with a glutathione S-transferase (GST) tag. (B) SDS-PAGE gel of the multi-domain wheat-germ cell free system produced recombinant multi-domain Pfs230 fragments used in (A). (C) Western blot with mAb 18F25.1 and recombinant single-domain Pfs230 fragments. (D) SDS-PAGE gel of the single domain wheat-germ cell free system produced recombinant Pfs230 fragments shown in (C). Note that part of the western blot in (A) and the western blot in (C) are also shown Figure 1, though for clarity are depicted here next to the SDS-PAGE results.




**Supplementary Figure 2. Overview of individual standard membrane feeding assays (SMFA) that form the basis for reported TRA values in Figure 3.**

18F25.1 (IgG1) and 18F25.2a (IgG2a) titrations were tested in two independent SMFAs with *P. falciparum* NF54 gametocytes and *A. stephensi* mosquitoes with active complement (A). The highest mAb concentration (30 µg/mL) was also tested in these assays with heat-inactivated complement (B). Complement-dependent α-Pfs230 monoclonal antibody 2A2.2a (2A2) was included as positive control in both SMFAs. Per condition, 20 individual mosquitoes were dissected and oocysts were counted. The line represents the mean number of oocysts per mosquito. The transmission reducing activity (% TRA) was calculated as the percentage in reduction in the number of oocysts per mosquito compared to the control in which no antibody was added (FCS control). Note that for 10 µg/mL 2A2.2a with inactivated complement in experiment 1, oocyst counts could be obtained for 16 mosquitoes instead of 20.




**Supplementary Figure 3. Overview of flow cytometry gating strategies.**

Exemplary plots that provide an overview of the gating strategy for (A) a binding assay with live female gametes and (B) a lysis assay with live female gametes. In the binding assay (A) gametes were gated for single cells (2nd column) and then live gametes were selected based on the absence of live-dead stain LD efluor 780, which stains dead cells (3rd column). (B) lysis assay with an inactive mAb (IgG1, top row) and active mAb (IgG2a, bottom row). Gametes were gated for single cells (2nd column), and then for Pfs47 positivity. Dead gametes were stained with live-dead stain LD efluor 780 to determine the percentage dead cells (4th column). Note that two gamete populations can be observed in the forward scatter plots (1st column) in A and B; the left population contains dead gametes, the right population contains live gametes. In the binding assay (A) we gated only the live population, while in the lysis assay (B) we gated both live and dead populations. SS-H = side scatter height, SS = side scatter, FS-H = forward scatter height, FS-A = forward scatter area, LD = live dead.

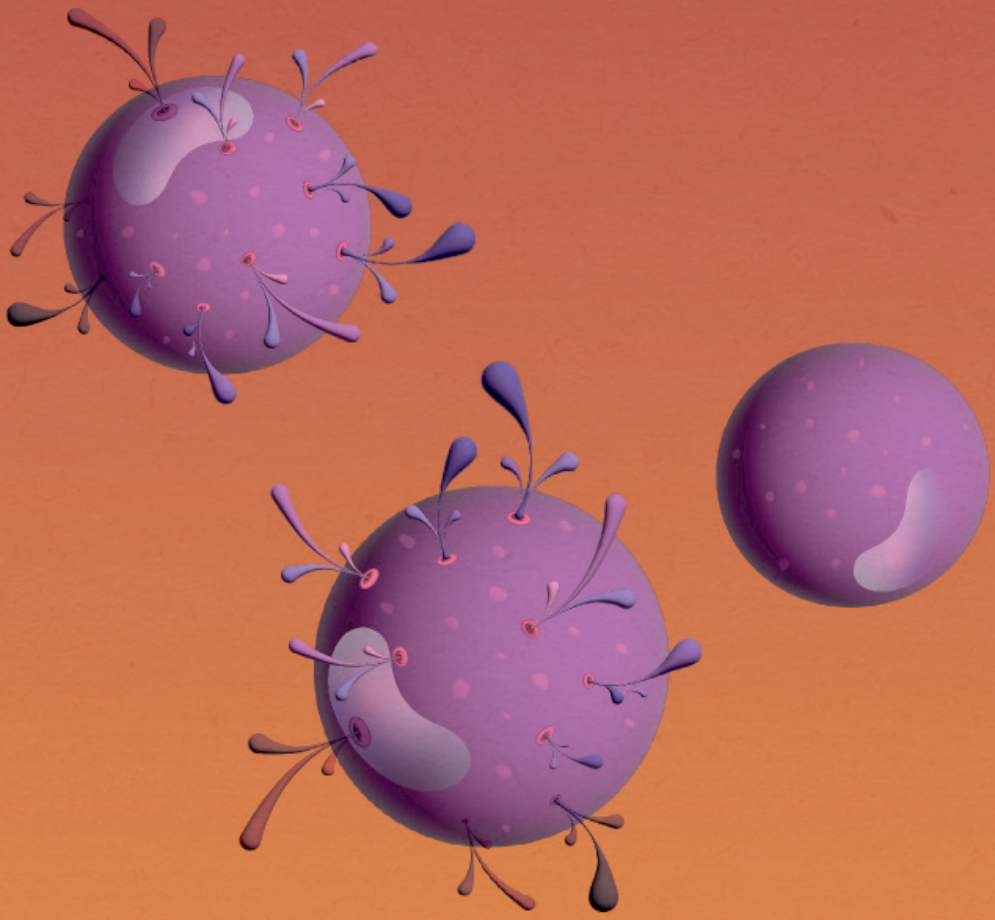
# Supplementary References

1 Tachibana, M. *et al.* Identification of domains within Pfs230 that elicit transmission blocking antibody responses. *Vaccine* **37**, 1799-1806, doi:10.1016/j.vaccine.2019.02.021 (2019).

2 Miura, K. *et al.* Functional comparison of Plasmodium falciparum transmission-blocking vaccine candidates by the standard membrane-feeding assay. *Infect Immun* **81**, 4377-4382, doi:10.1128/IAI.01056-13 (2013).

3 Farrance, C. E. *et al.* A plant-produced Pfs230 vaccine candidate blocks transmission of Plasmodium falciparum. *Clin Vaccine Immunol* **18**, 1351-1357, doi:10.1128/CVI.05105-11 (2011).

4 Singh, S. K. *et al.* Pfs230 and Pfs48/45 Fusion Proteins Elicit Strong Transmission-Blocking Antibody Responses Against Plasmodium falciparum. *Front Immunol* **10**, 1256, doi:10.3389/fimmu.2019.01256 (2019).



Chapter 4

# Pfs230 Domain 12 is a potent malaria transmission-blocking vaccine candidate

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## Abstract

Malaria transmission-blocking vaccines (TBV) target sexual stage parasites that are transmitted to mosquitoes and critical for spread of the pathogen. The clinically most advanced TBV candidate contains part of the Pro-domain (Pro) and Domain 1 (D1) of *Plasmodium falciparum* surface protein Pfs230. Subunit vaccines that contain other domains of Pfs230 have so far failed to induce functional antibodies. Here, we produced eight single domain fragments of Pfs230 in *Drosophila melanogaster* S2 cells and assessed their immunogenicity in mouse immunizations. In addition to D1-specific antibodies, antibodies raised against Domain 12 (D12) showed strong functional transmission-reducing activity in membrane feeding assays with cultured parasites, an activity that was complement-dependent. Murine D12-specific antibodies further reduced mosquito transmission of parasites acquired from naturally infected parasite carriers. The D12 antigen was recognized by sera from an all-age cohort of individuals who had been naturally exposed to *Plasmodium falciparum* with antibody levels increasing with age. In conclusion, we identified Pfs230D12 as a promising TBV candidate.

## Introduction

The burden of malaria has increased in recent years, with 450,000 fatal malaria cases in 2016 rising to 608,000 in 2022 [1]. Malaria is caused by *Plasmodium* parasites, of which *Plasmodium falciparum* is the deadliest, that are transmitted by *Anopheles* mosquitoes. Transmission to mosquitoes relies on the uptake of sexual stage parasites, female and male gametocytes, through a bloodmeal. Inside the mosquito midgut, gametocytes activate to become female macrogametes and exflagellating male microgametes that fertilize to form zygotes. From this point onwards *Plasmodium* parasites continue their lifecycle by developing into ookinetes that traverse the midgut epithelium and form oocysts on the midgut basal side underneath the basal lamina. Within these oocysts, sporozoites are formed that find their way to the salivary glands, resulting in infectious mosquitoes. Transmission to mosquitoes forms a bottleneck in the lifecycle of malaria parasites and is therefore an attractive target for interventions. Transmission-blocking vaccines (TBVs) target this bottleneck with the aim to reduce the number of mosquitoes that become infectious; TBVs thereby form valuable assets for malaria elimination strategies [2].



TBVs induce antibodies in the human host against surface antigens of gametes, zygotes and/or ookinetes. These antibodies are taken up by the mosquito via the bloodmeal together with gametocytes and human complement. Inside the midgut, where parasites egress from the red blood cells and become accessible to antibodies, the antibodies prevent further development of the parasite through neutralisation or activation of human complement that results in parasite lysis. The functional activity of malaria transmission-blocking antibodies is commonly quantified by measuring the reduction in oocyst numbers compared to a negative control, and expressed as the percentage transmission-reducing activity (TRA). The clinically most advanced TBV candidate is Pfs230, which is essential for fertilization and further development into oocysts [3]. Pfs230 is an abundant gamete surface protein that consists of fourteen 6-Cys domains (Fig. 1A) [4,5]. The large size of Pfs230 hampers recombinant expression of full-length Pfs230 and vaccine development has focused on expression of fragments of the protein [6]. Immunization studies found that the Pfs230 Pro-domain (Pro) and Domain 1 (D1) induced a functional response in rodents [7-12], which formed the basis for the development of Pfs230D1-EPA, the only TBV candidate to date that progressed to phase 2 clinical studies [13-15]. For many years, it has been unclear whether domains outside Pro and D1 contain epitopes for functional antibodies. Recent studies showed that functional monoclonal antibodies (mAbs) induced by whole parasite immunization or natural exposure target Pfs230 epitopes outside ProD1 [16-19]. However, recombinant

fragments containing non-ProD1 fragments of Pfs230, have so far failed to induce a functional response *in vivo* [7-9] and ProD1 thus remains the only Pfs230-based vaccine candidate with demonstrated *in vivo* efficacy described to date.

Here, we expressed all single Pfs230 domains in *Drosophila melanogaster* S2 cells that have been successfully used for expression of the 6-Cys domain protein Pfs48/45 [20,21]. We obtained eight pure single domain fragments that were used to immunize mice. Of these fragments, Domain 12 (D12) induced antibodies with strong TRA in membrane feeding assays with lab cultured parasites and in membrane feeding assays with naturally circulating parasites from human donors. We also show that people with natural exposure to malaria parasites possess antibodies that recognize Pfs230D12. These results position Pfs230D12 as a promising TBV candidate.

## Results

### Production of single domain Pfs230 protein fragments

We expressed single domain protein fragments with a C-terminal C-tag in *D. melanogaster* S2 cells (table S1-2). Nine of the fourteen constructs showed clear expression in S2 cell supernatants by western blot with an α-C-tag antibody (fig. S1A). The cell lines that showed clear expression, i.e. lines expressing D1, D3, D5, D6, D8, D9, D10, D12 and D13, were scaled up and proteins were purified using C-tag purification followed by size exclusion chromatography (Fig. 1B). Unlike the other domains, D3 and D9 showed strong aggregation by size exclusion chromatography. Using the mild detergent Empigen® BB, we resolved aggregation for D9. D3 remained largely aggregated in the presence of Empigen® BB and was excluded from further analyses.

We obtained pure D1, D5, D6, D8, D9, D10, D12 and D13 proteins as determined by SDS-PAGE (Fig. 1B) and western blot (Fig. 1C) analyses. The proteins showed a slight change in apparent mass between reducing and non-reducing conditions, indicating that they form intramolecular disulphide bonds, as can be expected for 6-Cys domain proteins (Fig. 1B, table S2). D6 appeared as a dimeric protein on SDS-PAGE, which was resolved by the addition of reducing agent, indicating intermolecular disulphide bond formation (Fig. 1B). All the antigens appear to be glycosylated, albeit to different extents (Fig. 1D). D12 ran as two different bands on SDS-PAGE gel and both appeared to be glycosylated. Deglycosylation resulted in a single band of smaller size, demonstrating that the two bands represent two

different glycoforms of D12 (fig. S1E). Altogether, we obtained eight Pfs230 single domain antigens in sufficient quantity and purity for mouse immunizations.

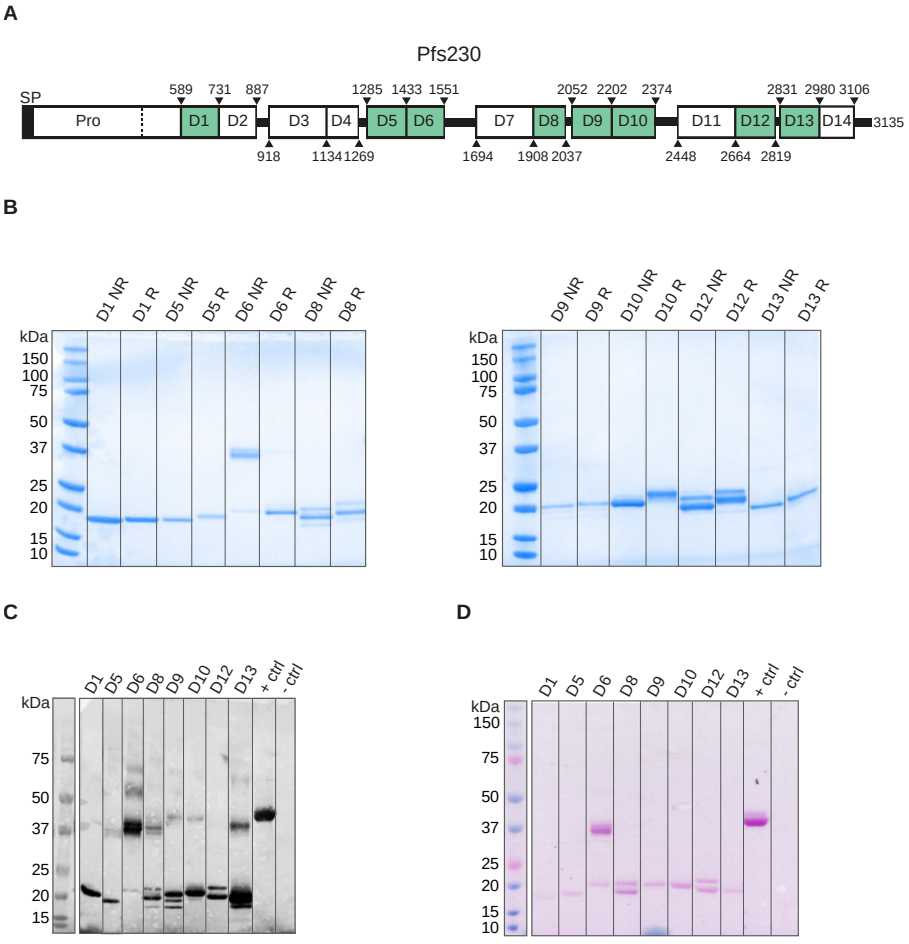



**Figure 1. Recombinant single domain Pfs230 proteins produced in Drosophila melanogaster S2 cells.**

(**A**) Overview of full-length Pfs230. Predicted domain boundaries are indicated by amino acid numbers and are based on predictions made by Gerloff et al. (5). Single domain fragments with these boundaries were expressed in *D. melanogaster* S2 cells. Green colouring indicates successful expression of the fragment. (**B**) Coomassie-stained SDS PAGE gel of purified single domain fragments under reducing conditions (R) and non-reducing conditions (NR). (**C**) Western blot of recombinant fragments with α-C-tag antibody under non-reducing conditions. + and – controls are C-tagged protein and untransfected S2 cell line, respectively. (**D**) Glycosylation-stained SDS PAGE gel of recombinant fragments under non-reducing conditions. + and – controls are control proteins provided with the Pierce™ glycoprotein staining kit.

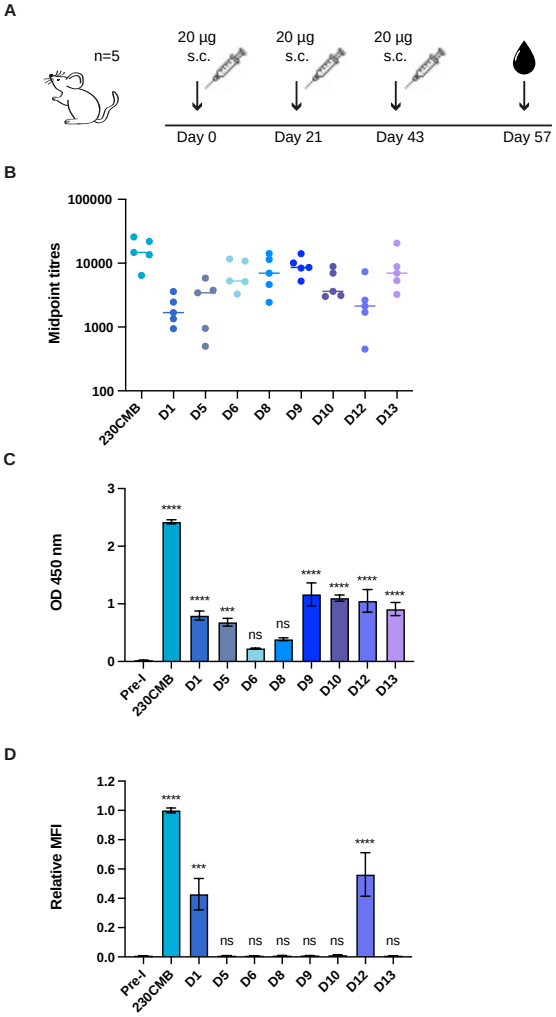



**Figure 2. Antigen and parasite recognition by mouse antibodies raised against single Pfs230 domains.**

(**A**) Overview of mouse immunisation regimen. Groups of five mice were immunised subcutaneously (s.c.) with 20 µg antigen formulated in Montanide ISA-720. One group of mice was immunised with 230CMB (amino acids 444-730) (21) and was included as positive control. Final bleed sera were collected at day 57 for analyses of antibody responses in panels B-D. (**B**) Midpoint titers from antigen specific ELISA. Each dot represents an individual mouse and bars represent median values. (**C**) Gametocyte extract ELISA with pooled mouse sera, tested at 1:100 dilution. Values are means from two independent experiments with three technical replicates each and error bars represent s.e.m. Pre-I, pre-immune serum. (**D**) Female gamete binding assay with pooled mouse sera, tested at 1:40 dilution. Values are means (MFI= Mean Fluorescence Intensity) from two independent experiments with two technical replicates each, and error bars indicate s.e.m. The data was normalised against 230CMB in each independent experiment, to allow averaging across experiments. Statistical analysis in (**C**) and (**D**) done by comparing test groups to pre-immune group using ordinary one-way ANOVA with a Dunnett's multiple comparison test using Pre-I as a reference (ns=not significant, ***=p<0.001, ****=p<0.0001).

## Pfs230D12 mouse antibodies recognize native Pfs230

For each selected Pfs230 protein construct, a group of five female mice was immunized three times with 20 µg antigen formulated in Montanide ISA720 and blood was collected 14 days after the third immunization (Fig. 2A). A positive control group was immunized with 230CMB, a plant-produced protein containing the Pro-domain and D1, that was previously shown to induce antibodies with strong TRA in rabbits [22]. All mice generated antibody responses against the immunogen they were immunized with (Fig. 2B, fig. S2). One mouse in the D12 group showed very low antibody responses (fig. S2) and sera from this mouse were therefore excluded from further analyses. Antibodies in pooled mouse sera recognized native Pfs230 in ELISA with gametocyte extract at different intensities (Fig. 2C). Sera raised against D1, D5, D9, D10, D12 and D13 showed statistically significantly higher recognition compared to pre-immune sera. Recognition by sera against D6 and D8 was weaker and not statistically significant. We also tested recognition of native Pfs230 on the surface of live female gametes. Strikingly, only sera generated against 230CMB, D1 and D12 bound to the surface of female gametes as assessed by flow cytometry (Fig. 2D) and microscopy (fig. S3). Together, the results indicate that while sera raised against most single domain constructs recognize Pfs230 in parasite extract, only sera against D1 and D12 recognize Pfs230 on live female gametes.



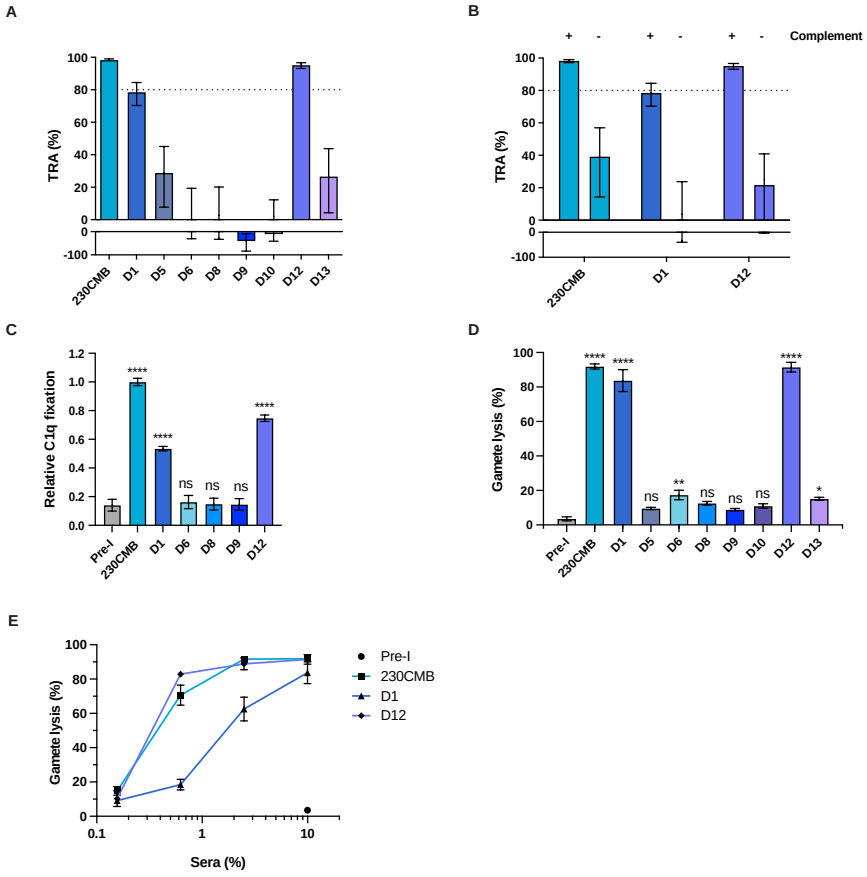
## Pfs230D12 antibodies block transmission of *Plasmodium falciparum* NF54

To assess functional activity of the domain-specific antibodies, we performed standard membrane feeding assays (SMFA). In these assays, we allowed laboratory-reared *Anopheles stephensi* mosquitoes to feed on a mixture of cultured *P. falciparum* NF54 gametocytes and sera from immunized mice, and after 6-8 days we counted oocysts in the mosquito midgut to calculate TRA. In line with previous studies, sera raised against 230CMB and D1 showed strong TRA when tested at 9-fold dilution, reducing oocyst formation by 98.3% (95% CIs: 97.2-99.0) and 78.5% (95% CIs: 70.3-84.4) respectively (Fig. 3A). Interestingly, D12 sera also showed strong TRA (95.2% TRA, 95% CIs: 93.1-96.6). Testing more dilute 230CMB and D12 sera showed that these sera retained TRAs of >80% at 72-fold and 36-fold dilution, respectively (fig. S4). At 36-fold dilution, D12 sera showed a strong TRAs of 92.1% (95% CIs: 89.64-94.0). Sera raised against other domains showed very low or no TRA, which is consistent with the binding assays where the antibodies in these sera failed to recognize the gamete surface (Fig. 2D). Mass spectrometry confirmed purity of the D12 immunogen (table S3-4), and western blots with gametocyte extract and single domain fragments expressed with the wheat germ cell-free system further confirmed that the functional antibodies were D12-specific (fig. S5).

Since the vast majority of functional Pfs230 antibodies described to date are complement-dependent, we tested sera against 230CMB, D1 and D12 in SMFA with either active or heat-inactivated human complement (Fig. 3B). Sera showed substantial TRA only in the presence of active complement, demonstrating the complement-dependency of antibodies against these domains. To confirm that the complement-dependent activity is mediated by classical pathway activation, the ability of the mouse antibodies to fix C1q on the parasite surface was assessed in a flow cytometry assay with live female gametes. Antibodies against 230CMB, D1 and D12 were able to mediate C1q deposition, while antibodies against D6, D8 and D9 failed to do so (Fig. 3C), in line with the SMFA results. We then tested whether the deposition of C1q leads to lysis of the female gamete in a flow cytometry lysis assay (Fig. 3D-E). In this assay purified female gametes are incubated with mouse sera and human complement, and after incubation the percentage lysis is determined by live/dead staining. Sera against 230CMB, D1 and D12 showed over 80% lysis when tested at 1:10 dilution (Fig. 3D). Other sera showed little, in most cases non-significant, lysis, which is consistent with results from the C1q deposition assay and SMFA. Titration of mice sera demonstrated similar potency for 230CMB and D12 sera, while D1 sera had slightly lower potency, similar to the trend observed in SMFA. Taken together, these data indicate that the D12 antigen can induce functional antibodies in mice capable of reducing transmission of lab-cultured malaria parasites to mosquitoes in a complement-dependent manner.

**> Figure 3. Functional activity of mouse antibodies against single Pfs230 domains.**

(**A**) Transmission reducing activity (TRA) of pooled mouse sera (day 57) in standard membrane feeding assay (SMFA) with cultured *Plasmodium falciparum* NF54 gametocytes and *Anopheles stephensi* mosquitoes. Values are estimates from two independent SMFA experiments with oocyst counts for 16-20 fully-fed mosquitoes per condition each. Dotted line indicates 80% TRA, which has previously been established as threshold for clinical development (22). Error bars indicate 95% confidence intervals. Sera were tested at a dilution of 1:9 in the presence of active human complement. (**B**) TRA of pooled mouse sera in SMFA, in the presence of active (+) or heat-inactivated (-) human complement. Pooled mouse sera were tested at a final dilution of 1:9. Values are estimates from two independent experiments with oocysts counts for 20 fully-fed mosquitoes per condition per experiment. (**C**) C1q deposition on the surface of female gametes in the presence of 2.5% pooled mouse serum, as assessed by flow cytometry. Values are means from two independent experiments with two technical replicates each and error bars indicate s.e.m. Data was normalised against 230CMB to allow averaging across experiments. Pre-I, pre-immune serum. (**D**) Female gamete lysis assay with 10% pooled mouse serum and active human complement. Values are means from two independent experiments with two technical replicates each and error bars indicate s.e.m. 0% lysis is defined as the number of live gametes after incubation with human complement only. Statistical analysis in (**C**) and (**D**) was done by comparing test groups to pre-immune group using ordinary one-way ANOVA and accounted for multiple comparisons by Dunnett's multiple comparison test (ns=not significant, *=$p<0.05$, **=$p<0.01$, ****=$p<0.0001$). (**E**) Pooled mouse sera were titrated in the gamete lysis assay. Values are means from two independent experiments with two technical replicates each and error bars indicate s.e.m.




## Murine Pfs230D12 antibodies reduce transmission of naturally circulating gametocytes

To assess the functionality of D12 antibodies against naturally circulating gametocyte strains, we performed direct membrane feeding assays (DMFA) using blood of naturally infected gametocyte carriers from Burkina Faso. After removal of autologous plasma, red blood cells were mixed with pooled mouse sera and normal human serum containing complement, and fed to mosquitoes. After seven days oocysts were counted and TRA was calculated. We tested 230CMB and D12 sera that both showed strong TRA in SMFA, and further included pre-immune and D5 sera as negative controls. While D5 sera did not reduce oocyst formation, in line with SMFA results, the 230CMB and D12 sera reduced oocyst numbers across three independent experiments (Fig. 4A-C). The estimated TRA values were 70.8% (95% CIs: 59.7-78.9) and 95.1% (95% CIs: 92.4-96.8) for 230CMB and D12 sera respectively (Fig. 4D). The DMFA results thus show that D12 antibodies have strong TRA against naturally circulating gametocytes.

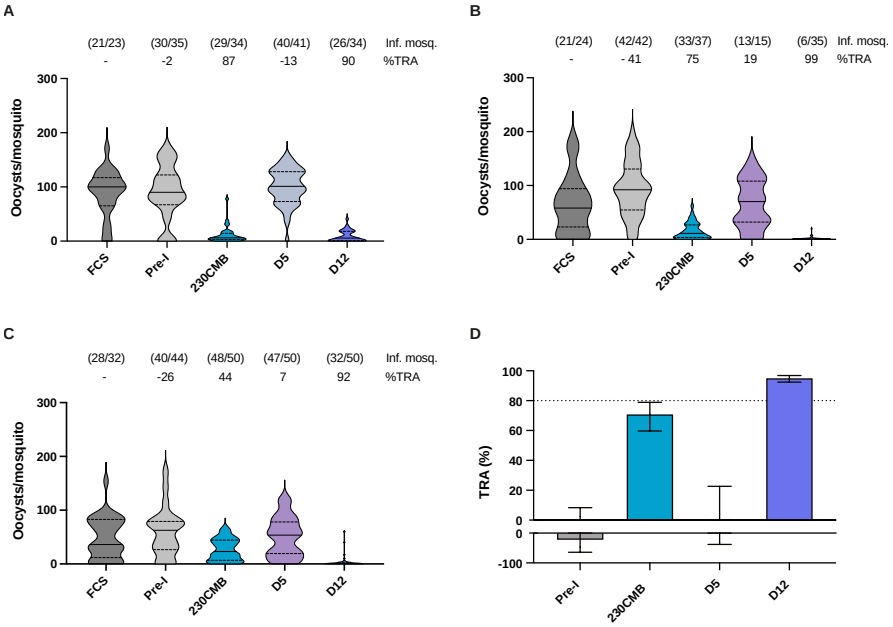



**Figure 4. Transmission-reducing activity of Pfs230D12 sera in direct membrane feeding assay (DMFA) with naturally circulating gametocyte strains from volunteers in Burkina Faso.**

(**A-C**) Gametocytes from three volunteers were fed to *Anopheles coluzzii* mosquitoes, in the presence of mouse sera (day 57) and human complement. Pre-I, pre-immune serum. Data are shown as violin plots with the median indicated as a line and the interquartile range (IQR) (Q1 and Q3 quartiles) as dotted lines. Values above bars indicate percentage TRA, and the number of infected mosquitoes and the total number of mosquitoes between brackets (# infected mosquitoes/# total mosquitoes). (**D**) Estimate TRA values from three independent experiments (**A-C**) combined. Bars are estimated means and error bars indicate 95% confidence intervals. Dotted line indicates 80% TRA, which has previously been established as threshold for clinical development (22).

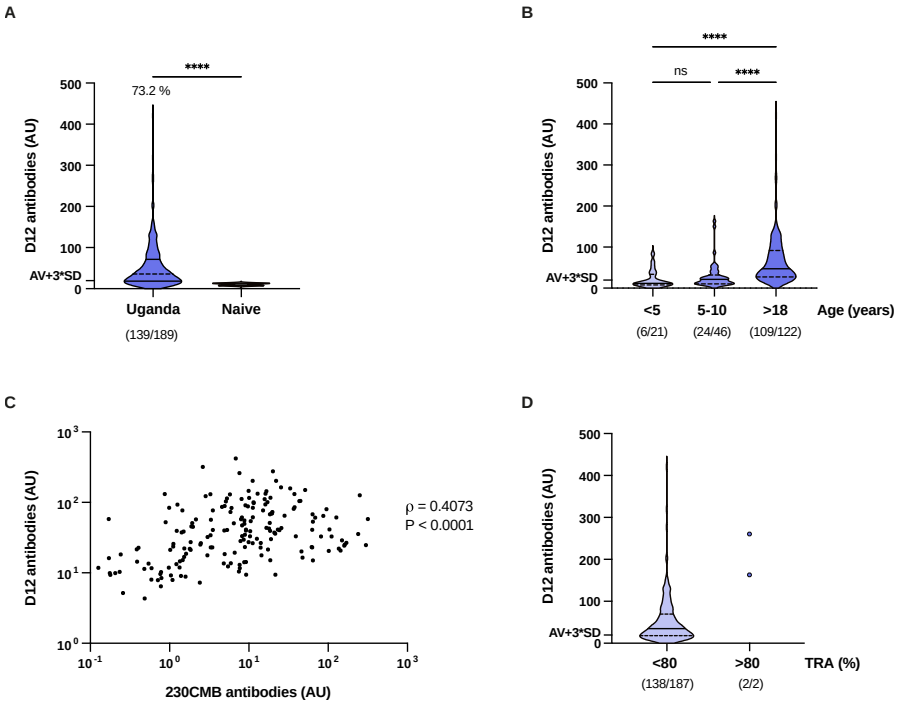



**Figure 5. Recognition of Pfs230D12 by plasma from volunteers naturally exposed to *Plasmodium falciparum*.**

(**A**) Antibody levels against D12 in Ugandan plasma samples, as determined by ELISA. AU are arbitrary units calculated using a highly reactive plasma pool from Tanzania as reference. Naïve samples are pooled plasma samples from malaria-naïve Dutch donors (n=8). Groups were compared by one-sided Mann-Whitney test (****=p<0.0001). The threshold for positivity is marked and defined as the mean of naïve controls plus three standard deviations (AV + 3*SD). Percentage of positive samples is indicated above the graph. The number of positive samples and the total number of samples are depicted in between brackets (# positive samples/# total samples). (**B**) Antibody levels stratified by age. Groups were compared by Kruskal-Wallis test with Dunn's correction for multiple testing (ns=not significant, ****=p<0.0001). Number of samples per age group are shown below the graph. (**C**) Correlation between antibody levels against D12 and 230CMB (Spearman's ρ = 0.4073, p < 0.0001). Arbitrary units against 230CMB are calculated using serum from an individual that had high levels of antibodies against D12 (Donor A (24)). Five individuals with 230CMB-specific antibody levels below 0.1 AU are not shown on graph. (**D**) Antibody levels stratified by TRA. Number of antibody positive over total tested individuals per group are shown below the graph. Data in (**A**), (**B**) and (**D**) are shown as violin plots with the median indicated as a line and the interquartile range (IQR) (Q1 and Q3 quartiles) as dotted lines.

### Pfs230D12 is recognized by sera from individuals naturally exposed to *P. falciparum*

To assess natural antibody responses to D12, we screened plasma samples from a cohort of individuals residing in Tororo, an area in eastern Uganda where, at the time of sampling (2013-2017), transmission intensity was intense and perennial [23]. Children ≤ 10 years of age and adults were eligible for enrolment. Purified IgG samples of these cohort participants were tested for transmission-reducing immune responses in the SMFA, as described above, as part of a larger study on naturally acquired transmission-reducing immunity. We detected significantly higher antibody levels in Ugandan samples compared to naïve control samples from Dutch donors (Mann-Whitney test, $p < 0.0001$) (Fig. 5A). 139 out of 189 (73.2%) individuals were seropositive for D12. Antibody intensity was significantly higher in adults compared to school aged children (5-10 years old) and younger children (<5 years old) (Kruskal-Wallis test, $p < 0.0001$) (Fig. 5B). We observed a statistically significant correlation between antibody responses against 230CMB and D12 (Spearman's, $\rho = 0.4073$, $p < 0.0001$) (Fig. 5C). Whilst numbers were too small for meaningful statistical comparisons, we observed very strong D12 responses in two individuals whose total IgG isolated from plasma showed strong TRA in SMFA (Fig. 5D). Together, we found that individuals living in a malaria-endemic country can generate antibodies against D12 and that antibody levels increase with age.

## Discussion

Here, we expressed eight individual domains of Pfs230, a *P. falciparum* protein that is essential for parasite transmission and forms the basis of current malaria TBV development. Antibodies raised against the D12 domain showed potent binding to live female gametes *in vitro* and have strong functional TRA. Functional activity was demonstrated against lab-cultured gametocytes and gametocytes from naturally-infected parasite carriers from Burkina Faso. Furthermore, sera from Ugandan donors naturally exposed to *P. falciparum* showed immune recognition of D12 in an age-dependent manner. Taken together, our results identify Pfs230D12 as a promising TBV candidate.

TBVs could be valuable tools for the elimination and eradication of malaria. Several TBV candidates have been identified, of which the ProD1 fragment of Pfs230 has progressed furthest in terms of clinical testing. This study aimed to comprehensively examine constructs outside ProD1 and found that D12 can elicit functional antibodies. Two earlier immunization studies included fragments containing D12,

produced in *Escherichia coli* [8] and wheat germ cell-free system [9]. These constructs induced antibodies that recognized native Pfs230 on gametes, but the antibodies did not reduce transmission to mosquitoes [8,9]. This is in sharp contrast with our results that show high TRA for mouse antibodies raised against D12 produced in *D. melanogaster* S2 cells. It is likely that the expression system plays an important role with *D. melanogaster* S2 cells producing (more) properly folded D12 that is critical for raising functional antibodies (Fig. 1B). This is in line with preclinical studies with the other 6-Cys family protein Pfs48/45 that showed that the host expression system and proper conformation of the antigen are essential for inducing functional responses [24].



While antibodies raised against the D12 antigen showed strong gamete recognition and functional TRA, we did not observe functional responses against D5, D6, D8, D9, D10 and D13 (Fig. 3A). Strikingly, antibodies against most of these domains recognized native Pfs230 in gametocyte extract, but did not recognize Pfs230 on live gametes (Fig. 2C-D) suggesting that epitopes on these domains are occluded by other parasite surface proteins that interact with, or are in close proximity of, Pfs230. Alternatively, epitopes on these domains may be close to the parasite membrane and therefore not accessible to antibodies. However, we cannot rule out that these domains of Pfs230 do contain functional epitopes, as the functional epitopes could have been absent in our recombinant antigens due to misfolding or masking by glycosylation in the *D. melanogaster* S2 cell expression system (Fig. 1C). Recently emerged cryogenic electron microscopy structures of the native full-length Pfs48/45:Pfs230 complex provide further insight into which Pfs230 domains are membrane distal and surface exposed, and as such more likely to contain functional epitopes [25,26].

Most if not all functional Pfs230 antibodies described to date are dependent on complement [4,12,13,16,27-29]. The antibodies we raised against D12 are no exception to this rule. The complement-dependency of the D12 antibodies was shown in membrane feeding assays where antibodies lacked TRA in the presence of heat-inactivated complement (Fig. 3B). The D12 antibodies can activate the classical pathway by fixing C1q (Fig. 3C) and induce complement-mediated gamete lysis *in vitro* (Fig. 3D-E), which is the presumed effector mechanism in the mosquito midgut.

The viability of D12 as vaccine candidate depends on several factors. In general, vaccine candidates should target conserved functional epitopes to generate cross-strain protection, should ideally be able to induce highly potent antibodies so lower overall antibody responses are needed for protection and should be immunogenic in humans. Like other sexual stage *Plasmodium* proteins, Pfs230 is well

conserved; D12 contains a similar number of non-synonymous single nucleotide polymorphisms, approximately 10 per kb coding sequence, as the leading TBV candidate ProD1 [17]. The D12-specific mouse antibodies not only block transmission of the reference strain *P. falciparum* NF54, but also block transmission of naturally circulating gametocytes that may be genetically diverse (Fig. 4). However, whether the functional antibodies indeed target conserved epitopes on D12 is currently unknown and should be the focus of future research. This could be addressed by isolating and characterising monoclonal antibodies, either from immunized animals or from human donors with naturally acquired immunity, and assessing their structure-function relationships as recently done for ProD1 [27,28,30]. Furthermore, we observed that many individuals in a Ugandan cohort were seropositive for D12, demonstrating that the D12 antigen is immunogenic in humans and that vaccine-induced antibody levels may be boosted by natural exposure and *vice versa*. Naturally acquired transmission reducing immunity is a rare phenomenon, at least at high levels of TRA that can be reproducibly demonstrated in the SMFA [31]. Whilst now allowing for a formal assessment of a possible role of D12-specific antibodies in naturally acquired TRA, we made use of a larger cohort study where plasma samples were available alongside TRA estimates. Two individuals with high levels of TRA also had high levels of antibodies against D12. The strong correlation between anti-D12 antibodies and anti-Pfs230CMB antibodies makes it impossible to determine whether D12 antibodies were causally responsible for TRA. Purification of D12-specific antibodies from plasma and assessment of their TRA in SMFA [31] would allow us to demonstrate causality but this was not possible with the plasma volumes available. Nevertheless, our findings demonstrate that D12 antibodies are naturally acquired in an age-dependent manner – probably reflecting cumulative exposure to *P. falciparum* gametocytes – in a manner that is similar to antibodies to other Pfs230 domains and that individuals with naturally acquired TRA can have high levels of D12 antibodies.

In our immunisation studies we included 230CMB [22], containing ProD1, as positive control. It is encouraging to see that our non-optimized D12 immunogen induced TRA levels that are close to those induced by 230CMB (Fig. 3A, Fig. 4C and fig. S4), and that D12 sera showed similar gamete lysis activity as ProD1 sera (Fig 3E). Future studies should assess whether the D12 antigen could be further optimized to induce stronger functional responses, for instance through coupling to carrier proteins, screening of glycosylation mutants, adjuvant screening and/or designing immunogen variants that are more stable or have improved epitope display. It will also be interesting to determine whether antibodies against D12 are synergistic or additive with antibodies against other TBV candidates such as Pfs230D1-EPA,

as combining antigens may be an attractive approach to increase overall vaccine efficacy, and to formally compare the potency of different vaccine candidates.

This study has several limitations. We aimed to assess all single domains of Pfs230 in our immunisation study but could not produce six of the fourteen Pfs230 domains. These included D4 and D7, which have been shown to be targets for functional mAbs raised against whole parasites [17,18]. Whether these domains can be produced recombinantly to induce functional antibodies thus remains unclear. Furthermore, one of the mice in the D12 group showed very low antibody responses to the immunogen (fig. S2). Whether this low response is linked to the immunogen itself, or whether this was caused by external factors is currently unclear and should be assessed in future immunisation studies. Finally, we could test the mouse sera in only two DMFA experiments due to logistical challenges. The results from these DMFA experiments should therefore be interpreted with caution and warrant more in-depth studies to assess cross-strain protection.



In conclusion, our work shows how employing a different expression system to produce recombinant domains of Pfs230 can lead to the identification of a malaria TBV candidate and that Pfs230D12 is a promising candidate for further preclinical investigation.

## Materials and Methods

### Protein construct design

Expression plasmids were created for all fourteen domains of Pfs230 (Fig. 1A). Boundaries of the domains were based on previous research [5] and the linkers between domains were excluded. Sequences were codon optimized for expression in *Drosophila melanogaster* and synthesized (BaseClear). The Pfs230 single domains were cloned with an N-terminal BiP signal peptide, $His_6$-tag and Alanine-serine linker, and a C-terminal glycine-serine linker followed by a C-tag into the pExpreS2.2 plasmid (ExpreS2ion Biotechnologies), downstream of the Actin+HSP70 promoter. The plasmids were verified by Sanger sequencing (Baseclear). The sequences of the inserts can be found in table S1.

### Drosophila melanogaster S2 cell transfection and culture

The *D. melanogaster* S2 cell line (ExpreS2ion Biotechnologies) was used for the expression of all Pfs230 protein constructs. S2 cells were cultured in shake flasks with vented cap in EX-CELL420 media (Sigma-Aldrich), supplemented with 1% penicillin and streptomycin, at 25°C shaking 115 rpm. Cells were counted twice a

week and resuspended to $8\times10^6$ cells/mL alternately by dilution or centrifugation. For transfections, 2.5 mL cell suspension was mixed with 6.25 µg plasmid DNA and 25 µL ExpreS2 Insect-TR 5x transfection reagent (ExpreS2ion Biotechnologies) in a T12.5 T-flask. The transfected cells were then incubated at 25°C, and 1 mL of FBS was added after 3 hours. 4000 µg/mL geneticin was added as a selection agent after 24 h. Approximately 26 days after the transfection, the cultures were scaled up to shake flasks. During this step FBS and geneticin were removed by centrifugation and resuspending cells in EX-CELL420 to $8x10^6$ cells/mL. Supernatant was harvested 5 days after the cells were diluted, for protein expression analysis on western blot and protein purification.

## Protein purification

The S2 cell supernatant was concentrated from 200-300 mL to approximately 50 mL using the Masterflex EasyLoad (Masterflex). The Pfs230 single domain protein constructs were affinity purified with CaptureSelect C-tagXL pre-packed columns (Thermo Scientific) on an ÄKTA start (Cytiva), using 20 mM Tris wash buffer (pH 7.4) and 20 mM Tris + 2 M $MgCl_2$ (pH 7.4) elution buffer. Peak fractions from the chromatogram were pooled and dialysed overnight in PBS. The sample was filtered and concentrated to approximately 600 µL. Subsequently, the sample was further purified using a Superdex75 10/300 GL column (Cytiva) with filtered and degassed PBS as running buffer. For Pfs230D9 and Pfs230D3 0.2% Empigen® BB (Sigma-Aldrich) was added to all purification buffers to decrease aggregation of the proteins. Superdex fractions containing pure monomer protein were pooled and the protein concentration measured using a Nanodrop spectrophotometer (Thermo Scientific). Samples were frozen in liquid nitrogen and stored at -70°C.

## SDS-PAGE analysis

For analysis of proteins from S2 expression, samples were mixed with 4x NuPAGE LDS sample buffer (Invitrogen), heated at 70°C for 10 min before loading on a 4-20% bis-tris polyacrylamide gel (GenScript). In the case of purified protein 1 µg protein was loaded per condition and the Precision Plus Dual Color protein marker (Bio-Rad) was used as size standard. The gels were stained for 30 minutes using Instant Blue Coomassie Protein Stain (Abcam). To reduce disulphide bonds, a final concentration of 10 mM dithiothreitol (DDT) was added in the preparation of the sample.

For protein analysis with parasite extract, *P. falciparum* NF54 gametocyte extract was prepared as described previously [32] and diluted to the equivalent of 500,000 gametocytes per well. A final concentration of 10 mM dithiothreitol (DTT) was added for reducing conditions. Gametocyte extract and 230CMB [22] samples were mixed

with 4× NuPAGE™ LDS sample buffer and heated for 10 minutes at 70°C before loading on a 4–20% Bis-Tris gel (GenScript). 20 ng 230CMB was loaded per well and the Precision Plus Dual Color protein marker (Bio-Rad) was used as size standard.

For analysis of proteins expressed in wheat germ cell-free system, purified proteins (described previously [9,18]) were mixed with SDS-sample buffer and TCEP-HCl (Pierce™), and denatured at 37°C for 30 minutes before loading on a 12.5% PAGE Tris gel (ATTO, Tokyo, Japan). 0.5 µg of each protein was loaded per well and Precision Plus Protein All blue standard (Bio-rad) was used as the size standard.



## Western blot analaysis

For western blot analysis of proteins expressed in S2 cells, a positive control with C-tag (Pro-CS3-6C, kindly gifted by Susheel Singh, 36.3kDa) and negative control (Pf3D7_1306500C no C-tag, transfected in S2 cells) was included. The bis-tris gels were blotted on a 0.45 µm nitrocellulose membrane using the TurboBlot system (Bio-Rad). The membranes were washed in between steps with PBS supplemented with 0.05% Tween20 (PBST), blocked overnight in 5% skimmed milk PBS (mPBS) at 4°C, and incubated for 1 hour at room temperature (RT) with the CaptureSelect Biotin Anti-C-tag conjugate (1/1000, Cat. No. 7103252100, Thermo Scientific) in 1% mPBST. Thereafter the membranes were incubated with 1/2500 IRDye Streptavidin 680LT (Cat. No. 926-68031, LI-COR) in 1% mPBST for 1 hour at RT. The blot was developed with Clarity Max Western ECL substrate (Bio-Rad) and imaged with the Odyssey CLX (LI-COR).

For western blots with gametocyte extract, gels were transferred to a 0.45 µm nitrocellulose membrane using the Trans-Blot Turbo transfer system (Bio-Rad). The blots were blocked with 5% skimmed milk in PBS before incubation with 1/5000 polyclonal serum from mice immunized with Pfs230D12. After washing, the strips were incubated with 1/3000 diluted polyclonal rabbit anti-Mouse IgG HRP (Cat. No. P0260, DAKO). Blots were developed with Clarity Max Western ECL substrate (Bio-Rad) and imaged on the ImageQuant™ LAS 4000 (GE Healthcare).

For western blot analysis of proteins expressed in wheat germ cell-free system, the SDS-PAGE gels were transferred to an Amersham Hybond P Low fluorescence 0.2 µm PVDF membrane (Cytiva) using the Trans-blot SD semidry transfer cell (Bio-Rad) (25 V, 126 mA/gel, 75 minutes). The blots were blocked with 5% skimmed milk in PBST before incubation with 1/1000 polyclonal serum from mice immunized with Pfs230D12 (diluted in PBST) for 1 hour at RT and overnight at 4°C. After washing, the blots were incubated with 1/10,000 polyclonal Sheep anti-Mouse IgG

HRP (Cat. No. NA931VS, Cytiva). Blots were developed with Immobilon Western chemiluminescent HRP substrate (Cat. no. WBKLS0500, Millipore) and imaged on the LAS-4000 (FUJIFILM, Tokyo, Japan) for 13 minutes.

## Glycosylation staining and deglycosylation

Glycosylation of Pfs230 domains was assessed using the Pierce Glycoprotein Staining Kit (ThermoFisher Scientific). 5 µg of each Pfs230 protein construct was loaded on gel and the gel was stained following manufacturer's instructions. D12 was deglycosylated with PNGase F (New England Biolabs) under denaturing/reducing conditions, following manufacturer's protocol. D12 was denatured with 0.5% SDS and 40 mM DTT for 10 minutes at 100°C. After cooling down the sample on ice, 1% NP-40, 50 mM Sodium Phosphate pH 7.5 and PNGase F were added and the sample was incubated for 1 hour at 37°C, followed by analysis on SDS-PAGE gel.

## Mass spectrometry

The identity of recombinant Pfs230D12 was confirmed by mass spectrometry. 5 µg of purified protein was denatured in 4M urea, 100 mM Tris-HCl (pH 8.0) and disulfide bonds were reduced using 10 mM DTT for 30 minutes at RT. Cysteines were alkylated using 50 mM Iodoacetamide for 30 minutes, and samples were diluted to 2M Urea, using 100 mM Tris-HCl (pH 8.0). Samples were digested overnight with 0.5 µg Trypsin at 25°C. Next day, samples were desalted using StageTips [33].

Peptides were analyzed using an Easy nLC 1000 equiped with a 30 cm reverse phase column, coupled on-line to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). A 60 minute gradient of buffer B (80% acetonitrile, 0.1% formic acid) was applied and the mass spectrometer was operated in TopS mode with a dynamic exclusion of 60 seconds.

RAW data was analyzed using Maxquant [34] version 1.6.6.0 with a *Drosophila* database supplemented with sequences for single domain constructs of Pfs230. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD039716 [35].

## Mice immunization

45 female 6-8 weeks old CD-1 mice (Charles Rivers), divided in groups of 5 mice, were immunized with 230CMB, D1, D5, D6, D8, D9, D10, D12 and D13. 230CMB, a construct comprising aa 444-730 of Pfs230 produced in a plant-based expression system [22] and known to induce transmission reducing capacity was included as positive control. Mice were injected subcutaneously with 100 µL of 0.2 mg/mL

antigen in 70% Montanide ISA720 (SEPPIC) at day 0, day 21 and day 43. Pre-bleed samples were collected at day -1 and final bleeding was performed at day 57 before sacrificing (Fig. 2A). Blood was allowed to clot at RT for 30 minutes, and serum was collected after centrifugation and was stored at -20°C. For each group of mice sera, samples were pooled for further analysis. All animal procedures complied with national regulations and were approved by the ethics committee of the Radboud University Medical Center.

## Enzyme-Linked Immunosorbent Assays (ELISA)



For antigen ELISA to assess antigen-specific antibody responses in mice, Nunc MaxoSorp 96-wells plates (ThermoFisher) were coated with 100 µL of 1 µg/mL antigen and incubated overnight at 4°C. Plates were washed three times with PBS in between incubation steps. Plates were blocked with 5% mPBS for 1 hour. Plates were incubated with serum samples diluted in 1% mPBST, for 3 hours at RT. Subsequently, the plates were incubated with polyclonal Rabbit Anti-Mouse HRP (1/3000 dilution, Cat. No. P0260, DAKO) for 2 hours at RT. The ELISA was developed by adding 100 µL tetramethylbenzidine (TMB). The color reaction was stopped by adding 50 µL 0.2 M $H_2SO_4$ and the optical density was read at 450 nm on an iMark™ microplate absorbance reader (Bio-Rad).

For the gametocyte ELISA, *P. falciparum* NF54 gametocyte extracts were prepared as described previously [32]. 100 µL lysate per well, equivalent to 75,000 gametocytes, was pipetted into Nunc MaxiSorp™ 96-wells plates (ThermoFisher) and incubated overnight at 4°C. The next steps of the ELISA were performed as described above.

D12-specific antibody levels in sera from individuals exposed to malaria parasites were assessed using an antigen ELISA as described above. Goat anti-Human IgG (H+L) HRP (1/40,000 dilution, Cat. No. 31412, Invitrogen) was used for detection. A titration of pooled hyperimmune serum from gametocyte carriers in Tanzania was used to calculate arbitrary units using ADAMSEL FPL (http://www.malariaresearch.eu/content/software).

## Gamete purification

To obtain purified female gametes for flow cytometry assays, we collected N-acetyl glucosamine treated 16-day old *P. falciparum* NF54 gametocyte cultures. The cultures were centrifuged for 10 minutes at 2,000×g at RT, to be resuspended in FBS using a volume that equals half the original culture volume. Gametocytes were placed on a roller bank for 45 minutes at RT for activation and thereafter centrifuged for 10 minutes at 2,000×g at 4°C. The pelleted gametes were resuspended in

1 mL PBS, loaded onto a 7 mL layer of 11% w/v Accudenz (Accurate Chemical) and centrifuged for 30 minutes at 7,000×g at 4°C without brake (Sorvall RC-5B Superspeed Centrifuge with HB-4 swing-out rotor). The female gametes present in the top layer were collected, transferred to a 50 mL tube and PBS was added up to 50 mL total volume. A final centrifugation for 5 minutes at 2,000×g at 4°C was done to pellet the gametes, which were resuspended in 1 mL PBS and counted using a Bürker-Turk counting chamber.

## Flow cytometry

For the assessment of gamete antibody binding, gamete C1q deposition and gamete lysis we used similar flow cytometry assays with specific adjustments that are described in this paragraph. All gamete incubations were carried out in PBS supplemented with 2% FBS and 0.02% sodium azide. For all three assays 50,000 purified gametes were used per well in a V-bottom non-treated 96-well plate (Costar) and were incubated for 1 hour at RT with mice sera. In the case of a lysis assay, there is an addition of 20% normal human serum (NHS) and the incubation is reduced to 30 minutes at RT. For a C1q deposition assay this is reduced to 10% NHS (30 minutes incubation at RT). Plates were centrifuged at 2,000×g for 3 minutes at 4°C and washed three times with PBS. The C1q deposition assay includes additional steps; first PBS supplemented with 10 mM EDTA is added for 5 minutes at 4°C to inactivate complement. Second, after 3 washes with PBS, 1/5000 anti-C1q goat anti-human polyclonal serum (Complement Technology) is added for 30 minutes incubation at RT. Gametes were washed and then incubated with either 1/200 Alexa Fluor™ 488 Chicken anti-Mouse IgG (H+L) (Invitrogen) (binding assay), 1/200 anti-Pfs47 (rat mAb 47.1) [36] labelled with DyLight™ 650 NHS ester (Thermo Scientific) (lysis assay) or 1/200 Alexa Fluor™ 488 Donkey anti-Goat IgG (H+L) (Invitrogen). 1/1000 eBioscience™ Fixable Viability Dye eFluor™ 780 (Invitrogen) was added in all assays and gametes were incubated for 30 minutes at RT. After washing with PBS, samples were resuspended in 150 µl PBS. Antibody binding to gametes, lysis of gametes and C1q deposition on gametes were assessed by flow cytometry by analysing a minimum of 2,000 gametes with the Gallios™ 10-color system (Beckman Coulter) and analyzed with FlowJo (BD, version 10.7.1) (gating strategy in fig. S6).

*SIFA*Fourteen day-old heparin-treated *P. falciparum* NF54 gametocyte cultures were spun down, reconstituted in half the volume FCS and incubated for 1 hour at room temperature to generate female gametes. Gametes were washed with SIFA buffer (0.5% FCS in PBS) three times. Cells containing female gametes were incubated with pooled mouse serum diluted in SIFA buffer for 1 hour at 4°C. Cells were washed three times with SIFA buffer, after which they were incubated with 1:200

diluted Alexa Fluor 488 goat anti-mouse IgG (H+L) (Invitrogen, A11029) for 1 hour at 4°C. Cells were washed again with SIFA buffer and imaged using a Axio Observer 7 Inverted LED microscope equipped with a Colibri 7 LED source and Axiocam 705 mono (Zeiss). Female gametes were first searched in the brightfield channel, after which fluorescence was assessed using the 475 nm LED module.

## SMFA

Mice sera were diluted in FCS to a final volume of 90 µL and mixed with 30 µL NHS and 180 µL mature *P. falciparum* NF54 gametocytes and RBCs. Indicated serum dilutions are calculated are relative to the final blood meal volume, e.g. a mouse sample tested at a nine-fold dilution contains 30 µL mouse serum in a total blood meal volume of 270 µL. To inactivate NHS for conditions where inactive complement is required, it was heated for 30 minutes at 56°C prior to mixing with the gametocytes. *Anopheles stephensi* mosquitoes from a colony maintained at Radboudumc (Nijmegen, the Netherlands) were fed blood meals as described previously [37]. Unfed and partially fed mosquitoes were removed. 20 mosquitoes per condition were dissected 6-8 days after the blood meal to collect their midguts. The midguts were stained with mercurochrome and oocysts were counted. TRA was defined as the reduction in oocyst intensity (oocysts per mosquito midgut) in a test condition compared to a negative control in which no mice sera (FCS control) was added. All samples were tested in two independent SMFA experiments for which the oocyst count data are shown in data file S1.

## DMFA

Gametocyte-infected blood from patients residing in the villages surrounding Bobo-Dioulasso was collected in heparin tubes, 5 mL per tube. Immediately after blood collection, the blood was centrifuged at 3000×g for 5 minutes, and plasma was removed. 120 µl of the remaining RBC pellet was transferred to tubes containing 90 µl of naïve AB serum and 30 µl of mice sera (or FCS as negative control). The total of 240 µl was carefully mixed by pipetting and the content of each tube was transferred to an individual feeder maintained at 37°C to allow *Anopheles coluzzii* mosquito feeding for 30 minutes. Unfed mosquitoes were removed; fullyfed mosquitoes were kept for 7 days post feeding. All surviving mosquitoes were dissected for each condition, midguts were stained with 0.5% mercurochrome for oocyst detection and oocysts were counted.

## Ethical statement

Asymptomatic gametocyte carriers, aged 7 and 10 years, were enrolled in November 2023 from the villages surrounding Bobo Dioulasso (Burkina Faso). Venous blood

samples of the two volunteers were collected after written informed consent was obtained from participants or their guardian(s). Ethical approval was provided by the Ethical Review Committee of the Ministry of Health, Burkina Faso (N°2022-05-093); Institutional ethics review committee for health science research Bobo Dioulasso (A014-2022-CEIRES). For the cohort study in Uganda, ethical approval was obtained from the Makerere University School of Medicine Research and Ethics Committee, the Uganda National Council for Science and Technology, the London School of Hygiene & Tropical Medicine Ethics Committee, the Durham University School of Biological and Biomedical Sciences Ethics Committee, and the University of California, San Francisco, Committee on Human Research.

## Statistical analysis

Transmission reducing activity (TRA) was calculated as the reduction in oocysts compared to a negative control, using an online tool [38]. All other statistical analyses were performed using GraphPad Prism (version 10.1.0).

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## Author contributions

MRI, RMdJ and MMJ designed research
MRI, RMdJ, DFD, LLH, MM, KT, ETB, SG, MvdV-B, G-JvG, RS, HN and CGS performed research
MRI, RMdJ, DFD, LLH, MM, HN, ET, CGS, AC, TB and MMJ analyzed data
ET, TT, MV, RKD, EA, AC and M.M.J. supervised the work
MRI, TB and MMJ wrote the original draft
All authors reviewed the manuscript

## Competing interests

MRI, RMdJ, TB and MMJ are inventors on a patent application about the use of Pfs230 Domain 12 as malaria transmission-blocking vaccine. All other authors declare they have no competing interests.

## Data and materials availability

Materials generated in this study are available upon request. Mass spectrometry data are deposited in the PRIDE database under number PXD039716. All data are available in the main text or the supplementary materials.

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# Supplementary Materials

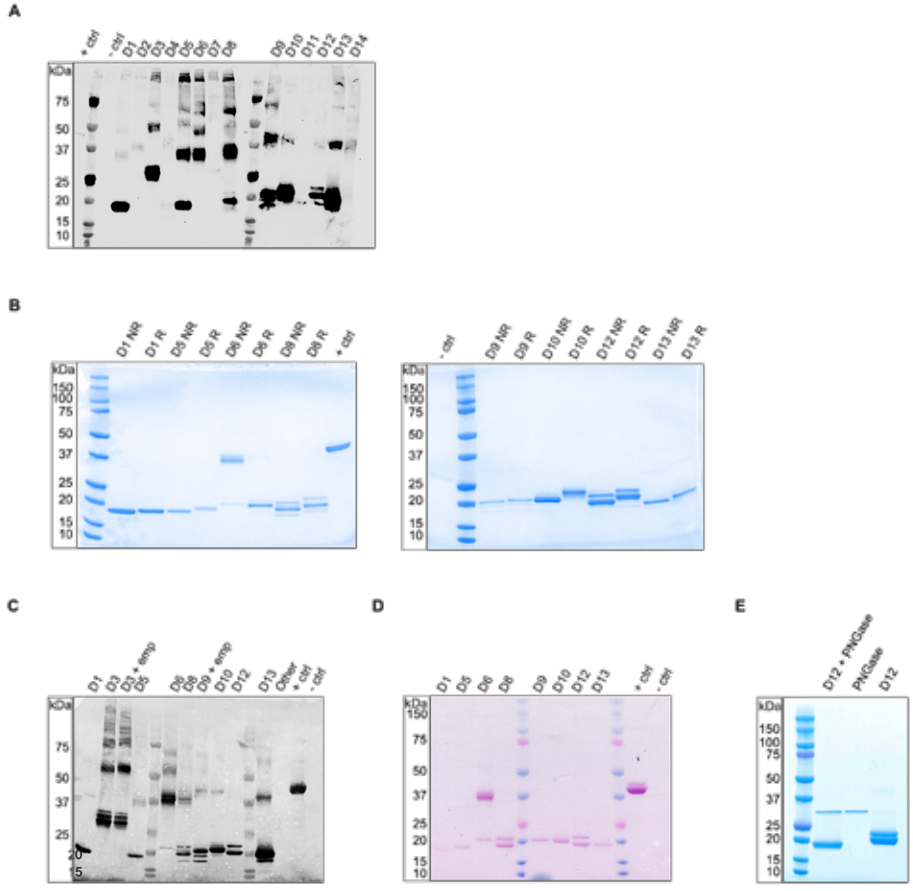



**Figure S1. Analysis of recombinant Pfs230 domain protein constructs produced in S2 cells.** (A) Western blot analysis of S2 cell supernatants. Supernatants of D1 till D14 were harvested from stable transfected cell lines. Supernatants were separated by SDS-PAGE, transferred to western blot, and stained with 1:1000 C-tag antibody and 1:2500 IRDye Streptavidin 680LT. + ctrl: Pro-CS3-6C; - ctrl: supernatant from un-transfected cells. (B) Coomassie-stained SDS-PAGE gels of Pfs230 single domain constructs purified from S2 cells and used for mice immunizations (original version of Figure 1B). NR: non-reduced conditions; R: reduced conditions. (C) Western blot of single domain Pfs230 constructs used for mice immunizations (original version of Figure 1C). (D) Glycoprotein staining after SDS-page (original version of Figure 1D). (E) Coomassie-stained SDS-PAGE gel of deglycosylated D12. D12 was deglycosylated with PNGase F under denaturing and reducing conditions and all samples were analyzed on gel under reducing conditions.

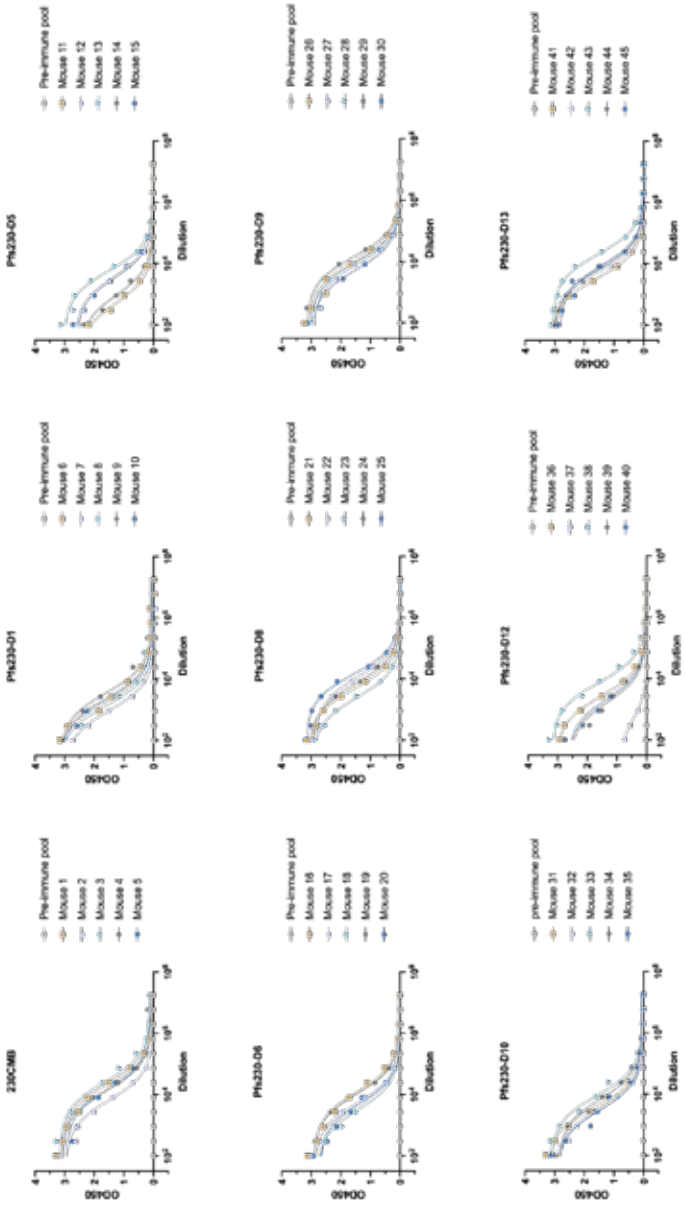



**Figure S2. Antigen specific ELISAs with sera from immunized mice.** The sera of each individual mouse were titrated (12-point titration, in singlicate) along with the pooled pre-immune sera. Sigmoidal curve fits were used to calculate $EC_{50}$ values that are shown in figure 2B.

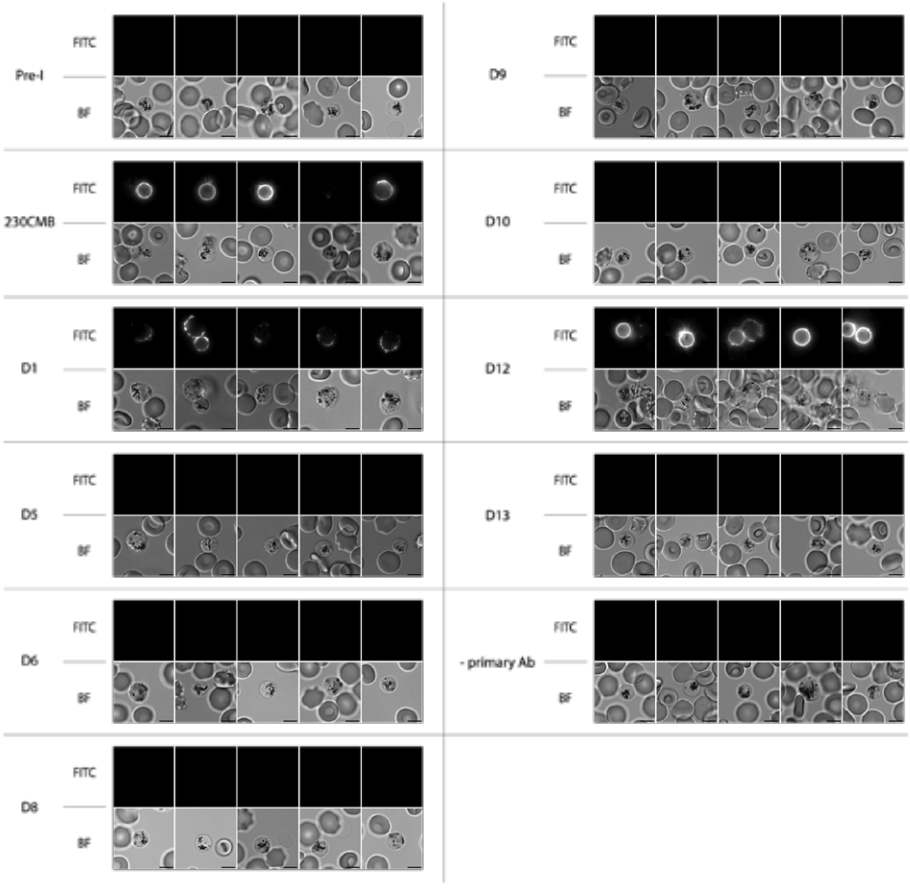



**Figure S3. Recognition of female gamete surface by mouse antibodies.** Pooled mouse serum was tested at 1:100 dilution in surface immuno fluorescence assay (SIFA) with live female gametes. Bound mouse antibodies were detected with a FITC labeled secondary antibody. BF = bright field image. Scale bar indicates 5 µm. Pre-I = pre-immune serum.

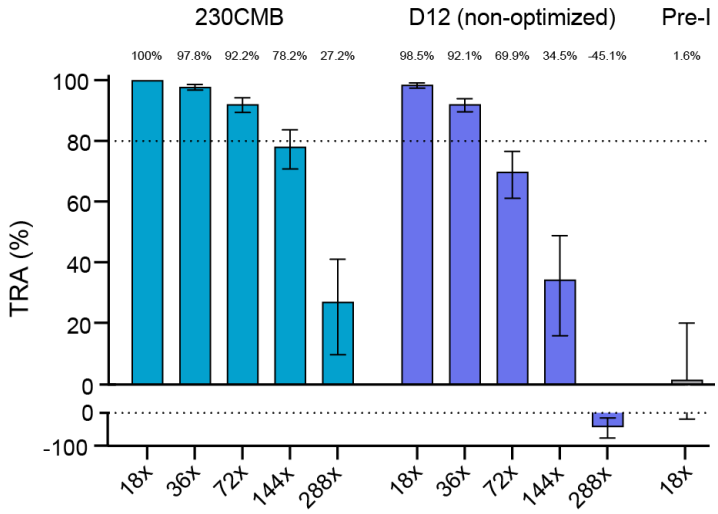



**Figure S4. SMFA with dilution series of 230CMB and D12 serum.**

Pooled mouse serum was tested in a dilution series, with each dilution tested in at least two independent SMFA experiments. Dilution factors are shown below the graph. Bars and values above indicate the estimated TRA. Error bars indicate the 95% confidence intervals. Pre-I = pre-immune serum. Raw oocyst count data are provided in Data S1.

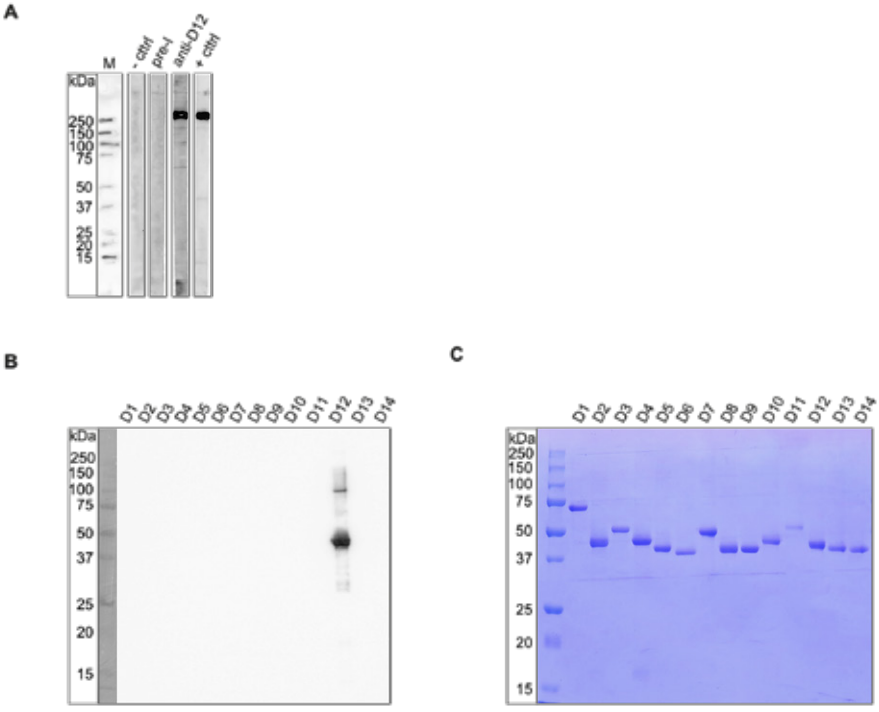
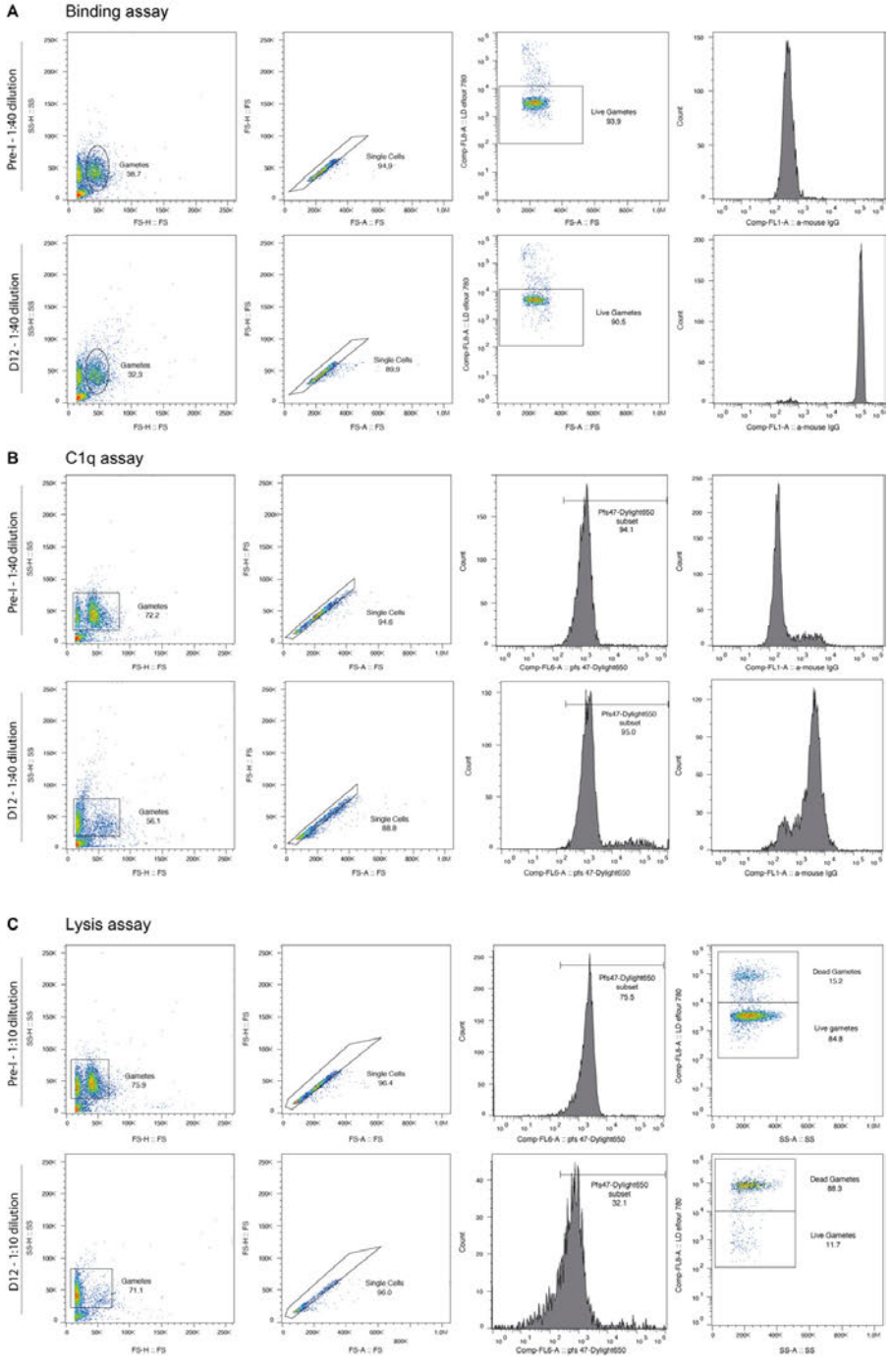



**Figure S5. Pfs230 and Pfs230-D12 specificity of the Pfs230-D12 induced antibodies.** Western blots incubated with mice sera induced by S2 cell-produced Pfs230-D12 showing specific recognition of **(A)** Pfs230 present in gametocyte extract and of **(B)** Pfs230-D12 recombinant protein produced by the wheat germ cell-free system. **(C)** The Pfs230 domains were separated by SDS-PAGE followed by Coomassie brilliant blue staining.


A
Binding assay
Pre-I - 1:40 dilution
D12 - 1:40 dilution
Gametes
Single Cells
Live Gametes
Count
B
C1q assay
Pre-I - 1:40 dilution
D12 - 1:40 dilution
Gametes
Single Cells
Pfs47-Dylight650 subset
Count
C
Lysis assay
Pre-I - 1:10 dilution
D12 - 1:10 dilution
Gametes
Single Cells
Pfs47-Dylight650 subset
Dead Gametes
Live gametes

**< Figure S6. Overview of flow cytometry gating strategies.**

Exemplary plots that provide an overview of the gating strategy for **(A)** an antibody binding assay, **(B)** a C1q fixation assay and **(C)** a lysis assay, all with live female gametes. For each of the assays a representative set of plots is shown for pre-immune serum and D12 serum. In the binding assay **(A)** gametes were gated for single cells (2nd column) and then live gametes were selected based on the absence of live-dead stain LD efluor 780, which stains dead cells (3rd column). For the C1q deposition assay **(B)** the gated single cells from the 2nd column were gated for gamete marker Pfs47 positivity, to then determine the anti-C1q deposition by FITC-labelled anti-C1q staining. For the lysis assay **(C)** gametes were gated for single cells (2nd column), and then for Pfs47 positivity. Dead gametes were stained with live-dead stain LD efluor 780 to determine the percentage dead cells (4th column). Note that two gamete populations can be observed in the forward scatter plots (1st column) in A and B; the left population contains dead gametes, the right population contains live gametes. In the antibody binding assay (A) and C1q deposition assay (B) we gated only the live population, while in the lysis assay (C) we gated both live and dead populations. Pre-I = pre-immune serum. SS-H = side scatter height, SS = side scatter, FS-H = forward scatter height, FS-A = forward scatter area, LD = live dead.

**Table S1. Overview of Pfs230 single domain sequences as they are expressed in S2 cells.**

| Pfs230 single domain | Nucleotide sequence<br>Underlined = kozak sequence<br>*Italic* = signal peptide<br>Blue = His-tag<br>Dark grey = linker (also restriction site Ngo MIV)<br>Grey = domain<br>Yellow = linker<br>Dark blue = C-tag |
|---|---|
| D1 | GCCACC*ATGAAGCTGTGCATCCTGCTGGCCGTGGTGGCCTTCGTGGGACTGAGCCTGGGA*CACC<br>ACCACCATCACCACGCCGGCAAAGAGTACGTCTGCGACTTCACCGATCAGCTGAAGCCAAC-<br>CGAGTCGGGCCCCAAAGTGAAGAAATGCGAAGTGAAAGTGAACGAGCCCCTGATCAAAGT-<br>CAAGATTATCTGCCCGCTGAAGGGCAGCGTGGAAAAGCTGTACGATAACATCGAGTACGTG-<br>CCCAAGAAAAGCCCCTACGTGGTGCTGACCAAAGAGGAAACGAAGCTGAAAGAGAAGCT-<br>GCTGAGCAAGCTGATCTACGGCCTGCTGATCTCCCCGACCGTGAACGAGAAAGAGAA-<br>CAACTTCAAAGAGGGCGTCATCGAGTTCACCCTGCCGCCAGTGGTGCATAAGGCCACCGT-<br>GTTCTACTTCATCTGCGACAACAGCAAGACCGAGGACGATAACAAGAAGGGCAACCGCG-<br>GCATCGTGGAAGTGTACGTGGAACCCTACGGATCAGAGCCCGAGGCCTAA |
| D2 | GCCACC*ATGAAGCTGTGCATCCTGCTGGCCGTGGTGGCCTTCGTGGGACTGAGCCTGGGA*CAC-<br>CACCACCATCACCACGCCGGCGGCAACAAGATCAACGGCTGCGCCTTCCTGGATGAGGATGAG-<br>GAAGAAGAGAAGTACGGCAATCAGATCGAAGAGGACGAGCACAACGAGAAGATCAAGAT-<br>GAAGACCTTCTTCACCCAAAACATCTACAAGAAGAACAACATCTACCCGTGCTACATGAAGCTG-<br>TACTCCGGCGATATCGGCGGCATTCTGTTCCCCAAGAACATCAAGAGCACGACCTGCTTCGAG-<br>GAAATGATCCCCTACAACAAAGAAATCAAGTGGAACAAAGAGAACAAGAGCCTGGGCAACCT-<br>GGTCAACAACAGCGTGGTGTATAACAAAGAGATGAACGCCAAGTACTTCAACGTGCAGTAC-<br>GTGCACATCCCCACCAGCTACAAGGATACCCTGAACCTGTTCTGCAGCATCATCCTGAAAGAG-<br>GAAGAGAGCAACCTGATCAGCACCTCCTACCTGGTGTACGTGTCCATCAACGAGGGATCAGAG-<br>CCCGAGGCCTAA |
| D3 | GCCACC*ATGAAGCTGTGCATCCTGCTGGCCGTGGTGGCCTTCGTGGGACTGAGCCTGGGA*CAC-<br>CACCACCATCACCACGCCGGCCACGATTATACCTGCGATTTCACGGACAAGCTCGACAAGAC-<br>CGTGCCGAGCACCGCCAATGGCAAGAAGCTGTTCATCTGCCGCAAGCACCTGAAAGAATTC-<br>GACACCTTCACGCTGAAGTGCAACGTGAACAAGACGCAGTACCCCAACATCGAGATCTTC-<br>CCAAAGACGCTGAAGGACAAGAAAGAGGTCCTGAAGCTGGATCTGGACATCCAGTACCAGAT-<br>GTTCAGCAAGTTCTTCAAGTTTAACACCCAGAACGCGAAGTACCTGAATCTGTACCCCTAC-<br>TACCTGATCTTCCCCTTCAACCACATCGGAAAGAAAGAGCTGAAAAACAACCCCACCTA-<br>CAAGAACCACAAGGACGTGAAGTATTTCGAGCAGTCCTCCGTGCTGAGCCCACTGAGTAGT-<br>GCCGATAGCCTGGGAAAGCTGTTGAACTTCCTGGACACCCAAGAGACAGTGTGCCTGAC-<br>CGAGAAGATTCGCTATCTGAACCTGAGCATCAATGAGCTGGGCAGCGATAACAACACCTTCTC-<br>CGTGACGTTCCAGGTGCCGCCGTACATCGATATCAAAGAACCCTTCTACTTTATGTTCGGCTG-<br>CAACAACAACAAAGGCGAGGGCAACATCGGCATAGTCGAGCTGCTGATTAGCAAGCAAGGAT-<br>CAGAGCCCGAGGCCTAA |
| D4 | GCCACC*ATGAAGCTGTGCATCCTGCTGGCCGTGGTGGCCTTCGTGGGACTGAGCCTGGGA*CAC-<br>CACCACCATCACCACGCCGGCGAAGAAAAGATTAAGGGCTGCAATTTCCACGAGTCCAAGCTG-<br>GACTACTTCAATGAGAACATCAGCAGCGATACCCACGAGTGCACGCTGCACGCCTATGAGAAC-<br>GATATCATCGGCTTCAACTGCCTGGAAACGACGCACCCCAACGAGGTGGAAGTGGAAGTTGAG-<br>GATGCCGAGATCTATCTGCAGCCCGAGAACTGCTTCAACAACGTCTACAAGGGCCTGAACTC-<br>CGTGGATATCACCACCATCCTGAAGAACGCCCAGACCTACAACATTAACAACAAAAAGAC-<br>CCCGACCTTCCTGAAGATCCCGCCATACAACCTGCTGGAAGATGTGGAAATCAGCTGCCAGTG-<br>CACCATCAAGCAAGTGGTCAAGAAAATCAAAGTGATCATCACCAAGAACGACGGATCAGAGC-<br>CCGAGGCCTAA |

**Table S1.** Continued

| | |
|---|---|
| D5 | GCCACC*ATGAAGCTGTGCATCCTGCTGGCCGTGGTGGCCTTCGTGGGACTGAGCCTGGGA*CACCACCACCATCACCACGCCGGCAAGATCTACAAGTGCGAGCACGAGAACTTCATCAACCCGCGCGTCAACAAGACCTTCGACGAGAACGTCGAGTACACGTGCAATATCAAGATCGAGAATTTCTTCAACTACATCCAGATTTTCTGCCCCGCCAAGGATCTGGGCATCTATAAGAATATCCAGATGTACTACGACATCGTGAAGCCGACGCGCGTGCCCCAGTTCAAAAAATTCAACAATGAGGAGCTCCACAAGCTCATCCCCAACTCCGAGATGCTGCACAAGACGAAAGAGATGCTGATCCTGTACAACGAAGAGAAGGTGGACCTGCTGCACTTCTACGTGTTCTTGCCCATCTACATCAAGGACATCTACGAGTTCAACATCGTGTGCGACAACTCCAAGACGATGTGGAAGAACCAGCTCGGCGGAAAAGTGATCTACCACATCACCGTCAGCAAGCGCGGATCAGAGCCCGAGGCCTAA |
| D6 | GCCACC*ATGAAGCTGTGCATCCTGCTGGCCGTGGTGGCCTTCGTGGGACTGAGCCTGGGA*CACCACCACCATCACCACGCCGGCTTCGATAACGAGCACGCCCACATGTTCAGCTATAACAAGACCAACGTGAAGAACTGCATCATCGACGCCAAGCCGAAGGATCTGATCGGCTTCGTGTGCCCAAGCGGCACACTGAAGCTGACCAATTGCTTCAAGGATGCCATCGTGCACACCAACCTGACCAACATCAACGGCATCCTGTATCTCAAGAACAACCTGGCCAACTTCACGTACAAGCACCAGTTCAATTACATGGAAATCCCCGCGCTGATGGACAACGACATCAGCTTCAAGTGCATCTGCGTGGACCTGAAGAAAAAGAAGTACAACGTCAAGAGCCCGCTGGGCCCCGGATCAGAGCCCGAGGCCTAA |
| D7 | GCCACC*ATGAAGCTGTGCATCCTGCTGGCCGTGGTGGCCTTCGTGGGACTGAGCCTGGGA*CACCACCACCATCACCACGCCGGCAACCGCCACGTGTGCGATTTCTCCAAGAACAATCTGATCGTGCCCGAGTCGTTGAAGAAGAAAGAGGAACTCGGCGGCAACCCCGTGAACATCCATTGCTATGCCCTGTTGAAGCCCCTGGATACGCTGTATGTGAAGTGCCCCACCTCCAAGGATAACTACGAGGCCGCCAAAGTCAACATCAGCGAGAATGATAACGAGTACGAGTTGCAAGTGATCTCCCTGATCGAGAAGCGCTTTCACAACTTCGAGACACTGGAAAGCAAAAAGCCCGGCAACGGCGACGTCGTGGTGCACAATGGTGTTGTGGATACCGGACCGGTGCTGGATAACTCCACGTTCGAGAAGTACTTTAAGAACATTAAGATCAAGCCCGATAAGTTCTTCGAGAAAGTTATCAATGAGTACGACGACACCGAGGAAGAAAAGGACCTGGAATCCATCCTGCCAGGCGCCATCGTGTCCCCAATGAAGGTGCTCAAGAAGAAGGACCCCTTCACCAGCTATGCCGCCTTTGTGGTGCCACCGATCGTGCCAAAGGATCTGCACTTCAAGGTGGAATGCAACAATACCGAGTACAAGGACGAGAACCAGTACATCAGCGGCTACAATGGCATCATCCACATCGACATCTCCAACAGCGGATCAGAGCCCGAGGCCTAA |
| D8 | GCCACC*ATGAAGCTGTGCATCCTGCTGGCCGTGGTGGCCTTCGTGGGACTGAGCCTGGGA*CACCACCACCATCACCACGCCGGCAACCGCAAGATCAATGGATGCGACTTTAGCACCAACAACTCCAGCATCCTGACCAGCTCCGTGAAGCTGGTTAACGGCGAGACAAAGAACTGCGAGATCAATATCAACAACAACGAAGTGTTCGGCATCATCTGTGACAATGAGACAAATCTGGACCCAGAGAAGTGCTTCCATGAGATCTACTCCAAGGACAACAAGACGGTCAAAAAGTTCCGCGAAGTGATCCCCAATATCGACATTTTCAGCCTGCACAACTCGAACAAGAAAAAGGTGGCCTACGCCAAGGTGCCCCTGGACTATATTAACAAGCTGCTGTTCAGCTGCTCCTGCAAGACCAGCCACACCAACACCATCGGCACGATGAAAGTGACCCTGAACAAAGACGAGGGATCAGAGCCCGAGGCCTAA |
| D9 | GCCACC*ATGAAGCTGTGCATCCTGCTGGCCGTGGTGGCCTTCGTGGGACTGAGCCTGGGA*CACCACCACCATCACCACGCCGGCAACGTGCACCTGTGCAATTTCTTCGACAACCCCGAGCTGACCTTCGACAACAACAAGATCGTGCTGTGCAAGATCGATGCCGAGCTGTTTAGCGAAGTCATCATTCAGCTGCCCATCTTCGGCACCAAAAACGTCGAGGAAGGCGTCCAGAACGAAGAGTACAAGAAGTTCAGCCTGAAGCCGAGCCTGGTGTTCGATGATAACAACAATGACATCAAAGTCATCGGCAAAGAGAAGAACGAGGTTTCCATCTCGCTGGCCCTGAAGGGCGTGTACGGCAACCGCATCTTCACCTTTGATAAGAACGGCAAGAAAGGCGAAGGCATCAGCTTTTTCATCCCGCCGATCAAGCAGGATACCGACCTGAAGTTTATCATCAACGAAACCATCGATAACAGCAACATTAAGCAGCGCGGCCTGATCTACATCTTCGTGCGCAAGAACGTGTCGGGATCAGAGCCCGAGGCCTAA |

**Table S1.** Continued

| | |
|---|---|
| D10 | GCCACC*ATGAAGCTGTGCATCCTGCTGGCCGTGGTGGCCTTCGTGGGACTGAGCCTGGGA*CAC-CACCACCATCACCACGCCGGCGAGAACTCGTTCAAGCTGTGTGATTTCACCACCGGCAG-CACCAGCCTGATGGAATTGAACAGCCAAGTGAAAGAAAAGAAGTGCACCGTTAAGATTA-AGAAGGGCGATATCTTCGGCCTGAAATGCCCCAAGGGATTCGCCATTTTTCCGCAAGCCT-GCTTCTCCAACGTCCTGCTCGAGTACTACAAGAGCGATTACGAGGACAGCGAGCACATCAAC-TACTACATTCACAAGGACAAAAAGTACAATCTGAAGCCCAAGGACGTTATCGAGTTGATGGAT-GAGAACTTCCGCGAGCTGCAAAACATTCAGCAGTACACCGGCATCAGCAACATCACCGAT-GTGCTGCATTTCAAGAACTTCAACCTGGGCAATCTGCCGCTCAACTTCAAGAATCACTACAG-CACCGCCTATGCGAAGGTGCCGGATACCTTCAACTCCATCATCAACTTCAGCTGCAACTGC-TACAATCCCGAGAAGCACGTCTACGGCACCATGCAGGTCGAGAGCGATAACGGATCAGAGC-CCGAGGCCTAA |
| D11 | GCCACC*ATGAAGCTGTGCATCCTGCTGGCCGTGGTGGCCTTCGTGGGACTGAGCCTGGGA*CAC-CACCACCATCACCACGCCGGCAATGAGCACATTTGCGACTACGAAAAGAACGAGTCGCT-GATCTCGACCCTGCCAAACGACACCAAGAAGATCCAGAAGTCGATCTGCAAGATTAACG-CGAAGGCCCTGGATGTGGTCACCATTAAGTGCCCGCATACCAAGAATTTCACCCCGAAG-GATTACTTCCCCAACAGCAGCCTGATCACCAACGATAAGAAGATCGTCATCACGTTCGATA-AGAAAAACTTCGTCACCTACATCGACCCCACCAAAAAGACGTTCTCCCTGAAAGACATCTACAT-TCAGAGCTTCTACGGCGTGTCCCTGGATCACCTGAACCAGATCAAAAAAATCCACGAGGAAT-GGGACGACGTCCACCTGTTTTACCCGCCGCACAACGTTCTGCACAACGTGGTCCTGAACAAC-CACATTGTGAACCTGTCCAGCGCCTTGGAGGGCGTGCTGTTCATGAAGTCCAAAGTGACCGGC-GACGAGACAGCCACGAAGAAGAATACCACACTGCCCACCGATGGCGTGTCCAGCATTCTGATC-CCGCCGTACGTGAAAGAAGATATCACCTTCCATCTGTTCTGCGGCAAGTCCACGACCAAGAAGC-CCAACAAAAAAAACACCAGCTTGGCCCTGATCCACATTCACATCAGCTCCAATGGATCAGAGC-CCGAGGCCTAA |
| D12 | GCCACC*ATGAAGCTGTGCATCCTGCTGGCCGTGGTGGCCTTCGTGGGACTGAGCCTGGGA*CAC-CACCACCATCACCACGCCGGCCGCAATATCATCCACGGCTGCGACTTTCTGTACCTGGAAAAC-CAGACCAACGACGCCATCTCGAACAACAACAACAACTCCTACAGCATCTTCACCCACAA-CAAGAACACCGAGAACAACCTCATCTGCGATATTTCGCTGATCCCCAAGACCGTGATCGG-CATCAAGTGCCCCAACAAGAAGCTGAACCCGCAGACCTGCTTTGACGAGGTGTACTACGT-CAAACAAGAGGACGTGCCGTCCAAGACCATCACCGCCGACAAGTACAATACCTTCAGCAAG-GATAAGATTGGCAACATCCTCAAAAACGCCATCAGCATCAACAACCCGGACGAGAAGGATAA-CACCTACACCTATCTGATCCTGCCGGAAAAGTTCGAGGAAGAGTTGATCGATACCAAAAAGGT-GCTGGCCTGCACGTGTGACAACAAGTACATTATCCACATGAAGATCGAAAAGTCCACCGGATCA-GAGCCCGAGGCCTAA |
| D13 | GCCACC*ATGAAGCTGTGCATCCTGCTGGCCGTGGTGGCCTTCGTGGGACTGAGCCTGGGA*CAC-CACCACCATCACCACGCCGGCGGCAAGGATATCTGCAAATACGACGTGACCACCAAGGTGG-CCACGTGCGAGATTATCGACACCATCGATTCGAGCGTGCTGAAAGAACACCACACCGTGCAC-TACTCGATCACCCTGTCGCGCTGGGATAAGCTGATCATCAAGTACCCGACCAACGAGAAAAC-CCACTTTGAGAACTTTTTCGTGAACCCGTTCAACCTCAAGGACAAGGTGCTCTACAATTACAA-CAAGCCCATCAACATTGAGCACATACTGCCCGGTGCCATCACCACCGATATCTACGATACGCG-CACCAAGATTAAGCAGTACATCCTGCGCATCCCACCGTATGTGCACAAGGATATTCACTTCTC-CCTGGAATTCAACAACTCCCTGAGCCTGACCAAGCAGAACCAGAACATTATCTACGGCAATGT-GGCCAAGATCTTCATCCATATCAACCAGGGCGGATCAGAGCCCGAGGCCTAA |
| D14 | GCCACC*ATGAAGCTGTGCATCCTGCTGGCCGTGGTGGCCTTCGTGGGACTGAGCCTGGGA*CAC-CACCACCATCACCACGCCGGCTACAAAGAGATCCACGGTTGCGATTTCACCGGCAAGTACAGC-CACCTGTTCACCTACTCCAAAAAGCCGCTGCCGAACGATGACGACATCTGCAATGTGACCATC-GGAAACAACACGTTCAGCGGATTCGCCTGCCTGTCGCACTTCGAGCTGAAACCCAACAACT-GCTTCTCGTCGGTGTACGATTACAACGAGGCCAACAAAGTGAAAAAGTTGTTCGACCTGTC-GACCAAGGTCGAGCTGGATCACATCAAACAGAACACCTCCGGCTACACCCTGTCCTACAT-CATTTTTAACAAAGAATCGACCAAGCTCAAGTTCTCCTGCACATGCAGCAGCAACTACTCCAAC-TACACCATCCGCATCACCTTCGATCCGGGATCAGAGCCCGAGGCCTAA |

**Table S2. Characteristics of all Pfs230 single domain constructs.**

| Pfs230 single Domain | Amino acid numbers within full-length Pfs230 | Molecular weight (kDa) | Number of cysteines | Predicted N-linked glycosylation sites | Predicted O-linked glycosylation sites |
|---|---|---|---|---|---|
| D1 | 589-730 | 17.8 | 4 | 0 | 2 |
| D2 | 731-886 | 19.8 | 4 | 2 | 0 |
| D3 | 918-1133 | 26.8 | 5 | 4 | 2 |
| D4 | 1134-1268 | 17.1 | 6 | 1 | 0 |
| D5 | 1285-1432 | 19.5 | 4 | 1 | 0 |
| D6 | 1433-1550 | 14.1 | 6 | 2 | 0 |
| D7 | 1694-1907 | 25.9 | 4 | 3 | 1 |
| D8 | 1908-2036 | 16.1 | 6 | 4 | 1 |
| D9 | 2052-2201 | 18.7 | 2 | 1 | 0 |
| D10 | 2202-2373 | 21.5 | 6 | 2 | 0 |
| D11 | 2448-2663 | 26.1 | 4 | 7 | 4 |
| D12 | 2664-2818 | 19.4 | 6 | 1 | 0 |
| D13 | 2831-2979 | 19.0 | 2 | 0 | 0 |
| D14 | 2980-3105 | 16.0 | 6 | 5 | 0 |

NetOGlyc version 4.0 (DTU Health Tech, Denmark) [1] and NetNglyc version 1.0 (DTU Health Tech, Denmark) [2] were used to predict the O-linked and N-linked glycosylation sites.

**Table S3. Mass spectrometry results confirm Pfs230-D12 identity**.
The table includes all Pfs230 recombinant domains produced in this study that were found in the peptide preparation.

| Protein ID | Intensity | % of total intensity | Peptides | Razor + unique peptides | Unique peptides | % coverage |
|---|---|---|---|---|---|---|
| Pfs230-D12 | $9.853x10^{11}$ | 98.226 | 29 | 29 | 29 | 89.9 |
| Pfs230-D5 | $5.715x10^{6}$ | 0.001 | 2 | 2 | 2 | 15.6 |

**Table S4. Peptides, covering recombinant Pfs230 fragments, identified by mass spectrometry.**

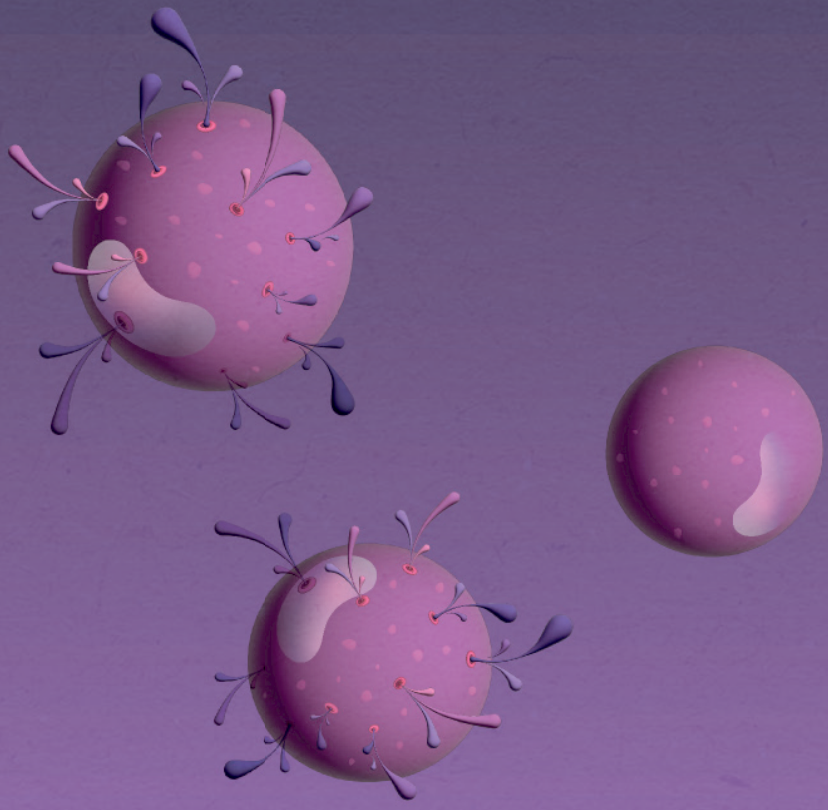
| Domain | Peptide |
|---|---|
| D12 | CPNKKLNPQTCFDEVYYVK |
| D12 | DKIGNILK |
| D12 | DNTYTYLILPEK |
| D12 | DNTYTYLILPEKFEEELIDTK |
| D12 | DNTYTYLILPEKFEEELIDTKK |
| D12 | FEEELIDTK |
| D12 | FEEELIDTKK |
| D12 | KLNPQTCFDEVYYVK |
| D12 | KLNPQTCFDEVYYVKQEDVPSK |
| D12 | KVLACTCDNK |
| D12 | KVLACTCDNKYIIHMK |
| D12 | LNPQTCFDEVYYVK |
| D12 | LNPQTCFDEVYYVKQEDVPSK |
| D12 | NAISINNPDEK |
| D12 | NAISINNPDEKDNTYTYLILPEK |
| D12 | NAISINNPDEKDNTYTYLILPEKFEEELIDTK |
| D12 | NIIHGCDFLYLENQTNDAISNNNNNSYSIFTHNK |
| D12 | NTENNLICDISLIPK |
| D12 | NTENNLICDISLIPKTVIGIK |
| D12 | QEDVPSK |
| D12 | QEDVPSKTITADKYNTFSK |
| D12 | TITADKYNTFSK |
| D12 | TITADKYNTFSKDK |
| D12 | VLACTCDNK |
| D12 | VLACTCDNKYIIHMK |
| D12 | VLACTCDNKYIIHMKIEK |
| D12 | YIIHMKIEK |
| D12 | YNTFSKDK |
| D5 | IENFFNYIQIFCPAK |
| D5 | TFDENVEYTCNIK |

**Data S1. Raw SMFA and DMFA data.**
Can be found online on the website of Science Advances (https://www.science.org/doi/10.1126/sciadv.adw8216) and in the Radboud Data Repository.

## Supplementary references

1 Steentoft, C. *et al.* Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. *EMBO J* **32**, 1478-1488, doi:10.1038/emboj.2013.79 (2013).

2 Gupta, R. & Brunak, S. Prediction of glycosylation across the human proteome and the correlation to protein function. *Pac Symp Biocomput*, 310-322 (2002)



Chapter 5

# Blood, sweat, and beers: investigating mosquito biting preferences amidst noise and intoxication in a cross-sectional cohort study at a large music festival

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## Abstract

**Introduction:** We live in a world split between mosquito magnets and those lucky enough to remain (nearly) untouched. The reasons why some people attract more bites than others remain largely mysterious. In the Mosquito Magnet Trial, we investigated differences in mosquito attraction amongst festivalgoers with varying levels of hygiene and intoxication.

**Methods:** Our study was conducted in a slightly steamy pop-up laboratory inside four connected shipping containers at Lowlands Festival in The Netherlands (August 18–20, 2023). Participants completed an anonymous questionnaire on hygiene, diet, and festival-related behaviour (including alcohol uptake and shared sleeping arrangements). Mosquito attraction was measured using a custom designed setup: a transparent cage with perforations where female *Anopheles* mosquitoes were offered a choice between a sugar-feeder and the participants arm. Mosquitoes could only smell, not bite, the participant's arm. Attraction was quantified through video imaging, measuring arm landings relative to total landings. Mosquito attraction was correlated with questionnaire responses and skin microbiota profiles collected from forearm skin swabs.

**Results:** Amongst the 465 included participants, mosquitoes showed a clear fondness for those who drank beer over those who abstained from the liquid gold (Fold Change 1.35, 95% CI 1.12–1.63, $P_{FDR}<0.001$). Attraction was also contagious: participants that successfully lured a fellow human into their tent the previous night also proved more enticing to mosquitoes (FC 1.34, 95% CI 1.14–1.58, $P_{FDR}=0.002$). Meanwhile, skipping the morning showering routine and using sunscreen reduced mosquito attraction (FC 0.52, 95% 0.38-0.70, $P_{FDR}<0.001$). *Streptococci* were more abundant on the skin of highly attractive individuals ($P_{uncorrected}=0.017$), and the overall abundance of malodour associated bacteria was high – possibly reflecting the container's smell.

**Conclusion:** The Mosquito Magnet Trial was conducted in a loosely controlled setting with a selection bias towards science loving festivalgoers. That said, using our custom designed experimental set-up, we found that mosquitoes are drawn to those who avoid sunscreen, drink beer and share their bed. They simply have a taste for the hedonists among us.

## Introduction

Do you get bitten by mosquitoes often, or are you lucky enough to escape their hunt for blood? The quest for factors behind observed differences in the preference of mosquitoes for certain humans has not only inspired scientists, it also forms a matter of public debate where factors as blood type, 'sweet blood', and alcohol consumption are regularly suggested. Mosquito nuisance was already described in the 5th century BCE by the Greek historian Herodotus – long before their role in disease transmission was understood[1]. Today, it is well known that mosquitoes can carry pathogens which may be transmitted to human hosts during blood feeding. This makes mosquitoes more than just annoying in endemic regions; individuals who are bitten frequently face greater exposure to pathogens, increasing their risk of contracting mosquito-borne illnesses such as malaria or dengue.



Mosquitoes rely on their senses in order to find a human to bite. The search for a blood meal typically starts with the perception of $CO_2$ that we exhale[2], which is considered the initial cue for mosquito activation. What then follows is a complex set of olfactory, visual, thermal, and physical cues that guide a mosquito to a human host[3,4]. Sensory factors contributing to mosquito attraction can be universal, including $CO_2$ and body heat, but also more specific to certain human individuals, like our smell. As most of us have experienced one way or the other, humans can have distinct odours. Odours depend on different skin microbiota and skin volatile compound compositions, which can influence mosquito attraction[5]. The skin microbiome of highly attractive people is reported to be of lower bacterial diversity[6] and producing higher amounts of volatile fatty acid metabolites[7] or long-chain carboxylic acids[8,9]. In contrast, presence of short-chain fatty acids may reduce the number of mosquito landings on an artificial bait[10]. Other studies explored the potential role of human blood type in host preference[11-13], but their findings remain conflicting and difficult to interpret – partly due to methodological challenges.

To this day it remains largely a mystery why some people receive more mosquito bites than others, even though bite frequency plays a key role in the risk of mosquito-borne diseases. Understanding factors behind mosquito attraction could improve prevention strategies in high-risk areas and possibly inform behavioural choices for those who would like to steer clear of mosquito bites. Existing cohort studies on mosquito attraction, however, are few, often use very small sample sizes, and show conflicting results, making them difficult to interpret. The present study, to our knowledge the largest of its kind, investigates why some people get bitten more than others in a large-scale 'Mosquito Magnet Trial' conducted during a

3-day music festival in the Netherlands. This setting was chosen because it pushes human behaviour, hygiene, and odour beyond the limits typically observed in observational studies.

# Methods

## Study setting and participants

A Campingflight to Lowlands Paradise (or Lowlands) is an annual 3-day festival with 65,000 attendees that takes place on the farmlands of Biddinghuizen, The Netherlands. Our study was conducted in 2023 (August 18–20) in a custom-designed laboratory set up inside welded-together shipping containers. Whereas the festival for many festivalgoers very much is a 72-hour non-stop experience, participants were invited to join the study between 12 to 8 pm, when a science fair (Lowlands Science) was open for visitors. After being briefed about the study procedures, participants (≥ 18 years old) could voluntarily participate in the Mosquito Magnet Trial, which took around 20 minutes to complete. We consulted the national medical ethics review committee (Oost-METC) to assess whether ethical approval was necessary for this study. They issued a statement confirming that ethical approval was not required.

## Mosquito rearing and transport

Not unlike the festivalgoers, *Anopheles stephensi* mosquitoes[14] were reared on a reverse day-night rhythm under controlled conditions (at 30°C and 70-80% humidity with a 12-hour day/night cycle) at Radboudumc in Nijmegen, The Netherlands. Mosquito rearing occurred approximately 100 kilometres away from the study container at Lowlands festival in Biddinghuizen. Every morning during the study period, non-blood fed mosquitoes were transferred to netted cages, put in Styrofoam boxes, and transported by car to the study container in Biddinghuizen. Over the course of three days, a total of 1,700 mosquitoes were transported. At the study container and before the start of the study day (<12:00 midday), 20-35 mosquitoes were transferred to each of the study cages using an aspirator in a designated section of the container, separated from its surroundings by nets. For the remainder of the study day, mosquitoes remained in their cages.

## Study procedures and experimental design

Participants were asked questions about their general health, diet, and hygiene during the festival (hoping that this was different from their normal routine), and substance use (idem) in a questionnaire that was anonymous and offered in either

Dutch or English. Subsequently, participants had their tympanic temperature taken and performed a breath alcohol test (Dräger Alcotest® 7000, Zoetermeer, Netherlands). Participants then continued to the mosquito attraction test. To quantify mosquito attractiveness, we used a custom-designed video-based assay consisting of transparent acrylic mosquito cage (15 x 15 x 15 cm) housing 20-35 *Anopheles stephensi* mosquitoes[14] (Figure 1).

On two opposing sides, the cage had rectangular areas (12.5 x 5.5 cm) perforated with an array of holes (diameter: 0.8 mm, spacing: 3.2 mm). An acrylic box with cotton pads soaked with 10% glucose solution was attached to the left side of the cage. The mosquito cage was placed on a transparent acrylic base, equipped with an armrest and a Raspberry Pi microcomputer with associated camera (piCamera V2) situated beneath the cage to record flight and landing dynamics. Participants were seated at this setup and asked to gently blow into the cage for 10 seconds through perforations in the front panel. Next, participants were asked to present their lower right arm by holding it against the right perforated side of the cage, while placing their arm on an acrylic support to prevent motion and fatigue. Perforations in the cage wall allowed skin odorants to diffuse into the cage, while being sufficiently small to prevent mosquitoes from biting through. A three-minute video was recorded and stored for analysis. After use, mosquito cages were stored and reused after a resting period of approximately 20 minutes in Styrofoam boxes free from external stimuli.



## Video analysis and mosquito tracking

Using the pose estimation python package SLEAP[15], we fine-tuned a neural network to detect centroid coordinates of mosquitoes. We engineered a custom tracking algorithm based on a Kalman filter and the greedy tracking algorithm to track mosquitoes over time. Using the output of the tracking algorithm, we quantified the number of mosquitoes that are in flight or stationary at a given time. To do so, we employed a simple state machine that would take a track's displacement between sequential frames and attribute the state of resting if the mosquito track did not change positions over a period of 7 frames, or attribute the state of flight to the mosquitoes that show displacement above a given threshold for more than 3 consecutive frames. With this data, not only did we quantify the number of resting and flying mosquitoes at a given point in time, we were also able to count the total number of landings on a given region of interest. On the 2D projection of the filmed cage, the left and right trapezoids represent each of our regions of interest (i.e. the area where the arm was presented and the sugar feeder area, Figure 1), while the remaining regions represent an out-of-region area (i.e. top and bottom trapezoids

and center square). By using the position of these regions of interest, we quantified how many resting and flying mosquitoes are at each region of interest at any given time. Absolute attractiveness was quantified as the number of mosquito landings at the arm-region of interest, relative mosquito attractiveness was obtained by dividing the number of mosquito landings in the arm-region of interest by the number of landings on the sugar feeder region of interest.

## Skin swab

After quantifying mosquito attractiveness, participants received instructions on how to perform a skin swab. To prevent contamination, participants performed the skin swab themselves by rubbing a cotton-swab back and forth across an area of approximately 15 cm on the inner part of their left forearm. Participants were told to rub for approximately 30 seconds, after which they put the swab in a designated tube labelled with their anonymous participant number and filled with SCF-1 solution (50 mM TRIS, 1mM EDTA, 0.5% Tween 20). Tubes were transferred to zip-lock bags and stored in a -20°C freezer in the study container. After the festival, swabs were transported in Styrofoam boxes to the -80°C freezer at Radboudumc, Nijmegen.

## Laboratory assays and sample selection

To pilot 16S rRNA (16S) microbial sequencing using genetic material from skin swabs, we randomly selected 8 swabs from participants in the top two quartiles of relative mosquito attractiveness (Supplementary Figure 1). Microbial genomic DNA was extracted from swabs using the ZymoBIOMICS DNA miniprep kit by vortexing the swab in a ZR BashingBead Lysis Tube with 750 µl of ZymoBIOMICS Lysis Solution for 30 seconds to bring the microbes into solution, followed by bead beating. Illumina 16S rRNA marker-gene (16S) amplicon libraries were generated and sequenced at BaseClear BV (Leiden, The Netherlands) on an Illumina MiSeq PE300 system. Sequencing of bacterial 16S rRNA is described in detail in the appendix. After confirming successful 16S sequencing in our pilot set, we selected an additional 85 swabs based on their relative mosquito attractiveness. This included 45 swabs from individuals with the highest daily attractiveness scores (15 per study day) and 40 swabs with the lowest daily attractiveness scores (12 from the first study day, and 14 from each of the next two days) after excluding samples with the daily lowest 10% of mosquito flight time and landings. The latter was done to avoid any bias from a very inactive cage resulting from overheating in the study container, or fatigued mosquitoes. The numbers used in this selection were arbitrary but avoided resource intensive analyses on skin swabs with a mediocre mosquito attraction score which were unlikely to inform our central question on whether skin microbiota differ between individuals who strongly attract mosquitoes and those who do not.

## Statistical analysis

The primary variable of interest was the number of arm-landings per mosquito. The relative attractiveness score (arm-landings divided by sugar feeding landings) was the secondary outcome variable of interest, and was used for the selection of skin swabs. We fitted generalized linear models with a log-link Gaussian distribution to investigate the relationship between arm-landings and a set of predictor variables divided in different categories. We excluded observations with a relative mosquito attraction score that fell in the daily bottom 10% of mosquito flight time and landing counts. This was done because, despite rotating the experimental cages to allow mosquitoes to rest, we observed a decline in mosquito responsiveness to participants' arms over the course of the study day (Supplementary Figure 2). The number of arm landings per mosquito was set as the model's outcome variable and a random effect with an interaction between time and day of the experiment was included to account for any potentially remaining temporal variation due to environmental temperature changes and mosquito fatigue. We additionally included an offset term with landings on the sugar feeder and anywhere else in the cage. This was done to model the effect on arm landings relative to the overall activity in the cage. Separate models were used to test fixed effects of variables categorized in i) demographics and health, ii) hygiene and care, iii) consumptions and diet, and iv) substance use. Participant age (in years) and time since last shower (in hours) were grouped into four quartiles and all categorical variables were included as factors. We selected fixed effects with a significant effect and included them in a final model to assess their combined effect. Since models shared the same outcome variable, we applied the Benjamini-Hochberg approach to adjust p-values for multiple testing ($P_{FDR}$). Model coefficients were exponentiated to obtain fold changes that represent multiplicative changes in the outcome value. Mann Whitney U tests were used to test if continuous densities (e.g. number of consumed beers) differed between groups. A two-sample z-test for proportions was used to compare prevalences between groups (e.g. proportion of substance users). Alpha diversity metrics by the Shannon index were obtained from "qiime2 diversity core-metrics-phylogenetic" with default settings [16]. Principal Coordinate Analysis (PCoA) with weighted UniFrac distances was computed via "qiime2 diversity core-metrics-phylogenetic"[16] and the 'vegan' R package[17]. Permutational Multivariate Analysis of Variance (PERMANOVA) was applied on the weighted UniFrac distances to compute differences in skin microbiota beta diversity between selected study groups [18, 19]. The difference in relative taxon abundance between two study groups was tested with either the Mann-Whitney U test (non-paired) or Wilcox signed rank test (paired) with continuity correction in the normal approximation for the p-value. All statistical analyses were performed in R (version 4.4.1) and supported by substantial coffee consumption.

## Results

Despite being in steep competition with acts like Billie Eilish, Bombino, Underworld, and the North Netherlands Orchestra performing a riveting rendition of Beethoven's 9th, our study attracted a total of 524 participants. Of these, seven individuals reported to have used mosquito repellents and were excluded from further analyses (Supplementary Figure 1). An additional 52 individuals were excluded because no video was recorded during the experiment (n = 17) or the mosquito activity fell in the daily bottom 10% of mosquito flight time and the daily bottom 10% of landing counts (n = 35).

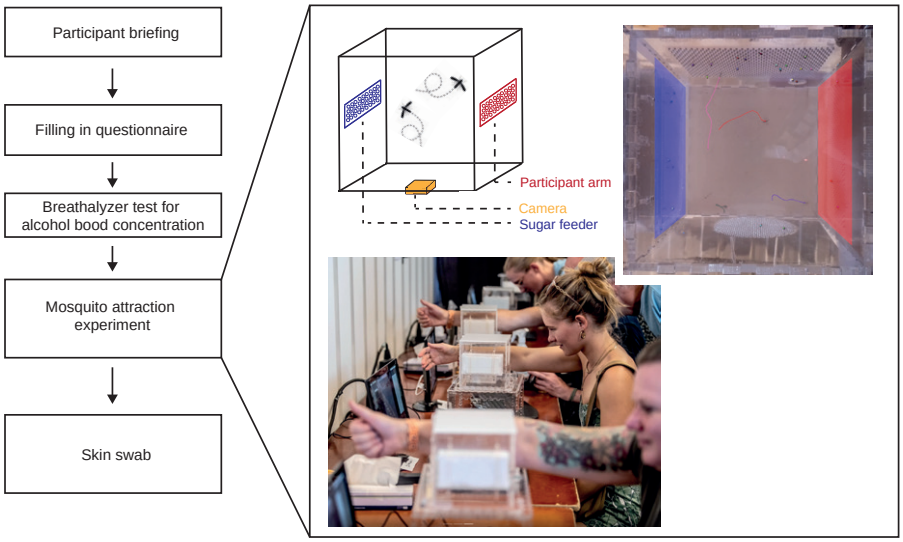


**Figure 1: Schematic overview of study procedures. Participants were invited to fill in an anonymous questionnaire after being briefed about the study purpose and procedures.** Alcohol blood concentration was measured using a Breathalyzer test. Mosquito attraction was quantified in a custom designed set-up consisting of an acrylic cubic cage housing 20-35 mosquitoes. Participants pressed their arms against the right side of the cage, where a grid of small perforations was located (highlighted in blue). Perforations were also located on the opposite side of the cage (the left side when facing the cage). Here, a sugar feeder was positioned (highlighted in red). A raspberry pi computer and associated camera were located beneath the transparent cage and recorded mosquito tracks for the duration of the experiment (3 minutes). Afterwards, participants were invited to perform a skin swab on their right forearm for skin microbiome assessments.

Among the remaining 465 participants, a majority identified as female (55.7%, 259/465) compared to those who identified as male (43.0%, 200/465, $P < 0.001$, Figure 2). One individual reported to be non-binary, and five individuals did not indicate their gender in the questionnaire. A single participant identified as

demigod; mosquitoes would not come near this participant and consequently she/he/they had to be excluded due to very low mosquito activity. Although the majority of individuals did not know their blood type (62.2%, 289/465), the most commonly noted blood type was type O (48.3%, 85/176). The proportion of males with blood type AB (14.1%, 9/64) was surprisingly high compared to the general national average of 3.0% [20]. Blood type was self-reported and not confirmed through laboratory testing, it thus may be subject to inaccuracies in participants' knowledge or recall. Sunscreen usage was high with over half of participants indicating to have used sunscreen on their forearm that day (52.7%, 244/463). The majority of participants did not have a special diet (77.2%, 338/438), followed by vegetarians (13.9%, 61/438), and pescatarians (5.7%, 25/438). Whether out of genuine love or mere convenience, canned sausages stole the culinary spotlight, appearing in 15.6% of people's latest meals (36/231). Participants used a wide variety of substances, with cannabis being the most frequently reported (19.6%, 91/465) followed by XTC (18.7%, 87/465) and cocaine (11.4%, 53/465) and no difference between males and females regardless of substance type (all $P > 0.279$). A non-negligible proportion of participants reported use of multiple substances with two reporting all listed substances except for amphetamines in the past 48 hours. The majority of participants drank at least one beer in the past 12 hours (65.6%, 299/456), this proportion was even higher amongst men (79.8%, 158/198). A higher proportion of beer abstainers reported sleeping alone last night (61.1%, 96/157) compared to beer drinkers (41.4%, 103/249, $P < 0.001$), yet all four individuals who consumed the most beers (> 20 beers in the past 12 hours) also slept alone. While beer consumption may not guarantee good decisions, it might at least facilitate company when consumed in moderation.



Mosquito attraction varied across participants, with the number of arm-landings per mosquito ranging from 0 to 14.8 (Supplementary Figure 3). Surprisingly, three of the four individuals with zero arm-landings came to the study container during the performance of Joost Klein, who was then famous for his song '*Friesenjung*', a hardcore take on a parody of Sting's *'Englishman in New York'*. Contemplating on whether mosquitoes could be repelled by upbeat electronic music, we continued assessing factors behind mosquito attraction using multivariate linear models (Figure 3). Mosquito attraction was similar between female and male participants ($P_{FDR} = 0.189$, Figure 3A). We found evidence for an interaction between time since last shower (in quartiles) and sunscreen on a participant's forearm (all $P_{FDR} < 0.007$). Amongst participants that showered recently (<6 hours ago), the presence of sunscreen on the forearm made them less attractive (Fold change [FC] 0.52, 95% CI 0.38-0.71, $P_{FDR} < 0.001$, Figure 3B). The repellent effect of sunscreen became

less pronounced when time since last shower increased. We found no effect of sunscreen usage when applied anywhere but on their forearms ($P_{FDR}$ = 0.992). We did not observe an effect of perfume when applied on participant's forearm or elsewhere (both $P_{FDR}$ > 0.641), potentially because of abundant olfactory triggers. Interestingly, participants who attracted a partner into their tent the night before also attracted more mosquitoes (FC 1.46, 95% CI 1.24-1.71, $P_{FDR}$ < 0.001), without evidence for an interaction with time since last shower (all $P_{FDR}$ > 0.182).

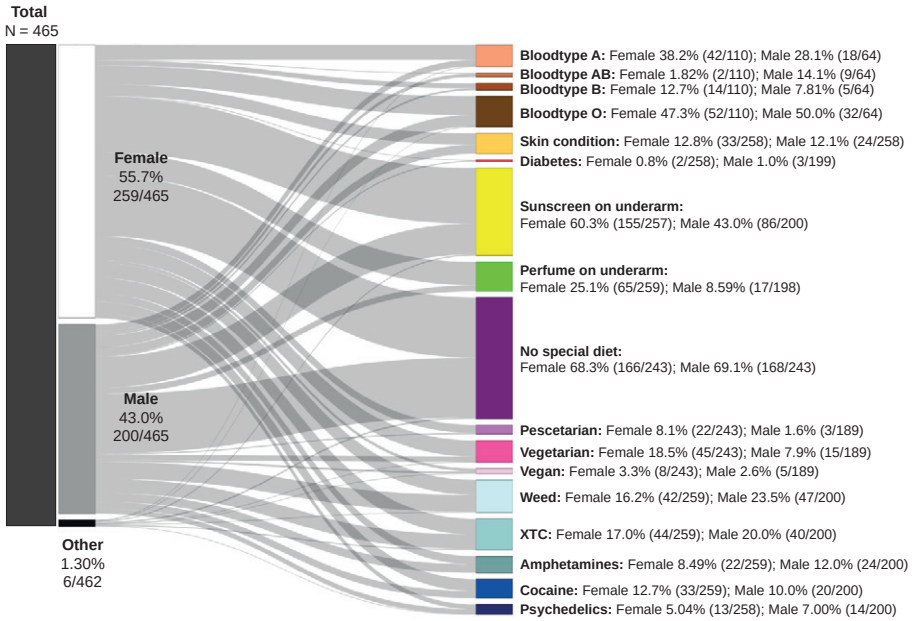


**Figure 2: General cohort characteristics. Sankey plot showing the proportion of participants grouped into different categories.** Bar sizes reflect the total proportion, whereas the proportion per participant sex (female/male) is indicted in text.

Mosquitoes showed a clear preference for the well-hydrated, on hops and grapes, that is. Arm landings were significantly higher in beer drinkers compared to those who had nobly abstained for at least 12 hours (FC 1.44, 95% CI 1.20-1.74, $P_{FDR}$ < 0.001, Figure 3C). Mosquitoes seemed to have a taste for wine drinkers too (FC 1.39, 95% CI 1.02-1.88, P = 0.035), but this effect sobered up after correcting for multiple testing ($P_{FDR}$ = 0.103). Measured blood alcohol concentration ranged from 0.00 to 1.82‰ and positively correlated with the self-reported consumed number of beers (Spearman rho = 0.46, P < 0.001) and glasses of wine (Spearman rho = 0.12, P = 0.011). No statistically significant effect of alcohol concentration was observed on mosquito attraction when included as a continuous variable (FC 1.04, $P_{FDR}$ = 0.853)

nor as a binned variable using the concentration of approximately two units as a threshold (< 0.5‰ versus ≥ 0.5‰, FC 1.21, $P_{FDR}$ = 0.344). Individuals reported to have smoked cannabis in the past 48 hours were more attractive to mosquitoes than individuals that did not smoke cannabis (FC 1.35, 95% CI 1.09-1.66, $P_{FDR}$ = 0.017, Figure 3D). Cannabis was the only substance for which an effect on mosquito arm-landings was found, the effects of other substances were statistically not significant (all $P_{FDR}$ > 0.569). There was no indication that the presence of a cannabis user made mosquitoes fly at higher altitudes or made them less aggressive.

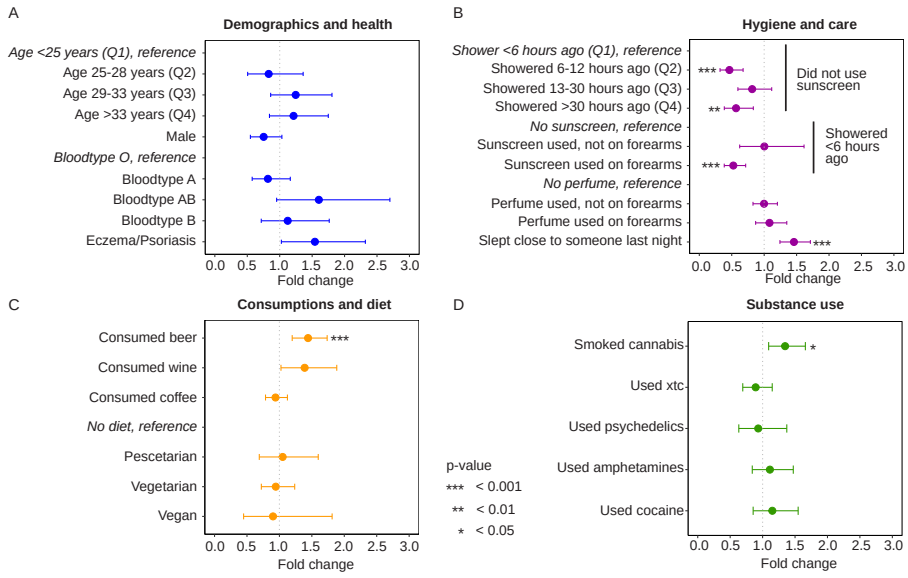


**Figure 3. Category-wise assessment of factors influencing mosquito attraction.** Forest plots showing fold changes in mosquito attraction (arm landings) for different participant characteristics relative to reference groups. Variables were grouped into different categories (A-D). A fold change greater than one indicates an increased attraction compared to reference. Dots represent the point estimates, horizontal bars indicate the 95% confidence intervals (CI). Asterisks represent significance levels of <0.05 (*), <0.01 (**), and < 0.001 (***), after adjusting for multiple testing.

We then assessed the effect of statistically significant variables from different categories combined in a single model. Mosquito arm-landings were still higher for individuals that drank at least one beer in the past 12 hours (FC 1.35, 95% 1.12-1.63, $P_{FDR}$ < 0.001, Figure 4); the effect of cannabis remained positive (FC 1.18, 95% 0.97-1.44) but lost statistical significance ($P_{FDR}$ = 0.216). Individuals who had slept with someone else the night before were 1.34 times more attractive than individuals who slept without close company (95% CI 1.14-1.58, $P_{FDR}$ = 0.002), without evidence for an interaction with beer consumption (P = 0.971). The interaction between time

since latest shower and application of sunscreen on forearms remained robust ($P_{FDR}$ < 0.001). Individuals that showered recently and used sunscreen on their forearms were still less attractive than individuals who did not use any sunscreen (FC 0.52, 95% 0.38-0.70, $P_{FDR}$ < 0.001).

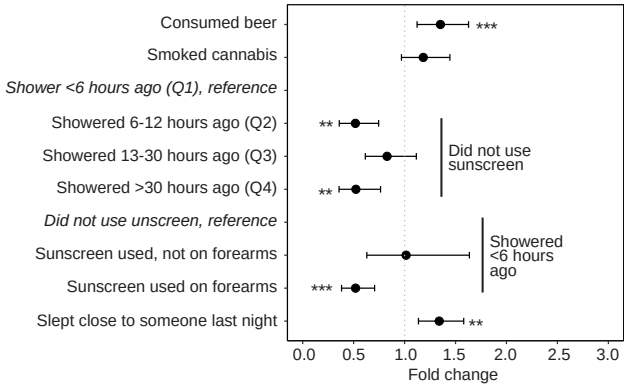


**Figure 4. Factors driving mosquito attraction.** Statistically significant variables (corrected for multiple testing) were selected from the category-wise models and assessed in a single model. Forest plot showing fold changes in mosquito attraction (arm-landings) for different participant characteristics relative to reference groups. A fold change greater than one indicates an increased attraction compared to reference. Dots represent the point estimates, horizontal bars indicate 95% confidence intervals (CI). Asterisks represent significance levels of <0.05 (*), <0.01 (**), and < 0.001 (***), after adjusting for multiple testing.

A variety of bacterial taxa were detected on forearm skin swabs from selected individuals (Supplementary Figure 4). The alpha diversity of the skin microbiota, indicated by the Shannon index, did not differ between the subset of highest and lowest attractive individuals (P = 0.77, Figure 5A). We also found no evidence of a different overall microbiota composition based on beta diversity between high and low attractive individuals (PERMANOVA P = 0.232, Supplementary Figure 5). The four most dominant genera in both high and low attractive individuals were *Cutibacterium, Sphingomonas, Corynebacterium,* and *Staphylococcus* (Figure 5B). All of these genera are recognized as skin commensals except for *Sphingomonas,* which is associated with water and other environmental sources [21, 22]. Its presence on the skin may indicate environmental exposure. The dominant presence of *Corynebacterium,* previously linked to human malodour[23,24], suggests a distinctive smell in our study container, though we will refrain from evaluating individual contributions. *Streptococcus* was found to be more abundant in high compared to low mosquito-attractive individuals (2.68% vs. 0.61% mean relative abundance, respectively), although the effect was modest and only statistically significant when uncorrected for multiple testing (Mann Whitney U P-uncorrected = 0.017).

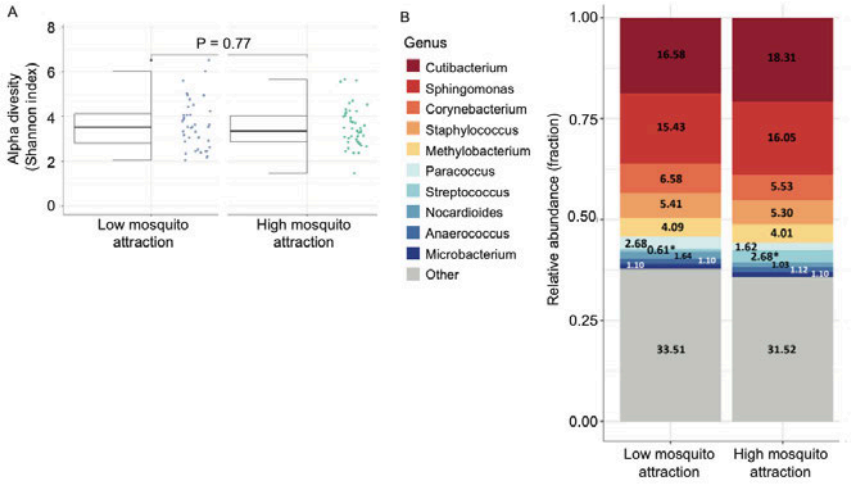


**Figure 5. Forearm skin microbiome diversity and composition in relation to mosquito attractiveness.** A subset of 85 participants with highest (n=45) and lowest (n=40) mosquito attractiveness were selected for skin microbiome assessments using forearm skin-swabs. A) Alpha diversity box plots of study groups with lowest (left) and highest (right) relative attraction scores. Alpha diversity was assessed using the Shannon index and no significant difference in diversity was observed. B) Stacked-bar graphs with the average genus-level relative abundance values for low (left) and high (right) attraction score individuals. Shown are the top 10 most abundant genera for this study contrast. The average successful classification rate up-to genus level was 88.6% ± 6.1% SD, for the complete dataset of n=97 samples (including 8 pilot samples). Note that this stacked-bar visualisation here has been normalized to 100%.

## Discussion

Some people are bitten by mosquitoes more often than others, but the underlying mechanisms remain poorly understood. In the present study, we developed an experimental setup to investigate differences in mosquito attraction in a cohort of festival goers in the Netherlands. Mosquito attraction was quantified by the number of landings on the participant's arm-side of the experimental cage, revealing substantial heterogeneity across the cohort.

Beer consumption significantly increased mosquito attraction. This observation is consistent with previous work studying mosquito attraction in a different experimental set-up with smaller sample sizes[25,26] and a considerably more modest intake. Measured blood alcohol concentration did not have an observed effect on mosquito attraction. Mosquito may simply be drawn to the distinctive smell of a

Heineken pilsner, and alcohol blood concentrations may also be high because of wine or liquor consumption. Given the number of beer drinkers in our study, it's clear that beer holds a special place for many. That said, while our findings are intriguing, there may be other contributing factors yet to be identified. Further research is certainly needed before we suggest rejecting a beer solely to avoid mosquito bites.

We also observed a negative association between the application of sunscreen on participants forearm and mosquito attraction. This association became less negative when participants showered a longer time ago. We did not ask participants when they applied sunscreen, but we assume it would be after their latest shower. The observed interaction suggests that sunscreen's negative effect on mosquito attraction may wane over time. The observed effect could stem from sunscreen blocking human odours naturally present on a participant's arm, or the sunscreen could contain a mosquito repellent component. This component is likely generally present in different sunscreen types and brands, given the consistently lower mosquito attraction observed and the likelihood that participants used a variety of sunscreen products. Even though further studies are needed to confirm and elaborate on the effect of sunscreen, it may be wise to bring some to the next summer barbeque.

We did observe a modest difference in skin microbiota of individuals with high versus low mosquito attractiveness. In line with a previous report[27], we observed *Streptococci* to be significantly more abundant in highly attractive individuals when uncorrected for multiple testing. Although using different set-ups like sampling feet instead of arms and excluding alcohol intoxication, earlier work found elevated levels of *Staphylococci* in the skin microbiome of individuals highly attractive to mosquitoes[6,27]. One of these studies did report an additional difference in bacterial diversity[6], whereas this was not confirmed by the other[27] and neither by our study, highlighting the complex role of the skin microbiome in human odour profiles and associated mosquito attraction. Importantly, others applying 16S rRNA sequencing to identify skin microbes have done this in a controlled lab setting where participants were, for instance, not allowed to shower on the day of sampling or use a sauna within 48 hours beforehand[28]. Our natural study setting with a 'pop-up' laboratory container situated in a steamy farmland populated by sixty thousand festival attendees limited control over these variables. Our participants were allowed to have showered, a privilege some clearly declined, and the music festival even had a sauna that people could have gone to.

Our study has several limitations. Although the large scale of our study distinguishes it from previous work, its setup also markedly differs from the controlled conditions used in earlier mosquito attraction studies[6,9,13,25-27], thereby limiting direct comparison of findings. Admittedly, though we like to think there's a curious inner scientist in everyone, our sample of science-loving festival-goers is not likely to be the average cross-section of humanity. Moreover, whilst substance use and alcohol uptake was already high in our cohort, it may be underestimated compared to the general festival crowd, considering the most active and perhaps least responsible partygoers (i.e. those frequenting the 24-hour stage) could still be asleep or too busy partying during our data collection hours.

The general picture that emerges from our study suggests that a sober life-style - abstaining from drugs and alcohol, sleeping alone, and applying sunscreen regularly - lowers one's chances of getting bitten by mosquitoes. While we found no evidence supporting popular myths such as blood type influencing bite frequency, we were unable to assess the existence of so-called 'sweet blood'. Ultimately, enjoy the next festival or camping trip as you like—but it seems mosquitoes may have a soft spot for those making less responsible choices.



**Acknowledgements**

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All authors declared to have no competing interests according to ICMJE uniform disclosure forms.

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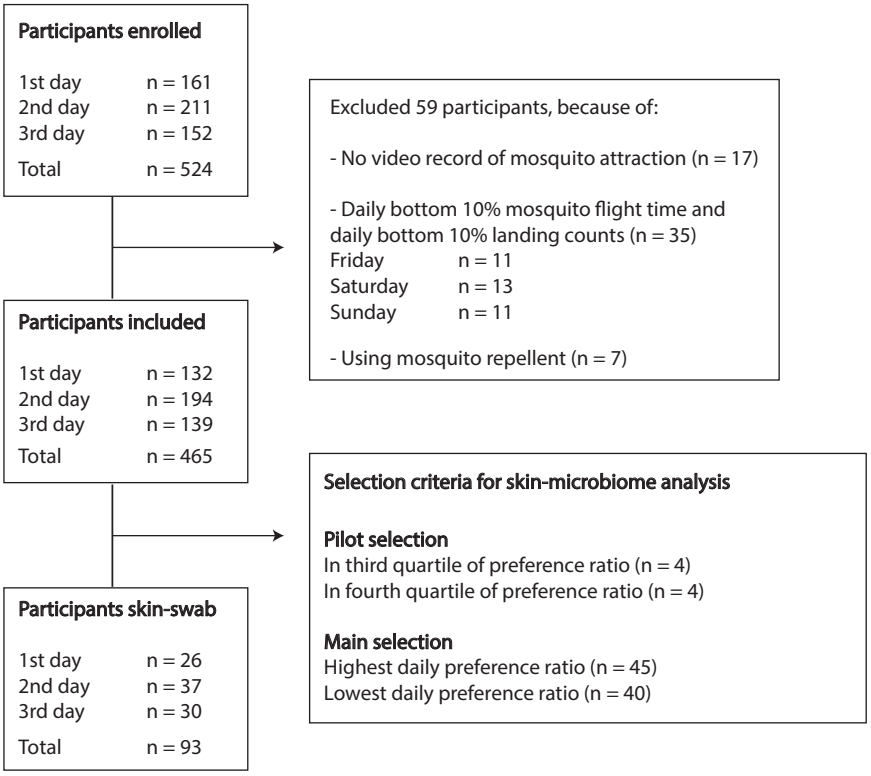
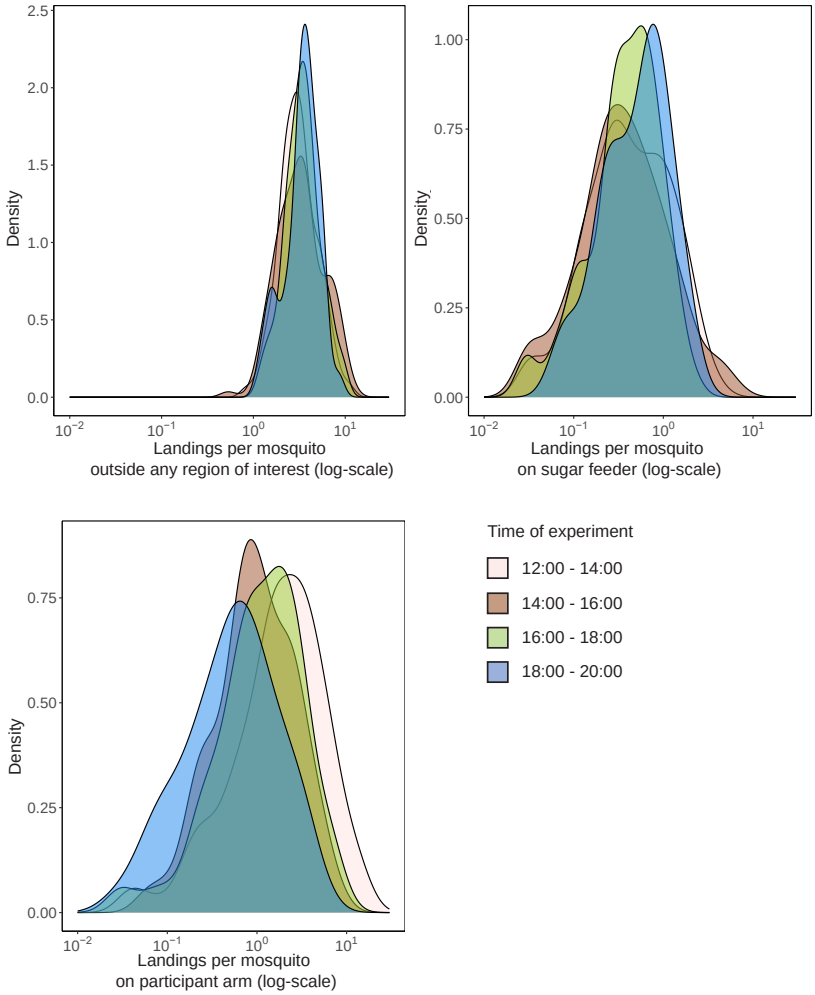
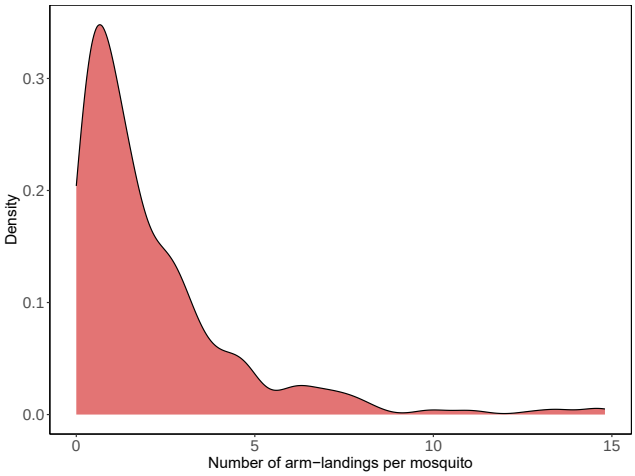
# Supplementary Figures



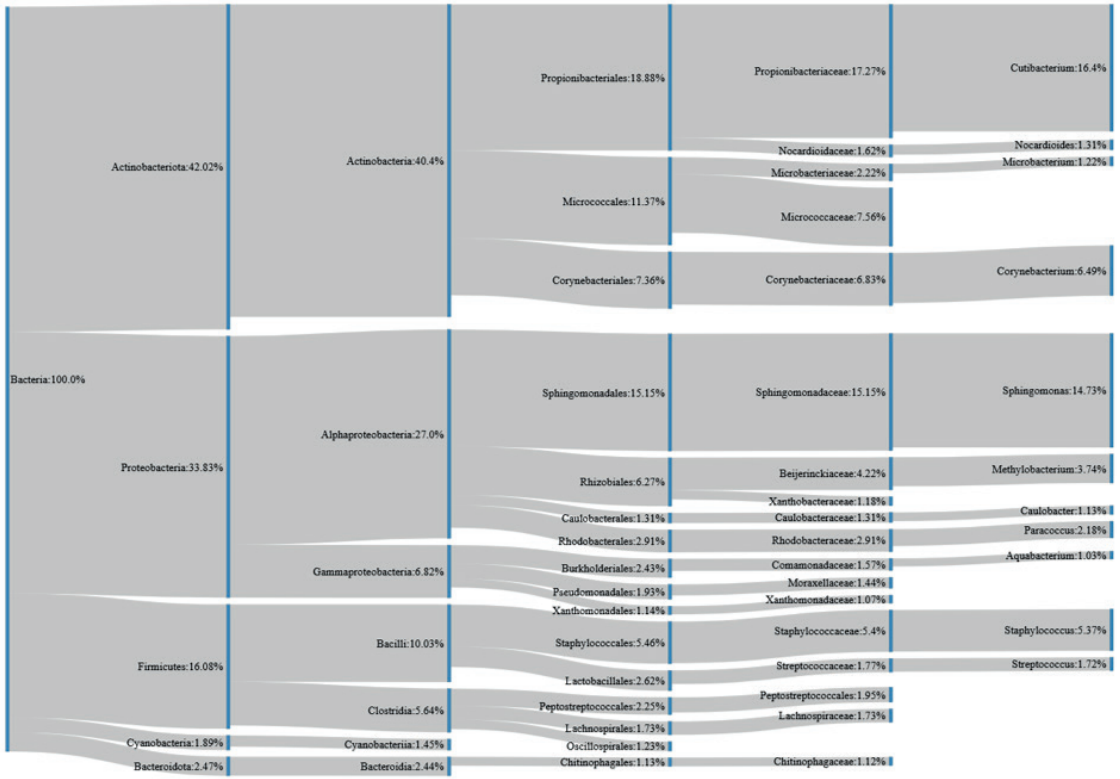

**Supplementary Figure 1. Sample selection procedures.** The number of participants included at start, after exclusion due to i) absence of video records, ii) low mosquito activity, or iii) usage of mosquito repellent, and those selected for skin-swab assessments.



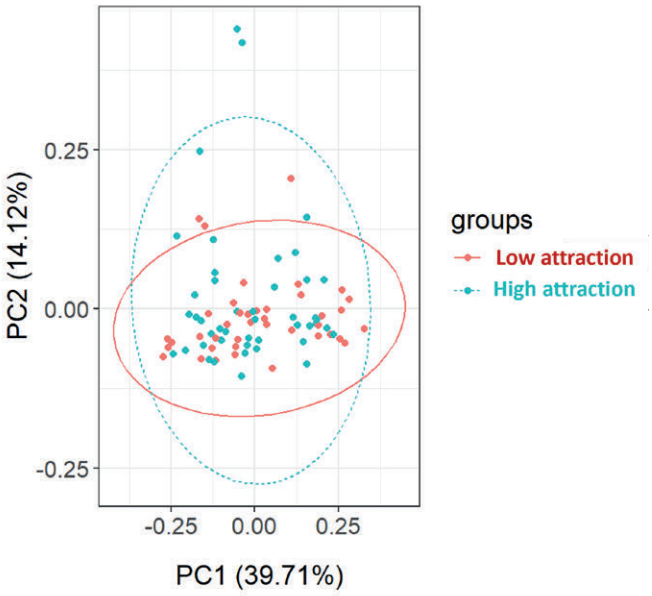

**Supplementary Figure 2. Density plots indicating the number of landings per mosquito outside any region of interest, landings on the sugar feeder and landings on the participants arm region, separated by time of the experiment (in slots of 2 hours).**



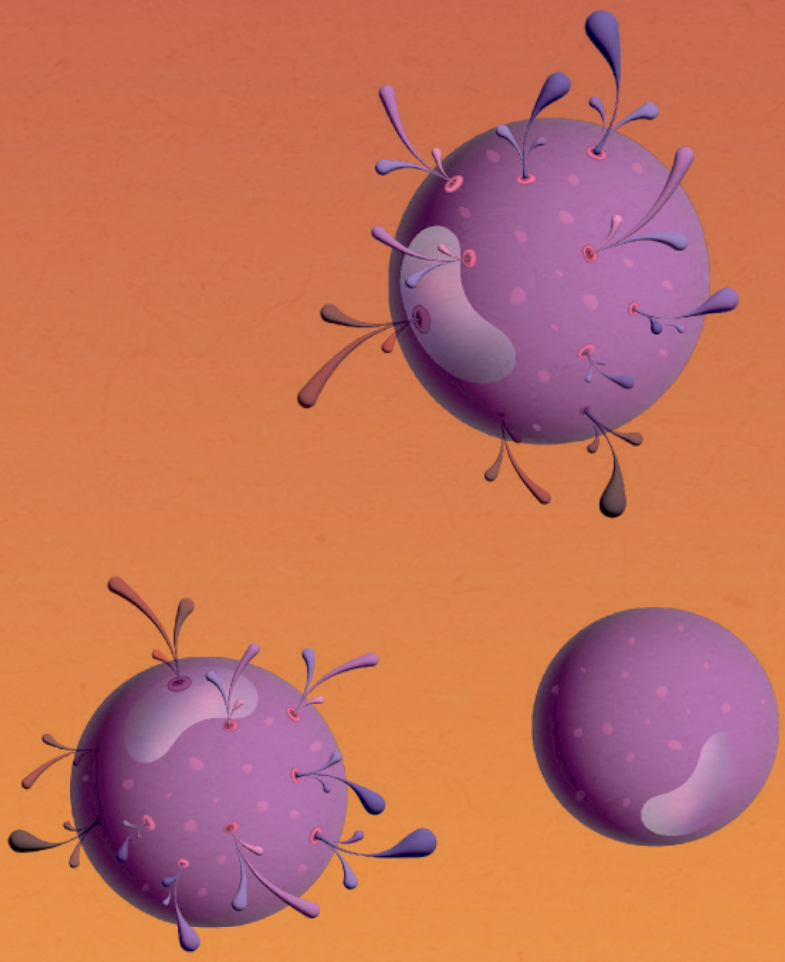

**Supplementary Figure 3. Density plot showing the number of mosquito arm-landings across all included participants.** The number of arm-landings per mosquito ranged from 0 to 14.8 in one measurement of three minutes.




**Supplementary Figure 4. The overall microbiota composition of forearm skin samples.** Sankey visualisation of the overall 16S microbiota composition of selected forearm skin samples (n=93). Values represent taxa average relative abundances over all samples, at different phylogenetic levels from phylum (left) up-to genus level (right).




**Supplementary Figure 5. 16S analysis of the forearm skin microbiota beta diversity.** Principal Coordinate Analysis (PCoA) of the Beta diversity based on weighted UniFrac distance, on study groups with low (red) and high (cyan) attraction score individuals (PERMANOVA p-value = 0.232)



Chapter 6

# General discussion

At the moment in time this thesis was completed, 47 countries had been certified as having eliminated malaria by the World Health Organization (WHO), defined as having achieved at least three consecutive years without local malaria transmission. However, we are still far removed from eradicating this life-threatening disease and to continue this effort, the WHO has recently recommended two vaccines aiming to reduce morbidity and mortality in children in areas with a significant malaria burden. These currently approved vaccines, RTS,S and R21/Matrix-M, directly reduce malaria incidence in vaccinees by lowering the likelihood of infection, though efficacy is limited and decreases substantially after 1 year[1]. Interventions that reduce exposure to infected mosquito bites (i.e. the force of infection) could complement current vaccines and further reduce the malaria burden. This is where a transmission blocking vaccine (TBV) could be a very valuable tool for preventing the spread of the malaria parasite by intervening during the sexual stage development, and thus lowering infection rates. The TBV Pfs230D1-EPA is currently on the forefront of the race. However, clinical trials performed with Pfs230-D1-EPA have not yet convincingly shown whether this TBV candidate will be efficacious enough to achieve herd immunity. Other leading TBV candidates, including R06C (Pfs48/45) and Pfs25, failed to induce transmission reducing activity (TRA) in human volunteers[2,3]. It is therefore essential to continue the search for new TBV candidates (**Chapter 3 and 4**), to optimise existing candidates and consider combining vaccine components directed at multiple parasite stages. The efficacy of Pfs230 antigens showcases the importance of the human complement system, since functional antibodies induced by Pfs230 almost all depend on complement for their activity. The involvement of complement thus provides the opportunity to tweak Pfs230 TBVs to higher efficacy by enhancing complement activation (**Chapter 2**). Exposure to mosquito bites is another crucial aspect of malaria transmission. Indeed, some of the most successful interventions to reduce transmission (e.g. bed nets) prevent the infectious bite in the first place, rather than disrupting the downstream infection. A detailed understanding of mosquito biting preferences is therefore another important piece of the transmission reduction puzzle. Attractiveness of one human can differ from the next, and studying the factors behind these differences can provide valuable insights **(Chapter 5)** that may ultimately form the starting point of interventions that aim to reduce exposure to (infected) mosquito bites.

This final chapter is divided in three parts. The first part discusses the important role of complement regarding antibodies targeting the transmission and pre-erythrocytic stages of *P. falciparum* and the role of complement in malaria vaccines. The second part provides a critical view on the discovery of TBV antigens, the process after identifying a new candidate, and finally how TBVs can contribute to malaria elimination. The third

and final part of this discussion delves into an unconventional approach to study human attractiveness to mosquitoes and the implications of this field of research.

# Part 1: Complement

## Complement-mediated antibody effector mechanisms

The human complement system is an essential part of the human innate immune system and consist of a complex protein network (**Chapter 2**). Antibody-mediated complement activation occurs via C1, which binds to antibodies on a foreign surface, and ultimately leads to cell lysis. The other effector mechanisms of complement are opsonization of pathogens and recruitment of immune cells by anaphylatoxins[4], however these mechanisms are not activated by TBV-induced antibodies that do not act in the human bloodstream but find their target in the mosquito midgut. The Pfs230D1M-EPA vaccine elicits antibodies that bind the gamete surface and activate the classical pathway of complement. This raises the question whether a Pfs230-based TBV and thus the induced antibodies would require complement for their activity. Interestingly, almost all anti-Pfs230 antibodies known to have TRA, require complement for their functional activity[5-11]. One exception was found by Dietrich et al.[10] who showed TRA by Pfs230 nanobodies that possibly experience less sterical hindrance due to their smaller size. The other exception was reported by Macdonald et al.[11] who noted functional activity by mAb 4F12 in standard membrane feeding assay (SMFA) without the addition of complement, although complement fixation by 4F12 was shown to significantly improve TRA[12]. There are several anti-Pfs230 antibodies that neither have TRA nor can activate complement, even when their antibody subclass can facilitate complement activation[6]. A clear explanation for this discrepancy has not yet been provided. Perhaps the cause is rooted in the spatial orientation of the antibody towards the antigen, preventing a proper foundation for the C1q complex. The tertiary structure of Pfs230 could prevent antibodies targeting certain epitopes to organise in such a matter that the classical pathway can be initiated. When speculating about the impact of Pfs230 protein folding, the complex it forms with Pfs48/45 should be considered[13,14]. Recent research provided valuable new insights by performing Cryo-EM on Pfs230:Pfs48/45 [15,16]. Bekkering *et al*. show that C-terminal Pfs230 domains are involved in the Pfs48/45-based attachment to the membrane, whereas it seems the N-terminal domains are unhindered in engaging in other protein interactions[15]. Complement dependent monoclonal antibodies 2A2 and 18F25 were used to structurally characterize epitopes on Pfs230D4 and Pfs230D7 (**Chapter 3**), respectively. Both these domains, as well as Pfs230D1 and D12, are shown to be oriented in the direction opposite the membrane[15]. This suggests that

the membrane distal orientation provides enough space for the antibody to bind and hexamerise, forming the proper foundation for activating the classical pathway.

This dependence on complement by a TBV seems exclusive to Pfs230. Pfs48/45 is a candidate capable of inducing antibodies that obstruct the critical function of Pfs48/45 in parasite development by binding their target[17]. It is suggested that accessibility and the unknown functional properties of Pfs48/45 contribute to the TRA efficacy of anti-Pfs48/45 antibodies[18]. Dietrich *et al*. argue that anti-Pfs48/45 antibodies dissociate the Pfs230:Pfs48/45 complex, posing that the potent mAb TB31F prevents the interaction between Pfs48/45D3 and Pfs230D13-D14[16]. This is however contradicted by a cryo-EM study showing that Pfs48/45 functional antibodies might prevent interaction with other proteins, since complex disruption is not directly correlated with functionality[15]. Other structural research on Pfs48/45D1 corroborates that several potent antibodies target Pfs48/45D1 and act independent of complement[19]. This prompts the question: what is the difference between antigens, particularly the gamete surface proteins Pfs230 and Pfs48/45, that determines whether they are targeted by functional complement-dependent or complement-independent antibodies? It is possible that antibodies against Pfs48/45 functional epitopes activate complement, though the blockage of Pfs48/45 function prevents the mechanism from being dependent on complement. Although, complement activation by Pfs48/45 antibodies would require a spatial orientation that can accommodate antibody hexamerisation. As suggested by the above mentioned cryo-EM studies, Pfs230 in complex with Pfs48/45 might cause sterical hindrance and prevent complement activation.

In summary, complement activation is an effective antibody effector mechanism for some vaccine candidates, but seems irrelevant for other vaccine candidates. Acquiring more knowledge on the difference between complement dependent and independent mechanisms of antibodies would provide valuable insights for vaccine candidate design.

## Complement dependency of malaria vaccines

Our understanding on the association between antibody-complement interactions and protection against malaria has increased over time[20-22]. It is suggested that these interactions can contribute to vaccine-induced immunity against malaria and that focussing on improving functional activity of antibodies, not antibody titre, might be a good alternative approach[23]. It is therefore important to not neglect the valuable knowledge on complement activation by antibodies when designing or improving malaria vaccines.

Designing a successful vaccine is almost entirely dependent on the induction of long-lasting protective antibodies (or long-lasting cellular responses). The development of human antibodies commences through the encounter of a naïve B cell with an antigen[24]. In the secondary lymphoid organs, B cells can differentiate within a germinal centre (GC). They exit the GC as either plasma cells or memory B cells. A third option for B cells is to continue recycling within the GC, which increases affinity maturation of their B cell receptors[25]. Within this process, B cells interact with T follicular helper (Tfh) cells, which steers differentiation towards one of these directions; plasma cell, memory B cell, or affinity matured B cell. T(f)h cells also undergo their own differentiation into Th1, Th2 or Th17, with the latter two being more capable in stimulating B cell differentiation and antibody secretion[26]. Interestingly, the Th1/Th2 phenotype can affect the predominant subclass of antibody production. IgG2a production in mice is linked to a Th1 response, whereas Th2 leads to IgG1 production[27]. Since complement fixation is dependent on the antibody subclass, with IgG1, IgG3 and IgM activating complement in humans (IgG2a, IgG2b and IgG3 in mice), this aspect of antibody production should not be overlooked for vaccine design. Especially in the case of the complement-dependent antibodies induced by Pfs230 (**Chapter 4**), it is worthwhile delving into steering the antibody production towards specific subclasses.



One aspect of vaccine design that lends itself for adjustment and thus potentially improvement, is the choice of adjuvant. For example, the $AS01_B$ platform for PfRH5 induced a response that was skewed towards Tfh2, which is desirable for antibody production[28]. An adjuvant can also steer the process of antibody production towards the complement-fixing subclasses. Adjuvants Alhydrogel and AS01 have been compared for Pfs230 with results showing that Pfs230D1-EPA induces higher antibody titres in AS01 [29]. Furthermore, Pfs230D1-EPA formulated in AS01 induced a response dominant for IgG2a and IgG2a (complement-fixing in mice), whereas a formulation in Alhydrogel induced more non-fixing IgG1[30]. SMFA results show that sera from immunization with Pfs230D1-EPA-AS01 is superior to formulation in Alhydrogel. It would be valuable to learn if the increased functional activity is causally linked to improved complement activation, or whether it is due to the higher antibody titre. More information might be available after results from the Phase 2 clinical trial involving Pfs230 formulated in AS01 are published [ClinicalTrials.gov: NCT03917654].

Furthermore, the complement evasion mechanisms employed by *P. falciparum* (**Chapter 2)** should not be forgotten in the process of vaccine design. It is possible that Pfs230 vaccine efficacy is negatively impacted by one of the gamete's

complement evasion mechanisms, recruitment of plasmin[31]. A bloodmeal containing functional anti-Pfs230 antibodies was supplemented with plasmin, which increased oocyst numbers, likely by degrading C3b on the gamete surface[31]. For sporozoites on the other hand, it has been shown that they evade complement evasion by recruiting C4BP to their most abundant surface protein, CSP[32]. In theory, it could be detrimental to the sporozoite if this evasion mechanism was blocked, depending on how essential C4BP recruitment is and if there are other classical pathway inhibitors recruited. Though, Ayman *et al*. only show a limited decrease in motility when sporozoites cannot recruit C4BP for evasion of the classical pathway, when activated by hyperimmune IgGs (purified from a serum pool)[32]. In this case, performing sporozoite lysis or hepatocyte invasion assays might be more informative on the viability of sporozoites. Including conditions with C4BP-depleted complement serum and an anti-C4BP mAb could provide answers on the implications of C4BP recruitment. Results for RTS,S indicate that the vaccine activates the classical pathway. Kurtovic *et al*. show that children vaccinated with RTS,S had significantly higher C1q-fixing antibodies to the NANP repeat and C-terminal region of CSP compared to the control group[22]. Though, if the C4BP evasion mechanism is still functional in people that received a CSP-based vaccine such as RTS,S, it might be causing a decrease in efficacy. It could therefore be beneficial for RTS,S efficacy rates to enhance classical pathway activation by preventing C4BP recruitment.

Besides C4BP, there are other examples of complement inhibitors or their binding proteins that could potentially be targeted as part of a more comprehensive malaria vaccine approach. Merozoites evade complement attack by recruiting down-regulatory complement proteins (Factor H and C1-INH) to their surface (**Chapter 2**), a mechanism that could be interrupted by a monoclonal antibody binding to the regulator-recruiting antigen. Bassi *et al*. demonstrated that a murine mAb against PfMSP3, binding partner of C1-INH, can interfere with C1-INH binding to the merozoite surface[33]. The authors show no further functional experiments but suggest using antigens such as PfMSP3 in multi-component vaccines along with a blood-stage antigen. There is certainly potential in preventing complement evasion, since active complement could aid in clearing out parasites by MAC formation and promote phagocytosis. It would also be interesting to investigate whether a mAb targeting a complement regulator binding antigen could enhance the efficacy of TBVs. Whilst conceptually attractive, it is challenging to combine vaccine antigens that each need to target functional epitopes, allow large-scale production and be amenable to co-administration. Overall, complement activation and evasion thereof have an important role in malaria vaccines and increasing our knowledge on this topic can be of great value for future vaccine design.

## Monoclonal antibodies against CSP

Instead of inducing antibodies with a vaccine, administering monoclonal antibodies could be a stand-alone strategy against malaria. Monoclonal antibodies (mAbs) have several advantages over a vaccine, including immediate action after inoculation, no reliance on effective activation of the host immune system and high specificity. Instead, mAbs allow achieving a high and predictable titre in all recipients. However, large scale production of mAbs comes with its challenges, with first and foremost the production costs. There may also be a need for repeated dosing (with timing depending on antibody half-life) and a boost effect by natural infection does not apply to a monoclonal antibody strategy. Though potentially a situation could occur in which naturally acquired antibodies work synergistically with an administered mAb. An example of a transmission blocking mAb is the highly potent anti-Pfs48/45 mAb TB31F, which requires a serum concentration of only 2.1 µg/mL in SMFA to reach 80% TRA and this single administration was estimated to retain activity up to 5 months[34]. Modelling that incorporates TB31F suggests that community wide administration with 80% coverage can lead to a 54% reduction in a high-transmission seasonal settings over the course of 3 years[35]. Another fair share of attention has been directed to monoclonals targeting CSP[36,37], which could be used as prophylaxis against malaria infection. The potential of this passive protection caused researchers to direct their aim at finding the most potent anti-CSP mAb. Besides being a stand-alone strategy, these mAbs could be a valuable tool for identifying functional epitopes or could be an attractive option for administering as a co-intervention. A combination strategy could consist of mAbs targeting different malaria stages, such as TB31F along with an anti-CSP mAb, which would prevent parasites slipping through the cracks due to resistance or a gap in efficacy. Interestingly, combining a vaccine, such as R21, with an anti-CSP mAb, like CIS43LS, could also have a synergistic effect, as shown by Wang *et al*.[38]. CIS43LS, which is currently in Phase 2 clinical trial (ClinicalTrials.gov: NCT04329104)[39], is one of several potent anti-CSP mAbs, including L9LS in Phase 2 clinical trial (ClinicalTrials.gov: NCT05304611)[40] and MAM01 in Phase 1 clinical trial (ClinicalTrials.gov: NCT05891236)[41].

As discussed earlier, complement activation by complement-fixing antibodies may contribute to the efficacy of RTS,S, and has been associated with the level of protection against malaria. Furthermore, anti-CSP antibodies from volunteers in malaria-endemic areas were shown to activate human complement on *P. falciparum* sporozoites inhibiting hepatocyte cell traversal[20]. This inhibition will prevent liver stage infection, essential for the malaria parasite's survival. Also, CSP-specific antibodies from volunteers immunized with whole sporozoites facilitate the deposition of C3 on the sporozoite surface, thereby causing damage to the

parasite[42]. Importantly, while antibodies induced by sporozoite vaccination induce functional CSP antibodies that can act without complement, the presence of complement does substantially increase sporozoite invasion[42,43]. Since complement fixation may thus play an important role in the activity of CSP antibodies, we investigated whether we could improve the efficacy of potent anti-CSP monoclonals by enhancing their complement activation potential.

Naturally, complement activating IgG antibodies will organise into hexamer structures on the cell surface once bound to their target antigen, providing a platform to bind C1q which recruits C1r and C1s to form the C1 complex, commencing the classical pathway[44]. This principle inspired the development of HexaBody® technology[45]. HexaBody antibodies contain a point-mutation (E345K or E430G) in the antibody Fc region, to improve antibody hexamer formation that is required for efficient fixation of C1q on the pathogen surface[45]. This technology has been implemented in several fields; developing a HexaBody of a chimeric anti-gonococcal mAb[46], but also of an IgG1 targeting CD38 to induce complement-dependent toxicity in patients suffering from multiple myeloma[47]. Here, we obtained HexaBody variants (point-mutation E430G) of several potent anti-CSP mAbs including 3SP2 (targets NANP repeat), CIS43 and MGG4 (both targeting the CSP junctional and NANP epitope), mAb311 and mAb317 (both target NANP repeat)[48-51] as well as parental human IgG1 antibodies that lacked the E430G mutation.

We aimed to determine the effect of the HexaBody design on complement fixation and if improved compared to the original mAbs, establish whether this would enhance functional activity against sporozoite liver cell invasion. For this aim, we used the HexaBody antibodies in several *in vitro* assays, including flow cytometry to assess affinity to live sporozoites, ELISA with CSP coating to determine enhancement of complement activation and flow cytometry to examine inhibition of hepatocyte invasion. We found that the recombinant CSP HexaBody antibodies bind CSP on the surface of whole sporozoites with a similar apparent affinity as the parental mAbs, except for HexaBody variants of 311 and 317 that seem to bind with lower affinity than their parental mAbs (**Fig. 1)**. We then set out to compare the C1q fixing capacity of the HexaBody mAbs and the parentals in ELISA coated with CSP. HexaBody variants showed superior C1q fixation compared to the parentals (**Fig. 2A**). However, a similar CSP-ELISA experiment designed to detect MAC formation, showed a very minimal difference between HexaBody and parental antibody (**Fig. 2B**). The HexaBody is only slightly superior in level of MAC detected for just one concentration (0.33 µg/mL), which interestingly is the concentration for which the HexaBody has the most C1q fixation compared to the parental (**Fig. 2**).

To assess whether increased capacity to fix complement activation translated to improved functional activity, we compared the Hexabody and parental antibodies in an *in vitro* invasion assay with sporozoites and HC-04 cells (hepatocytes). We tested antibodies in the presence of complement (NHS) or absence of complement (HIHS) to assess the effect of active complement on the functionality of the anti-CSP mAbs. The assay did not pick up any difference between the parental antibody and respective HexaBody, regardless of the presence or absence of active complement (**Fig. 3**). We therefore concluded that the anti-CSP HexaBody mAbs seem to enhance C1q fixation when bound to recombinant CSP, but that this does not translate to increased inhibition of sporozoite invasion of hepatocytes.

Multiple plausible explanations could explain the lack of increased activity of the HexaBody mAbs. To start, complement seems to have little to no effect on anti-CSP mAb activity in the invasion assay (**Fig. 3)**, which might indicate that this assay is not ideal for assessing the impact of potentially enhanced complement activation. Also, a sporozoite maturation assay could perhaps evaluate if the HexaBody targeted sporozoite is viable enough to invade a hepatocyte, though cannot further develop.



Since we do not show C1q fixation on live sporozoites, it is possible that such an experiment would not detect an increase in C1q between HexaBody and parental mAb. In that case, this could be explained by the spatial organization on the sporozoite surface not allowing an increase of deposited C1q. For example, PfEMP1 is suggested to be displayed on infected erythrocytes in such an orientation that it prevents activation of the classical pathway[52]. It would be interesting to perform flow cytometry experiments detecting C1q fixation and MAC formation on live sporozoites to determine the difference or lack thereof between HexaBody and parental mAb.

It is also possible that these highly potent mAbs left no room for improvement and have thus reached their maximum potential. The anti-CSP mAbs in our selection are primarily targeted at the NANP repeat of CSP, so it might be worthwhile to test other mAbs that target the same region though with a lower potency. It would also be interesting to see if antibodies targeting a different region, the N-terminus or the C-terminus, which is a target for RTS,S induced Fc-dependent functionality[22], might be susceptible to enhancement via HexaBody alterations. Interestingly, a recent study included mAb317 and CIS43 and suggests an Fc-dependence of protection by these mAbs and the authors show that Fc-engineering does have potential for increasing potency[53].

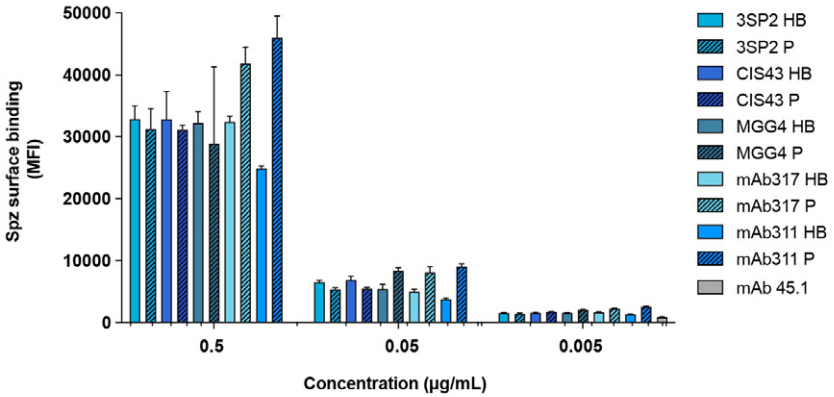
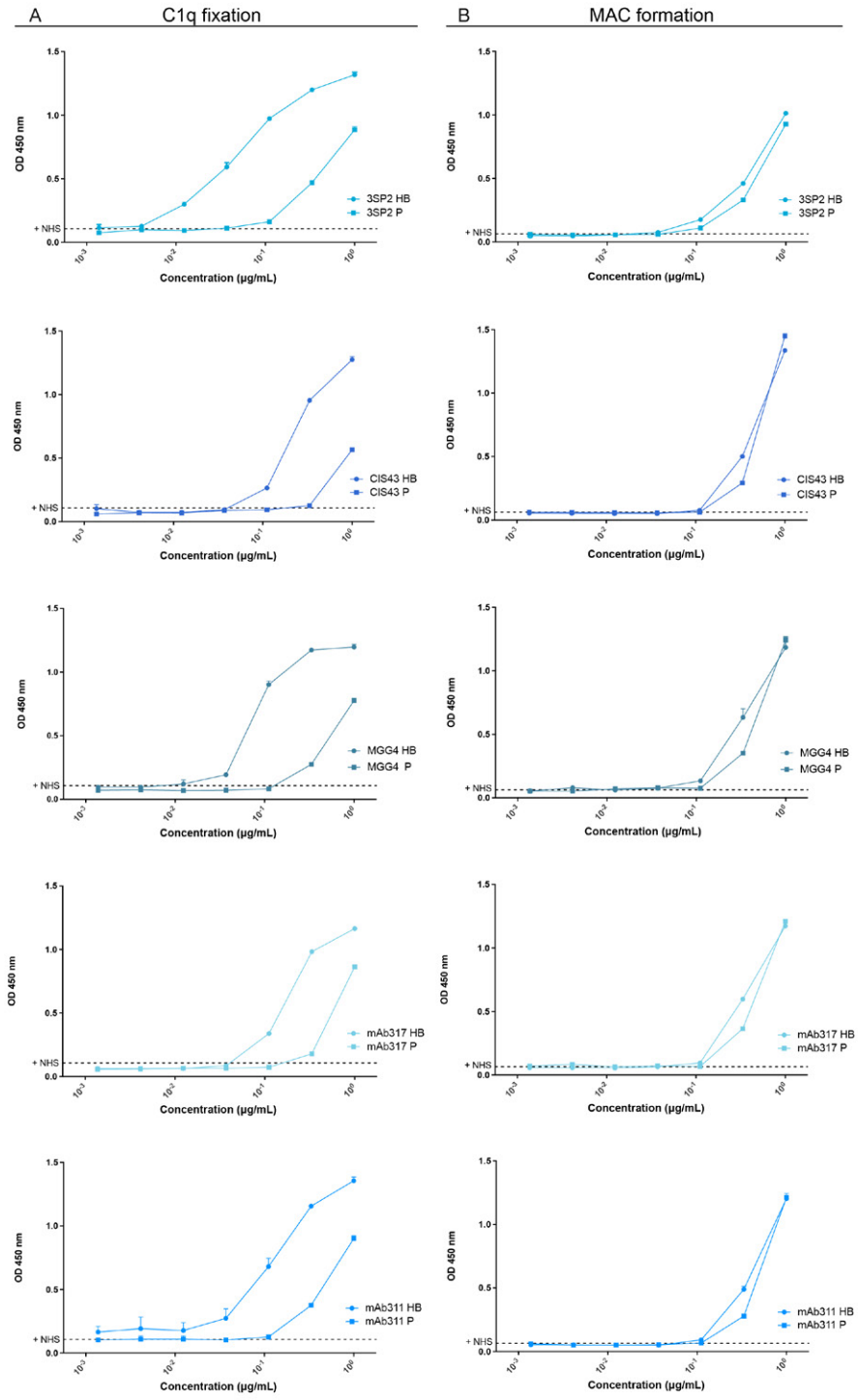



**Figure 1. Anti-CSP HexaBody and respective parental antibodies have similar affinity to sporozoites.** *P. falciparum* NF54 sporozoites were obtained from dissected salivary glands. Sporozoites were purified by centrifugation using a 17% Accudenz (Accurate Chemicals) gradient in PBS (MilliporeSigma), previously described by Kennedy *et al.*[54]. The sporozoites were incubated with 1µM Syto61 Red Fluorescent Nucleic Acid staining (Invitrogen; 1:5000 in PBS) for 30 min, before washing with PBS. Per condition, 50,000 sporozoites were incubated with HexaBody antibodies or parental antibodies (0.5, 0.5 and 0.005 µg/mL of 3SP2, CIS43, MGG4, mAb317 and mAb311 in 3% BSA in PBS). Anti-Pfs48/45 mAb 45.1 (IgG)[55] was used as a negative control. Sporozoites were incubated with antibodies for 45 min at 4ºC, before staining with 1:200 Alexa Fluor™ 488 Goat anti-Human IgG (H+L) (Invitrogen) for 45 min at 4ºC. Sporozoites were washed with PBS followed by centrifugation and fixed with 1% Paraformaldehyde. Antibody binding to sporozoites was assessed by flow cytometry with a Gallios™ 10-color system (Beckman Coulter) by acquiring data for 5000 Syto 61-positive cells (sporozoites). Further data analysis was performed using FlowJo (BD, version 10.7.1), employing the gating strategy as described previously[56]. Anti-CSP antibodies were a kind gift from GenMab (Aran Labrijn). Centrifugation steps were performed at 3220×g for 5 min at 4ºC. Bars show the mean antibody binding to the sporozoite surface measured in Mean Fluorescent Intensity (MFI) of three technical replicates and error bars indicate Mean ± SEM. HB = HexaBody mAb, P = Parental mAb.

**> Figure 2. Anti-CSP HexaBody antibodies show increased C1q fixation on recombinant CSP compared to their respective parentals, but only minimal difference in MAC formation.** ELISAs were previously described in full detail[42]. Briefly, Nunc MaxiSorp™ 96-wells plates (Thermo Fisher Scientific) were coated with 100 µL per well of 0.5 µg/mL full-length CSP (in PBS) and incubated overnight at 4ºC. After blocking, anti-CSP HexaBody and parental antibodies (3SP2, CIS43, MGG4, mAb317 and mAb311) were added in a threefold dilution series starting at 1 µg/mL. 10% NHS was added as source of human complement and complement activation was allowed by incubating at room temperature for 1 h. After washing, wells were incubated with either 1:30000 Goat anti-Human C1q pAb (Complement tech) or 1:6000 Mouse anti-Human C5b-9neo mAb (Dako) and finally incubated with 1:2000 Rabbit anti-Goat IgG (H+L) or Goat anti-Mouse IgG (H+L) HRP labelled Secondary Antibody (Invitrogen), respectively. Plates were developed using tetramethylbenzidine and the colour reaction was stopped with 0.2M $H_2SO_4$. The optical density (OD) was measured at 450 nm on an iMark™ microplate absorbance reader (Bio-Rad). Data points are means of three technical replicates and error bars indicate Mean ± SEM. The dotted line (+ NHS) indicates the signal without the addition of mAbs. HB = HexaBody mAb, P = Parental mAb.


A
C1q fixation
B
MAC formation
OD 450 nm
Concentration (µg/mL)
+ NHS
3SP2 HB
3SP2 P
CIS43 HB
CIS43 P
MGG4 HB
MGG4 P
mAb317 HB
mAb317 P
mAb311 HB
mAb311 P

Deploying the HexaBody strategy to antibodies with a different target antigen, such as Pfs230, could reap different results especially considering the complement dependency of almost all functional anti-Pfs230 antibodies. A complement-dependent anti-Pfs230 antibody that has low to moderate TRA, might thus be capable of forming the suitable oligomers for C1q fixation but leaves room for improvement. Mutating such an antibody to enhance hexamer formation could potentially make this an antibody with high TRA. The choice for an anti-Pfs230 mAb that has no functional TRA could be informed by structural studies such as the research by Bekkering *et al*.[15]. The related epitope would need to be positioned in such a way that the mAb can activate the classical pathway by creating a platform for C1q without steric hindrance from neighbouring Pfs230 domains or other interacting proteins.

In summary, anti-CSP antibodies have been associated with complement fixation and protection, though our study does not show an effect of complement fixation on anti-CSP mAb activity. Altogether, we highlight that complement has an equally important as intricate role regarding the functionality of anti-CSP mAbs. Delving further into this topic could improve strategies using anti-CSP monoclonal antibodies, prevent sporozoite invasion and help eliminate malaria.

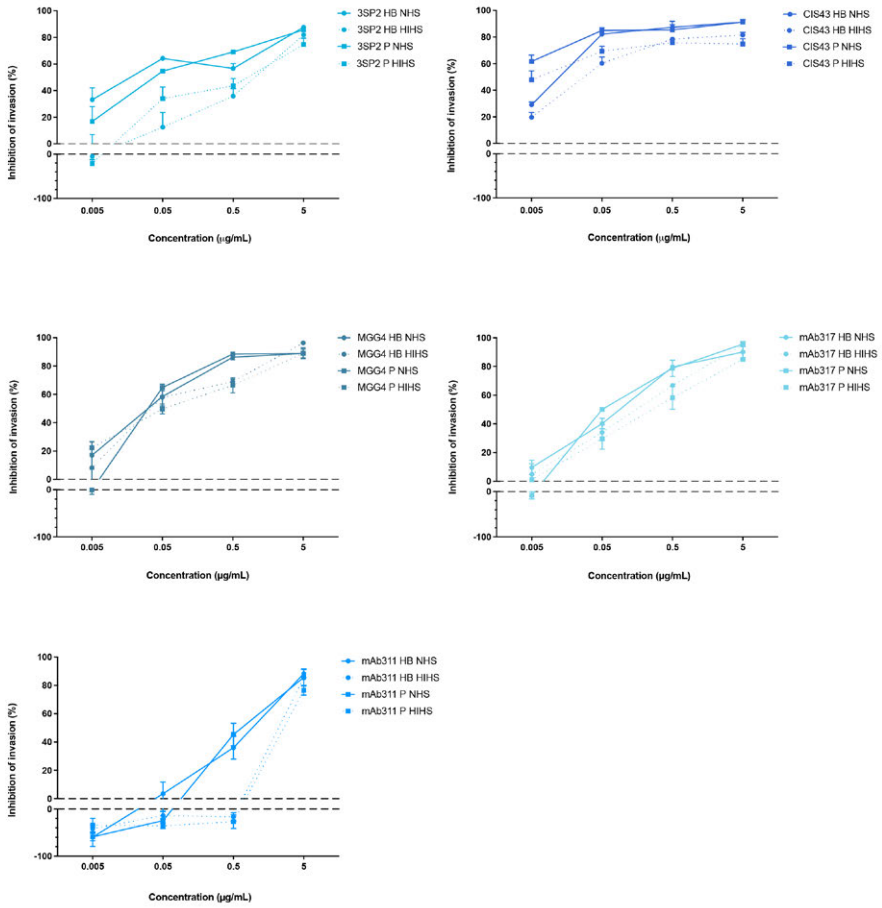



**Figure 3. Anti-CSP HexaBody antibodies are non-superior in blocking sporozoite invasion of hepatocytes compared to their respective parentals.** Inhibition of sporozoite invasion of the HC-04 hepatoma cell line was tested in a 3-hour assay as previously described [56]. Briefly, HC-04 cells were seeded overnight at 37ºC onto collagen treated 96-wells flat-bottom plates. *Pf*NF54 sporozoites were pre-incubated at 37ºC for 30 min with an IgG (HexaBody or parental 3SP2, CIS43, MGG4, mAb317 and mAb311) in a ten-fold dilution series starting at 5 µg/mL and with the addition of 10% NHS or HIHS (Heat Inactivated Human Serum). Culture media was removed from the HC-04 cells and replaced with the sporozoite mixture, after which the cells were incubated for 3 hours at 37ºC. Cells were washed with PBS and detached from the plate using trypsin, which was deactivated using 10% HIHS/PBS. After washing, cells were stained with 1:2000 Fixable Viability Dye eFluor 780 (eBioscience). Cells were then fixed and permeabilized with Foxp3 transcription factor staining buffer (eBioscience) and subsequently stained with mouse anti-CSP Alexa Fluor 488 labelled antibody (diluted 1:3200 in perm buffer). Finally, 1% PFA in PBS was used to fix the cells and data was acquired by flow cytometry with a Gallios™ 10-color system (Beckman Coulter) by acquiring data for 5000 HC-04 cells. Further data analysis was performed using FlowJo (BD, version 10.7.1). Values represent the percentage of invasion inhibition and were normalized against sporozoites without antibody added in the presence of either NHS or HIHS as applicable. Data points are means of three technical replicates and error bars indicate Mean ± SEM. HB = HexaBody mAb, P = Parental mAb.

# Part 2: Transmission blocking vaccines

## Finding a diamond in the rough – The search for new TBV antigens

The number of available TBV candidates is limited[57]. Of those identified, only Pfs25, Pfs48/45 and Pfs230 have been clinically tested. While initially looking promising in animal models, Pfs25 induced limited TRA in humans in multiple clinical trials[58], and an examination of the Pfs25-EPA formulation in healthy Malian adults indeed confirmed that no durable functional activity was induced[2]. While a first generation Pfs48/45 based vaccine, R0.6C, induced no TRA in Dutch or Burkinabe volunteers, a second-generation vaccine, ProC6C-AlOH/Matrix-M, induced IgG with 80% or higher TRA in 13 out of 20 volunteers[3,59]. These findings indicate that results are difficult to predict and that optimalisation is often required, though success is not guaranteed. Pfs230 is the only candidate of which we currently await Phase 2 clinical trial results [ClinicalTrials.gov: NCT03917654]. The most recently reported Phase 1 trial results show that Malian adults receiving 3 doses of Pfs230D1-EPA/AS01 have TRA percentages above 80% only after the third and fourth dose as measured by SMFA (ClinicalTrials.gov: NCT02942277)[60]. Healy *et al*. also performed direct skin feeding assays (DSF)[61] that require the volunteer to directly expose skin for feeding by laboratory-reared mosquitoes, to measure prevalence and magnitude of infection based on oocysts counts. The percentage of positive DSFs (with a minimum of 1 infected mosquito) for the Pfs230D1-EPA/AS01 receiving group was 0.42%, though interestingly this percentage for the comparator group was as low as 1.51% [60]. Taken together, these clinical results from the most advanced TBV candidates provide incentive to continue the search for more potent TBV candidates or new candidates that can be combined to enhance activity of current candidates.

Several decades ago, the TBV candidates that are still the most prominent today were discovered by immunisation of rodents with whole sexual stage parasites, to thereafter isolate (functional) mAbs and determine the target antigens by immunoprecipitation[62-64]. The TBV candidates Pfs25, Pfs48/45 and Pfs230 were the only candidates found by this method, most likely because these are the most abundant parasite surface antigens, the most immunogenic ones or a combination of both. If researchers would only deploy this method, important antigens might be overlooked even though there could be potential TBV candidates amongst them. This is exemplified by the vaccine candidate Rh5 that could have been identified earlier, if not for the distraction by other more immunogenic blood stage antigens[65]. Therefore, it is important to search for TBV candidates via alternative approaches.

Investigating the natural antibody response to sexual stage proteins is a different approach to discover new antigens for TBV development. This method evaluates the anti-gametocyte response in individuals who have been naturally exposed to malaria[66-68]. An interesting study by Stone *et al*. employed large scale immune-profiling to attempt linking naturally occurring antibody-mediated TRA to Pfs230, Pfs48/45 and a set of novel gametocyte proteins[69]. They included 315 antigens on the protein microarray, a valuable tool for antigen discovery that can be adapted for a high-throughput assessment of plasma samples obtained from naturally exposed individuals[70-72]. They assessed if the antibody responses against the protein targets could be associated with SMFA-determined protective immunity (TRA), aiming to identify potential TBV candidates[69]. A drawback of this method is that it can identify antigens that correlate with a protective response but are in fact not causally related. Antibody depletion or antigen-specific antibody purification against identified candidates can demonstrate causality, as done for Pfs48/45 and Pfs230 [69]. Alternatively, immunization with identified candidates would confirm whether an antigen induces a functional response. Two studies that expressed microarray-identified antigens in either *Drosophila melanogaster* S2 and *Lactococcus lactis* cells (for the antigens described by Stone *et al*.[69,73]) or the human embryonic kidney cell (HEK293) expression system[74] failed to induce any functional response in mice. It does remain a possibility that these antigens are TBV candidates, since there are several other important factors involved, such as improper folding of the recombinant antigen or interfering glycosylation introduced by the recombinant host.



The currently most advanced TBV candidate, Pfs230D1-EPA, consists of domain 1 and a small portion of the Pro Domain of Pfs230. Over the years several expression systems, including *Escherichia coli* and the wheat-germ cell free system[11,75-79], failed to produce a Pfs230 domain (other than D1) capable of inducing a functional response. Considering that Pfs230 consists of 14 cysteine-rich domains, we wondered; are none of the other domains targeted by functional antibodies and capable of inducing a functional response? We thus adopted a different plan of action in both **Chapter 3 and Chapter 4**. In **Chapter 3** a non-functional antibody (18F25), previously induced in mice by immunization with whole gametes, was found to target Pfs230 domain 7 (Pfs230D7). Interestingly, the subclass switched variety of this antibody showed complement-dependent TRA in standard SMFAs. Furthermore, the complement-fixing 18F25 is a potent antibody, reaching TRA of 93% upon addition of 30 µg/mL complement-fixing 18F25 in SMFA. Bekkering *et al*. included 18F25 in their structural studies, elucidating the structure of the Pfs230:Pfs48/45 complex whilst discovering the conserved epitope of 18F25 [15].

Taken together, we identified Pfs230D7 as new TBV candidate. In several studies Pfs230D7 failed to induce any functional response after immunization[11,75-79]. Since the functional mAb 18F25 targets a conformational epitope on Pfs230D7, it is plausible that recombinant D7 antigens have so far failed to induce functional responses due to improper folding. We attempted the expression of D7 in the *D. melanogaster* S2 (**Chapter 4**) and HEK293 cell systems, both systems failed to produce the antigen. A slightly divergent option to circumvent challenges with obtaining the confirmational epitope, would require the incorporation of Pfs230D7 in a self-assembling ferritin nanoparticle[80]. The design of a nanoparticle can increase protein stability in solution, thereby improving expression. The design could also be adjusted to avoid (post-translational) modifications that negatively affect recombinant expression, for example by eliminating undesirable glycosylation[81].

The *D. melanogaster* S2 cell system was previously used for an extensive list of *P. falciparum* antigens, including 6-Cysteine protein family member Pfs48/45, though not for Pfs230 [73]. In **Chapter 4** we managed to express eight Pfs230 single domain constructs and use them for mice immunization. Sera against Pfs230D1 showed TRA in SMFA, corroborating previous studies, but interestingly, sera against Pfs230D12 also potently reduced transmission. Pfs230D12 (**Chapter 4)** has few non-synonymous SNPs, comparable to the leading candidate Pfs230D1, indicating it is a conserved antigen[82]. In line with this, we observed strong TRA of the anti-D12 mice sera against potentially genetically diverse parasites from naturally infected gametocyte carriers (Burkina Faso) in a direct membrane feeding assay (DMFA). Additionally, the Pfs230D12 antigen was recognized by sera from individuals naturally exposed to *P. falciparum* with intensity of anti-Pfs230D12 responses increasing with age, demonstrating that this antigen is immunogenic in humans and that a D12 vaccine could potentially benefit from natural boosting. These results show that the choice of expression system can have critical impact on the outcome of immunisation studies with new antigens. The S2 cell system however was not ideal for the expression of Pfs230 domains, since 6 domains could not be expressed and purified for mouse immunization. The cryo-EM structure of the Pfs230:Pfs48/45 complex[15,16] with domains known to be targets for functional antibodies positioned distal of the membrane, provides leads for other target domains within Pfs230 such as D8 and D9. Furthermore, the different modules of the Pfs230:Pfs48/45 complex, for example Pfs230D1-6, can provide new insights for design of immunogens. These recent advances in Cryo-EM and tools such as AlphaFold (Google DeepMind), that predict the 3D structure of proteins from their amino acid sequences, can inform earlier in the TBV discovery process about potential issues, for example by determining domain boundaries for expression.

We have thus identified two new targets for transmission blocking antibodies, domain 7 and domain 12, with the latter being a promising new TBV candidate based on its conserved nature, the level of TRA comparable to the established Pfs230D1 and the recognition by plasma from Ugandan individuals naturally exposed to *P. falciparum*.

## Pre-clinical development of TBVs

When a TBV candidate is identified, it is just that, a candidate. There are several important pre-clinical steps that follow the identification of a new TBV candidate to prepare for clinical trials. First, the vaccine should be optimised, which can involve (1) identification of an optimal expression system yielding high quality and quantity antigen, (2) optimisation of the antigen to focus the immune response to epitopes that induce potent antibodies, and (3) identification of the optimal vaccine delivering technology and (immune modulatory) adjuvant. These different steps involve *in vivo* studies in e.g. mice. Lead candidates should be selected for head-to-head comparison with the current leading TBV candidate.



As said, optimising the vaccine candidate is a vital step when continuing TBV development process and producing the antigen is without a doubt crucial. Unfortunately, heterologous expression of recombinant TBV antigens often poses a great hurdle in TBV development. This is illustrated by Pfs230D7, identified as a new TBV candidate in **Chapter 3**, that has yet to be expressed in such a manner that the recombinant protein can induce a functional response upon immunization, limiting the process of optimisation for this candidate. *Lactococcus lactis*, *Escherichia coli*, the wheat germ cell-free (WGCF) system, *Pichia pastoris*, *Saccharomyces cerevisiae*, *Drosophila melanogaster* are all commonly used for recombinant expression and the choice of expression host can greatly impact protein yield, protein folding, and post-translational modification such as glycosylation[57,58]. Eukaryotic systems have the advantage over prokaryotic systems, since they are better equipped with machinery to form disulphide bonds, common in *P. falciparum* surface proteins, and properly fold the protein. It is however essential that the protein is guided to extracellular expression by a signal peptide. The chosen expression system can greatly impact the protein folding, for example the lack of certain disulphide formation catalysing enzymes in the WGCF system could have a negative impact on the extent to which the product resembles the native protein. As for post-translation modifications, glycosyl unit placement that is divergent from natural *P. falciparum* glycosylation could obstruct TBV candidate discovery due to tampering with functional epitopes. In **Chapter 4** the S2 cell system produced Pfs230D12, which was confirmed to be glycosylated, though the effect on protein immunogenicity, solvability and yield

is currently unknown. An interesting approach would be to mutate glycosylation sites within the D12 fragment sequence to potentially benefit the induced immune response, since the glycosylation might be masking functional epitopes.

Another important aspect of pre-clinical development is antigen optimisation to focus the immune response to epitopes that induce potent antibodies and away from non-functional epitopes. One attractive way to achieve this would be determining the epitopes that are targeted by transmission reducing antibodies and should therefore be included in a vaccine construct, while on the other hand determining epitopes that elicit non-functional antibodies and should therefore be excluded from the vaccine construct. This direction of optimisation requires monoclonal antibodies that can be derived from animal immunisation studies or from naturally exposed or vaccinated human donors. Monoclonal antibodies from animal studies can be generated by isolating B cells after protein immunization and fusing these with myeloma cells to generate hybridoma clones that each produce a monoclonal antibody[83], which can be sequenced to conduct further in-depth analysis[84]. The mAbs from human donors can be generated through B-cell immortalisation with Epstein-Barr virus[85] and subsequent cloning, single B-cell sorting and stimulation, or antigen-specific B-cell sorting. Antigen-specific B cell sorting has been successfully used to obtain human mAbs against TBV candidates Pfs230 and Pfs48/45 epitope maps[5,6,17]. In these studies, human antibodies were crystallised with their antigen target to reveal potent and non-potent epitopes. Pfs230D12 (**Chapter 4**) could be a candidate for optimising via this route of immunofocusing via structure-based design[86], a development made possible in the last decade by scientific advances in B cell technology and structural biology, paving the way for improved vaccines.

Altering the constitution of a vaccine by coupling the antigen to another entity can enhance the immunogenicity. A conjugated carrier protein is thought to elicit an increased T cell response, possibly attributable to its T cell epitopes. The conjugate may also benefit the internalization and processing by antigen-presenting cells. In 2006, the protein-protein conjugate approach for TBV candidates was first tested by chemically conjugating Pfs25 to the outer-membrane protein complex (OMPC) of *Neisseria meningitidis* with improved TRA as a result[87]. OMPC has also been explored for Pfs230[88], before research interest shifted to linking TBV antigens with immunogenic carrier proteins that create cross-linked multimers forming nanoparticles[89]. In the past years, the TBV field has researched the potential of recombinant Exoprotein A (EPA) from *Pseudomonas aeruginosa*. EPA has been coupled to both Pfs25 [90] and Pfs230D1 [2,8,29,60] with the latter inducing functional TRA in Malian adults and currently

being the most progressed TBV in clinical trials. In addition to chemically conjugating Pfs230, the antigen has also been incorporated into virus-like particles (VLPs), though no immunogenic benefits were detected[91,92]. It is also possible to use the SpyTag/SpyCatcher technology to chemically conjugate an antigen to a VLP, which has been studied for Pfs48/45 based vaccine candidates, but not for Pfs230 [93]. More recently, Pfs230D1 has been fused to a *H. pylori* ferritin particle enabling self-assembly into a 24-copy immunogenic nanoparticle with the potential benefit of an improved production pipeline and cost reduction[94]. Other conjugate proteins that have been extensively studied include Tetanus Toxoid (TT)[95] and CRM197 [96], both involved in licensed polysaccharide conjugate vaccines. TT, CRM197 and EPA were compared for Pfs25 and Pfs230D1 and used for mice immunization, with TT and CRM197 showing equal or superior functional activity to EPA, depending on the adjuvant used[30]. We have discussed the multitude of options to conjugate and TBV candidate such as Pfs230D1. Considering the structural resemblance between the disulphide forming Pfs230D1 and D12 (**Chapter 4),** conjugating the latter to EPA or fusing the domain to a ferritin particle might be a promising route.



As was just briefly implied, the choice of adjuvant can have a substantial effect on the immune response elicited by a TBV. The Pfs230D1-Ferritin nanoparticle shows high TRA in different adjuvants, though a formulation in AddaS03 outperformed Alhydrogel when TRA was measured 4 months post-vaccination while Pfs230D1 exhibited poor immunogenicity when adjuvanted with AddaS03[94]. Carrier proteins TT, CRM197 and EPA were also compared for Pfs230D1, and the authors detected differences depending on the adjuvant used[30]. They also investigated the generation of different antibody subclasses, showing that the carrier protein had no impact, whereas the choice of adjuvant (GLA-LSQ or Alhydrogel) did. GLA-LSQ induced a more diverse IgG profile with substantial amounts of IgG2a and IgG2b produced, steering towards a mixed Th1/Th2 response (mentioned in more detail in Part 1 of this discussion). Another illustration of the impact of an adjuvant on antibody clonotype diversity, binding and function was given by the comparison between AS01 and Alhydrogel. Pfs230D1 in AS01 induced a broader range of germline sequences, though a larger percentage of mAbs was nonbinding[97]. Interestingly, the percentage of functional antibodies (arbitrarily defined as > 75% TRA following vaccination with 100 µg/mL) however was similar for both adjuvants. Considering the impact an adjuvant can have on the induced immune response and long-term TRA, a comparison of different adjuvants in an Pfs230D12 (**Chapter 4**) immunization study would be a logical next step in and should include all above mentioned adjuvants. Also, the complement dependent functionality of both Pfs230D1- and Pfs230D12-specific antibodies argues for the use of adjuvants that

generate strong human IgG1/3 or mouse IgG2 responses. Overall, the identification of the optimal adjuvant and conjugate protein or particle can be a critical step for the preclinical development of a promising TBV candidate such as Pfs230D12.

Finally, once an optimal antigen design and formulation for a new candidate have been identified, a head-to-head comparison with the currently leading TBV should be conducted in animal immunisation studies. Head-to-head studies are preferred as standalone studies may have slightly different conditions that can influence study outcome. A mouse immunization study can provide the framework for assessing the TRA of both formulations in SMFA and can give the opportunity of isolation mAbs from B cells. The prediction based on studies in mice can be misleading, as illustrated by Pfs25 which induced TRA in mice but not in non-human primates and humans[8]. It is therefore worth considering using non-human primates as an animal model for immunization studies. If the candidate in question would be superior to the current vaccine, progressing to vaccinating human volunteers with both formulations would be a next step, considering a human immune response can still diverge from the response in animals. Each step in the above-described pre-clinical optimisation process of TBVs has the potential to contribute to the development of new and improved candidates.

## Challenges, advantages and potential impact of TBVs

TBV development and implementation face several challenges, some of which are specific to this type of vaccination. The major challenges for TBV development are ethical concerns, complex clinical trial design and uncertainty about the threshold efficacy needed to justify deployment.

The ethical concerns are related to their mode of action; TBVs are not directly beneficial to the vaccinee, as they do not provide direct protection against infection and disease whilst the vaccinee is at risk of experiencing adverse effects from the vaccine. One could argue that the vaccine has an indirect benefit for the vaccinee by protecting family members and other close social contacts, and thereby (indirectly) the vaccinated individual him or herself. Interestingly, *A. gambiae* mosquitoes may have a dispersal range that does not exceed a few hundred meters to a kilometre if sources of blood meals can be identified over that range[98]. This focal nature of transmission indicates that TBVs could confer protection to small and settled communities, where household members, family and neighbours will be the primary beneficiaries. It should also be noted that there are other examples where vaccinations are primarily deployed for community benefit; there is an altruistic component in vaccinating male recipients with vaccines against Human Papillomavirus (HPV) where the largest clinical benefit is in females[99] and vaccines

against COVID-19 were deployed in populations that were not primarily at risk of the gravest consequences of infection[100]. Nevertheless, it would be ideal if a vaccine confers not only community but also personal benefit; ethical concerns for TBV may be circumvented by adding an anti-infection or anti-disease component to the vaccine that do direct benefit to the vaccinee.

Assessing TBV efficacy is a complex endeavour. In Phase 1 and 2 studies, efficacy may be measured at the individual level, using SMFA, DMFA and/or DSF. SMFA, using cultured parasites to assess efficacy, and DMFA, in which whole blood of vaccinees is fed to mosquitoes, are somewhat artificial and it is unclear how they translate to natural transmission through a mosquito bite. DSFs are therefore a favourable option to evaluate the efficacy of TBVs. DSFs have been used in Phase 1/2 clinical trials that assessed efficacy of both Pfs230 and Pfs25 TBVs[2,60]. Healy *et al*. recently published their Phase 1/2 TBV clinical trial results and revealed that only 1.51% of the DSFs performed was positive for the comparator group, so consequently the numbers of DSFs required for reasonable statistical power will be enormous (Healy *et al*. performed a total of 9923) and such efforts are extremely laborious[60]. Mosquito dissection and oocyst quantification on a large scale is time-consuming and labour-intensive, and unfortunately applies to all three methods: SMFAs, DMFAs and DSFs. It is most likely that a good replacement for these established transmission assay will remain elusive, though they measure a surrogate endpoint. Connecting DSF or DMFA data to the contribution of a mosquito to malaria transmission and finally reduction of human infection remains uncharted territory.



The success of TBVs relies on the development of herd immunity and determining whether this immunity has indeed developed is a complex and time-consuming process[58]. This has significant consequences for the outline of a potential Phase 3 TBV clinical trial. To properly assess herd immunity development and considering that TBVs confer immunity to communities, a cluster-randomised trial (CRT) design seems the best way forward. Such a CRT includes independent units of transmission, areas where transmission is local and not affected by a neighbouring cluster. These clusters would then be randomised to receiving either a TBV or a control. Whilst this is a relatively straight forward trial design, the number of clusters is likely to be very large, especially since TBVs are predicted to have the largest impact in areas of lower transmission, meaning that the number of events is lower and variation between clusters is likely to be very high. These cluster randomised trials for TBVs are thus major endeavours that include a large population and a considerable cost. Not only human movement but also mosquito travelling during the clinical trial requires consideration. Therefore, it is suggested that a CRT has a sentinel group within a

cluster, surrounded by a buffer region that would help reduce potential confusion caused by movement[101]. The clinical benefit by TBVs needs to be established by reduction in infection incidence or reduction of clinical incidence of malaria within the community[101,102]. To maximise the impact of TBVs on transmission, a large proportion of, or ideally the entire, infectious reservoir needs to be targeted. Not only children, who are the largest contributors to transmission[103,104], but also adolescents and adults, that are still susceptible to infection and can transmit to mosquitoes, will likely need to be included in CRTs to maximize the impact of the TBV on overall infection[105].

Finally, it is difficult to predict what efficacy and coverage is needed to achieve impact. This impact can be a large reduction in clinical burden or, ideally, malaria elimination. The coverage required to avert cases of malaria can be determined by mathematical modelling and includes estimates on the duration of responses and their efficacy as well as the target population and intervention setting[106]. Duration of the efficacy is also an important factor for vaccines in general, as well as their cost-effectiveness. Development of TBVs thus requires further optimisation and more clinical studies before TBVs can be rolled out on a large scale. These steps require considerable financial resources, and in the past TBV research did not receive much economic and commercial interest[107]. Malaria vaccines in general are not where the industry's main (commercial) interest lies, and TBVs are no exception. The potential market is in the endemic regions, including some of the poorest countries, that do not necessarily have the economic means to invest in development, purchase or distribution of vaccines. Taken together, setting up a Phase 3 TBV clinical trials poses a significant challenge and will undoubtedly demand noteworthy financial funds.

There are several important advantages of TBVs compared to vaccines targeting other stages. TBV candidates are highly conserved in comparison to other vaccine candidates[108-110], which is concluded after analysing the genomic Pf7 dataset comprising more than 20,000 *P. falciparum* samples from 33 countries[111]. In line with this, there are experimental leads that mAbs can block transmission of genetically diverse strains, can target epitopes that are conserved across field isolates, and that their binding is not strongly affected by natural SNPs[6,17,82].

Another advantage of several vaccines, including pre-erythrocytic, blood stage and transmission blocking vaccines, is the potential of an immunity boost by naturally acquired and potent antibodies against gametocytes, which would be beneficial in the case of fading immunity. The concept of boosting vaccine responses with natural parasite exposure was demonstrated and confirmed in mice, which is promising for administration of a TBV in field settings[112]. Boosting however does

not apply to Pfs25, since it is exclusively expressed in the mosquito stage of the malaria lifecycle. For Pfs230 and Pfs48/45 it has been repeatedly shown that these antigens naturally induce antibodies that are associated and causally associated with transmission reducing activity[69,113]. In **Chapter 4**, we show that Pfs230D12 is recognized by plasma from Ugandans with natural exposure to *P. falciparum*, which has positive implications in the case of D12 as a TBV, as vaccine responses could potentially be boosted by natural exposure. Whether the acquired antibodies are causally linked to naturally acquired TRA remains to be determined. Confirmation of such a causal relationship would be very relevant, since immune priming and boosting before/after vaccination could enhance TRA[112].

Antibodies targeting the transmission stages also positively stand out in terms of their potency in comparison to antibodies directed to the other malaria stages, suggesting that lower antibody titres are required for similar efficacy[114]. This suggests that if such potent antibodies can be induced in humans, the antibody concentrations required for efficacy will be relatively low compared to for example anti-CSP antibodies.



Finally, if simulations can demonstrate that a TBV, given the potency and durability of responses, can have a meaningful beneficial community impact is an important motivation for more TBV research, advanced clinical trials and finally vaccine implementation. Challenger *et al*. used data gathered from naturally infected mosquitoes in Burkina Faso to estimate efficacy in the field and used transmission dynamics to predict a TBV's impact on public health while taking existing interventions into account[106]. The authors conclude that a TBV targeted towards children, the group responsible for the largest portion of the infectious reservoir, would be most effective and that community-based interventions will provide the greatest benefit to this group. The combination of a TBV with a pre-erythrocytic vaccine substantially increased the estimated number of averted cases, also when targeting school-aged children.

Overall, we argue that the benefits of an efficient transmission blocking vaccine outweigh the challenges that TBVs impose. Since the lack of funding for expensive trials is likely to prevent reaching the milestone of an approved TBV, it is essential that we convince stakeholders and policymakers that there is a future for TBVs.

***Moving forward with multi-stage vaccines***

When the goal is progress, it's important to avoid getting trapped in tunnel vision, for example by solely focusing on transmission blocking vaccines. Especially since the combination of different tools against malaria might prove to be the ultimate

solution. The current most advanced vaccines against malaria are leaving gaps in efficiency with current vaccines against CSP and candidates against Rh5 and Pfs230 showing incomplete protection. These gaps could be filled by a multi-component vaccine targeting multiple stages in the lifecycle. A vaccine formulation with a pre-erythrocytic or blood stage antigen combined with a transmission blocking antigen could target both in-human parasite development and transmission via mosquito. A multigenerational population model shows that combining vaccines can increase efficacy and suggests that anti-sporozoite and anti-transmission interventions can act synergistically[115]. Not only could a combination vaccine be more effective, it could also help overcome the ethical issues linked to TBVs since the other vaccine component would prevent symptoms of infection.

In 1998, a study by Ockenhouse *et al*. tested the concept of a multi-stage vaccine for the first time in humans[116]. With colleagues, he inserted 7 *P. falciparum* genes from all stages of the life cycle into a *vaccinia* virus genome and immunized volunteers, though results were variably immunogenic, leaving plenty of room for improvement. In recent years there have been advances in clinical studies exploring the potential of multi-stage vaccines. ProC6C is a vaccine that includes sequences from three different *P. falciparum* antigens, CSP, Pfs48/45 (6C domain) and Pfs230 (Pro Domain). The CSP sequence contains six NANP repeats and 3 copies of NVDP, both originally part of the immune dominant central repeat region of CSP that is a target for functional antibodies[117]. The Phase 1 in human trial was conducted in Burkina Faso by 3 immunizations of healthy adults (n = 125) with 30 µg or 100 µg ProC6C and R0.6C formulated in Alhydrogel alone or in combination with Matrix-M[59]. The results after 70 days show that the group receiving 100 µg of ProC6C Alhydrogel and Matrix-M had significantly higher TRA compared to the other groups, with 13 out of 20 individuals reducing transmission with more than 80% in SMFA performed with purified IgG at physiological concentration (15 mg/mL). At day 180 post immunization, samples of only 7 out of 20 individuals showed high levels of TRA, and results suggest that only vaccine-induced anti-Pfs48/45-6C IgG is positively correlated with TRA, supported by the independency of complement in SMFA[118]. SMFAs were performed with purified IgG at a concentration of 15 mg/mL, based on the average physiological concentration of total IgGs for African American adults[59,118], therefore the observed TRA is potentially not applicable to younger or school-age children or other populations with a lower IgG concentration[119]. Knowledge on the efficacy of the CSP component of ProC6C is currently limited, with ProC6C PfCSP antibodies suggested to be functional after an *in vivo* assessment[120]. Though, in case ProC6C PfCSP antibodies provide protection, the ProC6C construct might provide opportunities for further

optimalisation. The selection of the Pro domain for ProC6C instead of another Pfs230 domain with higher immunogenicity[76,77], was made due to lack of cysteine residues in this domain that would possibly interfere with disulfide formation within the 6C domain[121]. Replacing the Pro domain with Pfs230D1 [2] or D12 (**Chapter 4**), if production is successful, could lead to a construct inducing both anti-Pfs230 and anti-Pfs48/45 transmission reducing antibodies.

Rh5 is a blood stage vaccine candidate that provides several possibilities for use in a multi-stage vaccine. Rh5 in formulation with Matrix-M has recently been studied in a Phase 2b clinical trial in children from Burkina Faso[122]. The promising results show that Rh5/Matrix-M induces high levels of growth-inhibiting antibodies against *P. falciparum*, and the vaccine has demonstrated a notable efficacy of 55% (95% CI 20–75%) against clinical malaria[122]. It could however benefit from further improvement, and one option worth exploration is combining Rh5 with a transmission-blocking antigen such as Pfs230D1. Both antigens display low diversity and little to no immune selection in functional epitopes[108]. While the Rh5 vaccine would aid in prevention of clinical symptoms, for example Pfs230D1 or Pfs230D12 (**Chapter 4**) would reduce transmission by acting in the mosquito part of the parasite's lifecycle. Another possibility lies in the combination of Rh5 with a pre-erythrocytic antigen. Currently studied in a Phase 1 clinical trial, is Rh5 with the established R21 vaccine (ClinicalTrials.gov: NCT05357560). Such a combination could prevent infection, and the surviving parasites would come across another hurdle once emerged from the liver cells. When the goal is to target high transmission areas, where children are responsible for a large fraction of ongoing transmission, the addition of a TBV to a multi-stage vaccine is an attractive option.



In general, multi-stage vaccines are subjected to their own limitations. Designing a multi-stage vaccine that consists of a single and complex construct, such as ProC6C, is particularly difficult due to structural interference between components. It is important to choose the optimal adjuvant for immunogenicity and to prevent aggregation or complex formation, which could hide functional epitopes. Co-administrating vaccines can help overcome these challenges, but it would not be practical from a logistical standpoint for production, distribution, administration, as well as the costs related to using multiple GMP products. Co-administration would also require the compatibility of vaccine products, as for example the addition of CSP can lead to lower response for to other components[123]. Finally, the analysis of a multi-stage vaccine in clinical studies is far more complex compared to a singular vaccine, since it is important to assess the effect of each separate component to establish the benefits of its inclusion.

Combatting a parasite with such an intricate lifecycle as *Plasmodium* requires a multi-facetted approach. The progress made in the field of multi-stage vaccines shows a promising path forward, with several options to explore with a potential important role for TBVs or TBV components. Depending on the type of multi-stage vaccine there are limitations that could stand in the way of highly efficacious multi-stage vaccines, hence this niche field of research requires substantial investment of both efforts and funds to establish further development and optimisation.

## Part 3: Human attractiveness to mosquitoes

### No bite, no transmission

Transmission blocking vaccines should be administered to individuals that form the infectious reservoir. Since an individual carrying gametocytes will provide this sexual stage of the parasite for transmission to the mosquito. When vaccinated with a TBV, this individual would have developed transmission reducing antibodies as well, and once both parasite and antibodies have arrived in the mosquito midgut, the antibodies can act. The attractiveness and thus exposure of humans to mosquitoes is an important part of this process, since without exposure, there is no occurrence of transmission. TBVs would work hand in hand with a strategy to decrease exposure of humans to mosquitoes, which as a stand-alone method (for example ITNs) has proven its efficiency[124]. The human attractiveness to mosquitoes is however not uniform across individuals and currently not well understood. It would be interesting to develop new approaches that prevent exposure and gaining knowledge on the factors driving exposure would be very valuable for this field of research.

The anthropophilic vector *An. stephensi* has peak periods in its host-seeking behaviour. Mainly during the hours that humans are asleep, the potentially with *Plasmodium* infected mosquito is searching for a host[125]. The mosquito is first lured in by $CO_2$ plumes, followed by a mixture of olfactory, visual, thermal and physical cues that influence the mosquito's flight trajectory[126,127]. Since these factors can influence whether a mosquito bites and potentially transmits parasites, it is important that these specific factors are identified as they might aid us in avoiding the mosquito's infectious bite.

There is substantial variability between humans and how strongly they attract mosquitoes[128]. Many different factors such as alcohol consumption, pregnancy, and blood type have been suggested to mediate attraction[129-132], though these

studies generally rely on small cohorts and sometimes report conflicting results. In **Chapter 5** we discuss the Mosquito Magnet Trial, a study that tested more than 400 festivalgoers for their mosquito attractiveness by quantifying the number of *An. stephensi* that participants attracted using a custom-built assay. In addition to the mosquito attraction assay, participants answered a questionnaire on behavioural and dietary choices and personal attributes. Relating the questionnaire to the measured mosquito attraction score revealed several behavioural choices and personal characteristics that affected mosquito attraction. In line with previous studies, mosquitoes preferred individuals who drank beer. Furthermore, participants that shared a bed with a fellow festivalgoer also received the attention of more mosquitoes, whilst participants that were considered less attractive by mosquitoes did not shower in the morning and used sunscreen. As outlined in detail in **Chapter 5**, the conditions of the Mosquito Magnet Trial were not tightly controlled. Except for excluding individuals below the age of 18, there were no restrictions for participating and the pop-up lab was situated in a large and very hot container. It is interesting to consider repeating a similar trial with more controlled circumstances, perhaps at the start of a large event instead of several consecutive days, instructing participants beforehand with a list of requirements. Also, a follow-up study could be conducted after selecting outliers from the Mosquito Magnet Trial results, just as we selected individuals for the microbiome analysis (**Chapter 5**). Nonetheless, the large number of participants in the Mosquito Magnet Trial and the significant differences observed, provide leads that future research can delve into and potentially benefit from.



The human skin microbiome may be an important factor for attractiveness, since it affects the presence of skin volatile compounds and human odour. The mosquito magnet trial in **Chapter 5** detected a higher abundance of *Streptococci* on the forearm of participants that were highly attractive, and the overall abundance of malodour associated bacteria was high. A limitation of the Mosquito Magnet Trial was the lack of a method for detecting volatile compounds. Carboxylic acids, that are a constituent of human odour by secretion via skin sebaceous glands and microbiota, have been associated with human attractiveness to mosquitoes[133,134]. There are also indications that the length of the carboxylic acid chain is important, as short chain fatty acids on the human skin seem connected to repelling mosquitoes, whereas longer chains attract[134]. Interestingly, the strength of attraction of a person is likely to fluctuate over time, indicated by repeated measurements of the presence of carboxylic acids on individuals and the difficulties experienced when determining who consistently attracted more mosquitoes[134]. Giraldo *et al*. gathered their insights on body odour and carboxylic acids by performing whole

body volatilomics, though the cohort consisted only of 6 humans[133]. Due to the small cohort, it is likely that substantial variability was excluded from their results, providing an argument for future studies including whole body volatilomics with larger human cohorts.

The unique opportunity to conduct a trial at the Lowlands festival created a chance to share the relevance of studying mosquito biology and increase awareness of the harmful diseases they can transmit. Simultaneously it provided an interesting first screening mechanism at an unprecedented scale to identify people that could later be invited for studies in a controlled environment. By proposing the Mosquito Magnet Trail at Lowlands festival, we agreed to the location and population choice that naturally accompanies the festival. Therefore, the participants of the trial do not represent the target population at risk for malaria in endemic regions, though in a future trial similar to the Mosquito Magnet Trial, an endemic setting could be chosen to obtain data more relevant in the context of malaria transmission. Nevertheless, the initial screening could be very valuable for future follow up studies and provide a possible framework for studies in field settings.

Our trial and researching human attractiveness to mosquitoes is relevant for several reasons. Understanding the motivation of a mosquito to choose a certain host is valuable information and could help assess the risk of infection, aid in design of interventions, and possibly lower the coverage required for TBVs to succeed. If the presence of a certain compound on the skin causes either less or more mosquito attraction, this could potentially be adapted as an extra addition to the anti-malaria toolkit[135-137]. It will require intensive future studies to reach the stage of implementing such a tool but organising large cohort studies are an important first step towards this goal and lays the groundwork for follow up studies to narrow down why mosquitoes clearly have their favourites. Whilst the relevance of the findings in **Chapter 5** for clinical use may be limited, one may imagine that vector control measures that are based on traps with attractive human odours[138] may one day be combined with TBVs to jointly target transmission.

## Concluding remarks

The fight against malaria is still raging on and the grip of malaria on public health remains strong. This thesis has hopefully positively complemented this fight by identifying Pfs230D7 as a new TBV target and Pfs230D12 as a new transmission blocking vaccine candidate, while highlighting the role and impact of the human complement system. Raising awareness for mosquito-borne disease and simultaneously aiming to help unravel what makes a human attractive to

a mosquito, forms a great complementing endeavour to this thesis. There is a long path ahead towards implementing an efficacious TBV, though there is great potential, especially when combining a TBV with other components into a multi-stage vaccine. I strongly hope that the knowledge gathered in this thesis regarding the complement system, transmission-blocking vaccines, and human attractiveness to mosquitoes will have a role in guiding future research and will complement this fight that has persisted long enough.

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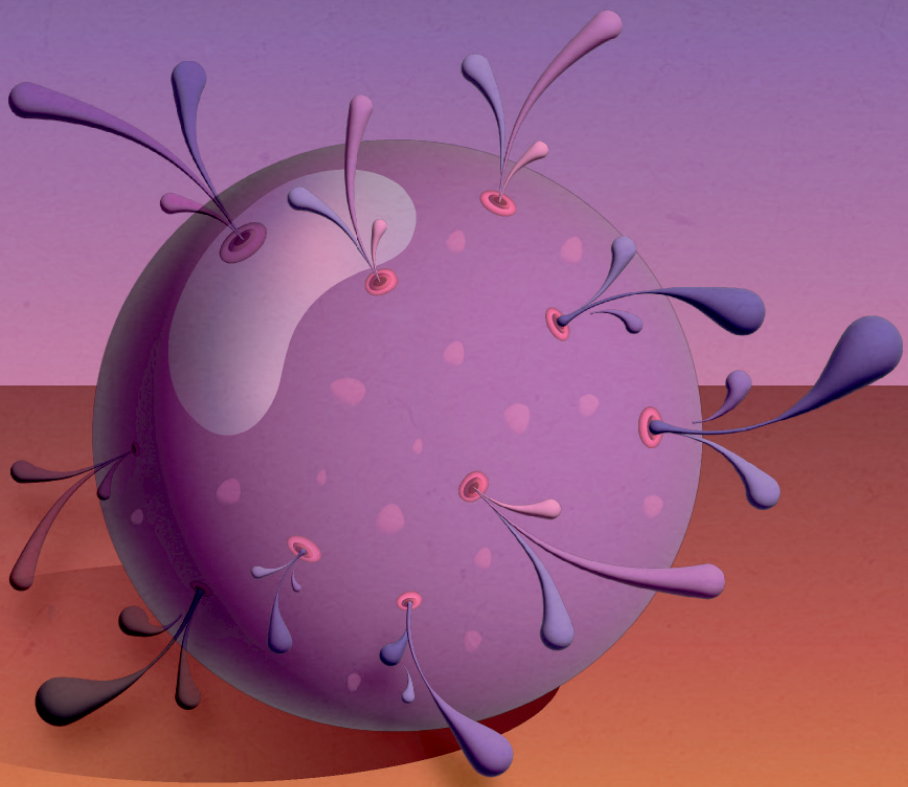

Chapter 7

# Appendices

Summary
Samenvatting
Research Data Management
List of publications
Acknowledgements
About the author
Portfolio

# Summary

Malaria has had a firm grip on public health for thousands of years. Nowadays, the disease caused by a parasitic infection still prevails in many regions, primarily on the African continent. The parasite species *Plasmodium falciparum* is dominant on the African continent and responsible for the gravest malaria burden. The *Anopheles* mosquito efficiently transmits this malaria parasite at a level that is a real concern for public health.

The infectious stage of the parasite, the sporozoite, resides in the salivary glands of the *Anopheles* mosquito waiting for the mosquito to find a human to feed on. This human then becomes the next host of the parasite. There are several cues informing the mosquito where to go, starting with $CO_2$, followed by a complex set of olfactory, visual, thermal, and physical factors. Some humans are mosquito magnets, whereas others are lucky enough to remain (nearly) untouched. The reasons why some people attract more mosquito bites than others remain largely unknown. In the Mosquito Magnet Trial (**Chapter 5**), we investigated differences in mosquito attraction amongst 465 festivalgoers with varying levels of hygiene and intoxication. The trial was in part a serious scientific undertaking and in part an entertaining outreach activity and was conducted in a pop-up laboratory at Lowlands Festival. After completing an anonymous questionnaire on hygiene, diet, and festival-related behaviour, the participants were measured for their mosquito attraction using a transparent cage with perforations where female *Anopheles* mosquitoes were offered a choice between a sugar-feeder and the participants' arm. Attraction was quantified through video imaging and correlated with questionnaire responses and skin microbiota profiles collected from forearm skin swabs. We found that mosquitoes are drawn to individuals that avoid sunscreen, drink beer and share their bed. These results could provide interesting leads for further research on human attractiveness to mosquitoes.

Upon entering the human bloodstream, the malaria parasite encounters the human complement system, part of the innate immune system. During its lifecycle, the *Plasmodium* parasite is exposed to both the human and mosquito complement systems. In recent years, we have gained new insight into the fascinating interactions of the malaria parasite with complement. It has become clear that evasion of these innate immune responses is important for parasite survival: parasites that circulate in the human blood stream (blood-stage parasites) and parasites that are activated after ingestion by mosquitoes (gametes) recruit regulators to neutralize human complement activation, while another parasite stage in the mosquito

inhibits mosquito complement activation by disrupting processes in the midgut. In **Chapter 2** of this thesis, we describe an in-depth overview of the evasion mechanisms currently known and we speculate on the existence of yet unidentified ones. We discuss how these mechanisms could provide novel targets for urgently needed malaria vaccines and therapeutics.

When the time has arrived for human-to-mosquito transmission, the mosquito engulfs a blood meal containing gametocyte-infected red blood cells from an infected human. In the mosquito midgut, these female and male gametocytes activate and develop into gametes. Malaria transmission-blocking vaccines (TBVs) are designed to induce human antibodies against antigens on the sexual-stage parasite or mosquito midgut surface. When taken up together with gametocytes, these antibodies can interrupt sexual development in the mosquito midgut and reduce parasite transmission. As a result, mosquitoes do not become infected and do not become a risk to humans. Because of this vaccine-induced transmission reducing activity (TRA), TBVs can play an important role in efforts to eliminate malaria. The clinically most advanced TBV candidate contains part of the Pro-domain and Domain 1 (Pro-D1) of gamete surface protein Pfs230; other functional targets within Pfs230 remained elusive for a long time. In **Chapter 3**, we show that the murine monoclonal antibody (mAb), 18F25.1, targets Pfs230 Domain 7. We generated a subclass-switched complement-fixing variant, mAb 18F25.2a, using a CRISPR/Cas9 engineering method. This subclass-switched mAb 18F25.2a potently reduced *P. falciparum* infection of *Anopheles stephensi* mosquitoes in a complement-dependent manner, as assessed by assays where we offered cultured malaria parasites to mosquitoes in the presence of antibodies. We also immunized mice with eight (out of fourteen) single domain fragments of Pfs230 that we produced in insect cells (**Chapter 4**). Antibodies raised against Domain 12 showed strong and complement dependent TRA in mosquito feeding assays with cultured parasites. We also demonstrated that Domain 12-specific antibodies were able to reduce transmission of genetically diverse parasites that were naturally acquired by malaria-exposed individuals in Burkina Faso. We also found that the D12 antigen was recognized by sera from an all-age cohort of naturally exposed individuals. Together, our data (**Chapter 3 and 4**) identify Pfs230 Domain 7 as target for transmission-blocking antibodies and Domain 12 as a new TBV candidate, providing strong incentive to study domains outside Pfs230Pro-D1 as TBV candidates.

In this thesis I discuss the important role of complement regarding antibodies targeting the transmission and pre-erythrocytic stages of *P. falciparum* and the role of complement in malaria vaccines. New functional targets within Pfs230

were uncovered and a critical view is provided on the discovery of TBV antigens, the process after identifying a new candidate, and how TBVs can contribute to malaria elimination. Finally, a large-scale trial studying human attractiveness to mosquitoes was conducted at the Lowlands festival, and I delve into the methods of studying human attractiveness to the mosquito and the implications of this field of research. The insights collected in this thesis regarding the complement system, transmission-blocking vaccines, and human attractiveness to mosquitoes hopefully will help guiding future research and thereby complements the fight against malaria.

# Samenvatting

Malaria heeft al millennia lang een aanzienlijke impact op de volksgezondheid. Ook vandaag de dag is de ziekte, veroorzaakt door een parasitaire infectie, nog steeds endemisch in diverse regio's, met name op het Afrikaanse continent. In die regio is *Plasmodium falciparum* de dominante parasietensoort en daarmee verantwoordelijk voor de zwaarste last door malaria. De *Anopheles* mug is essentieel voor de transmissie van de malariaparasiet en deze transmissie is dermate efficiënt dat het aanzienlijke druk uitoefent op de volksgezondheid.

Het infectieuze stadium van de parasiet, de sporozoïet, bevindt zich in de speekselklieren van de *Anopheles* mug en wacht daar tot de mug een mens heeft gevonden voor haar volgende bloedmaal. Deze persoon wordt dan de volgende gastheer van de parasiet. Er zijn diverse signalen die het gedrag van de mug sturen, te beginnen met $CO_2$, gevolgd door een complex geheel van olfactorische, visuele, thermische en fysieke prikkels. Sommige individuen zijn bijzonder aantrekkelijk voor muggen, terwijl anderen nauwelijks worden gestoken. De onderliggende oorzaken van dit verschil in aantrekkelijkheid zijn tot op heden grotendeels onbekend. In de Mosquito Magnet Trial (**Hoofdstuk 5**) is onderzocht hoe 465 festivalgangers van elkaar verschillen in aantrekkingskracht tot muggen en hoe dit samenhangt met variatie in hygiëne en intoxicatie. Deze studie was deels een wetenschappelijke onderneming en deels een publieksgerichte activiteit, en vond plaats in een pop-up laboratorium op het Lowlands Festival. Deelnemers vulden een anonieme vragenlijst in over hygiëne, dieet en festival gerelateerd gedrag, waarna hun aantrekkelijkheid voor muggen werd gemeten met behulp van een transparante kooi waarin vrouwelijke *Anopheles* muggen konden kiezen tussen suikervoeding en de arm van de vrijwilliger. De mate van aantrekkelijkheid werd gekwantificeerd via video-analyse en gecorreleerd met de antwoorden op de vragenlijst en huid microbioom data, verkregen via swabs van de onderarm. De resultaten toonden aan dat muggen zich in het bijzonder aangetrokken voelen tot personen die zonnebrandcrème vermijden, bier consumeren en hun bed delen. Deze bevindingen kunnen aanknopingspunten bieden voor vervolgonderzoek naar menselijke aantrekkelijkheid voor muggen.

Na het betreden van de menselijke bloedbaan wordt de malariaparasiet geconfronteerd met het complementsysteem, een belangrijk onderdeel van het humane aangeboren immuunsysteem. Gedurende zijn levenscyclus wordt de *Plasmodium* parasiet blootgesteld aan het complementsysteem van zowel de mug als de mens. Interessante nieuwe inzichten van de laatste jaren hebben aangetoond dat

het ontwijken van deze immuunresponsen cruciaal is voor het voortbestaan van de parasiet: parasieten die in de menselijke bloedbaan circuleren (bloedstadium parasieten) en parasieten die activeren na het inslikken door de mug (gameten) rekruteren regulatoren om activatie van complement te neutraliseren, terwijl een ander parasietstadium de activatie van muggen complement onderdrukt door processen in de muggenmaag te verstoren. In **Hoofdstuk 2** wordt een uitgebreid overzicht gegeven van de bekende ontwijkingsmechanismen, maar ook gespeculeerd over potentiële strategieën. Het hoofdstuk bediscussieert hoe deze mechanismen kunnen leiden naar nieuwe doelwitten voor de ontwikkeling van vaccins en andere therapeutische strategieën.

Wanneer transmissie van mens naar mug plaatsvindt, neemt de mug een bloedmaaltijd die gametocyt-geïnfecteerde rode bloedcellen bevat. In de maag van de mug ontwikkelen deze gametocyten zich tot gameten. Transmissie-blokkerende vaccins (TBV's) zijn ontworpen om antilichamen op te wekken tegen antigenen op het oppervlak van de muggenmaag of de seksuele stadia van de parasiet. Wanneer deze antilichamen gelijktijdig met gametocyten worden opgenomen door de mug, kunnen zij de ontwikkeling van de parasiet onderbreken en daarmee transmissie reduceren. Met als resultaat dat muggen niet geïnfecteerd raken en geen bedreiging vormen voor de mens. Door deze vaccin-geïnduceerde transmissie-reducerende activiteit (TRA) kunnen TBV's een rol spelen in bestrijding van malaria. De klinisch meest gevorderde TBV-kandidaat bevat delen van het Pro-domein en Domein 1 (Pro-D1) van het gameten antigen Pfs230; andere functionele doelwitten binnen Pfs230 bleven lange tijd onbekend. In **Hoofdstuk 3** wordt aangetoond dat het monoklonale antilichaam 18F25.1 bindt aan Pfs230 Domein 7. Met behulp van CRISPR/Cas9 engineering hebben we 18F25.2a geproduceerd, een ander subklasse antilichaam die complement kan fixeren. De *P. falciparum* infectie van *Anopheles stephensi* muggen werd sterk gereduceerd door 18F25.2a op een complement-afhankelijke wijze, zo werd gemeten in experimenten waarbij gekweekte parasieten en antilichamen aan muggen werden aangeboden. In **Hoofdstuk 4** werden bovendien muizen geïmmuniseerd met acht (van de veertien) fragmenten bestaande uit een enkel Pfs230 domein, die werden geproduceerd in insectcellen. Antilichamen tegen Domein 12 vertoonden sterke en complement-afhankelijke TRA in muggenexperimenten met gekweekte parasieten. We lieten ook zien dat Domein 12-specifieke antilichamen transmissie reduceerden van genetisch diverse parasieten afkomstig van individuen uit Burkina Faso die zijn blootgesteld aan malaria. We stelden ook vast dat het D12 antigen werd herkend door sera van een breed cohort van individuen in endemisch gebied. De bevindingen uit **Hoofdstuk 3 en 4** identificeren dus Pfs230 Domein 7 als doelwit voor transmissie-

blokkerende antilichamen en Domein 12 als een nieuwe vaccinkandidaat, wat het belang benadrukt om ook Pfs230 domeinen buiten het al uitgebreid onderzochte Pro-D1-domein te onderzoeken.

In deze thesis bespreek ik de rol van het complementsysteem in relatie tot antilichamen gericht op transmissie- en bloedstadia van *P. falciparum*, en bediscussieer ik bovendien de implicaties voor malariavaccines. Ik beschrijf het identificeren van nieuwe functionele doelwitten binnen Pfs230 en geef een kritische beschouwing van het proces van antigeen-identificatie, het selecteren van een vaccinkandidaat en de bijdrage van TBV's aan malaria-eliminatie. Bovendien bespreek ik een grootschalige studie naar menselijke aantrekkelijkheid voor muggen, uitgevoerd op het Lowlands Festival, waarbij methodologische aspecten en implicaties voor toekomstig onderzoek worden besproken. De inzichten die in deze thesis zijn verkregen wat betreft het complementsysteem, transmissie-blokkerende vaccins en menselijke aantrekkelijkheid voor muggen geven hopelijk richting aan toekomstige studies en complementeert daardoor de strijd tegen malaria.

# Research Data Management

## Ethics and privacy

Chapter 5 of this thesis is based on the results of research involving human participants, which were conducted in accordance with relevant national and international legislation and regulations, guidelines, codes of conduct and Radboudumc policy. A statement that the study was not subject to the Dutch Medical Research Involving Human Subjects Act (WMO), was obtained from the recognized Medical Ethics Review Committee 'METC Oost-Nederland'. Furthermore, the privacy of the participants in this study was warranted by using fully anonymous data.

## Data collection and storage

Research data for Chapter 3, 4 and 5 of this thesis was obtained through laboratory experiments involving anonymous or non-human materials and from experiments involving animals. The animal studies in Chapter 4 were approved by the Central Animal Facility from the Radboud University. Data and documentation (including research protocol and experimental set-up) from Chapter 3,4 and 5 were stored and analysed on the MMB department server and are only accessible by project members working at the Radboudumc. Specifically, the video dataset from Chapter 5 is stored on the Radboudumc archiving drive. Storage locations of plasmids and bacterial stocks that are generated for Chapter 3 are documented in communal excel files on the MMB department, which is backed up monthly and accessible for other department members. These secure storage options safeguard the availability, integrity and confidentiality of the data.

## Data sharing according to the FAIR principles

The reuse of research data is facilitated by the guidelines to enhance to Findability, Accessibility, Interoperability and Reuse of research data (FAIR principle). Chapter 3 is published open access. Chapters 4 and 5 have been submitted for publication and are currently under review. The data used for chapter 3 is published in the Radboud Data Repository (DOI: 10.34973/kxjz-qq56) and openly accessible for reuse. The experimental data from chapter 4 and 5 will be uploaded in Data Sharing Collections in the Radboud Data Repository upon publication and will be openly accessible for reuse (DOI: 10.34973/cb7t-nw52 and 10.34973/yzgh-kd41). Data Sharing Collections remain available for 15 years. The mass spectrometry dataset from Chapter 4 will be deposited to the ProteomeXchange Consortium via the PRIDE partner repository (dataset identifier PXD039716) upon publication.

# List of Publications

Blanken SL, **Inklaar MR**, Wan Z, Evers F, Smit MJ, Kalyuzhnyy V, Verhoef JMJ, Bekkering ET, Schinkel M, Mulder S, Andrade CM, van Gemert GJ, Gusinac A, Zeeuwen P, Ederveen THA, Bousema T, Hol FJH. Blood, sweat, and beers: investigating mosquito biting preferences amidst noise and intoxication in a cross-sectional cohort study at a large music festival. *British Medical Journal* (under review).

**Inklaar MR**, de Jong RM, Da DF, Hubregtse LL, Meijer M, Teelen K, Bekkering ET, Grievink S, van de Vegte-Bolmer M, van Gemert GJ, Stoter R, Nagaoka H, Tsuboi T, Takashima E, Spruijt CG, Vermeulen M, Dabire RK, Arinaitwe E, Cohuet A, Bousema T, Jore MM. Pfs230 domain 12 is a potent malaria transmission-blocking vaccine candidate. *Science Advances* (2025).

Bekkering ET, Yoo R, Hailemariam S, Heide F, Ivanochko D, Jackman M, Proellochs NI, Stoter R, van Gemert GJ, Maeda A, Yuguchi T, Wanders OT, van Daalen RC, **Inklaar MR**, Andrade CM, Jansen PWTC, Vermeulen M, Bousema T, Takashima E, Rubinstein JL, Kooij TWA, Jore MM, Julien JP. Cryo-EM structure of endogenous Pfs230:Pfs48/45 complex with six antibodies reveals mechanisms of malaria transmission-blocking activity. *Immunity* (2025).



Kucharska I*, Ivanochko D*, Hailemariam S*, **Inklaar MR**, Kim HR, Teelen K, Stoter R, van de Vegte-Bolmer M, van Gemert GJ, Semesi A, McLeod B, Ki A, Lee WK, Rubinstein JL, Jore MM, Julien JP. Structural elucidation of full-length Pfs48/45 in complex with potent monoclonal antibodies isolated from a naturally exposed individual. *Nature Structural & Molecular Biology* (2025).

**Inklaar MR***, de Jong RM*, Bekkering ET, Nagaoka H, Fennemann FL, Teelen K, van de Vegte-Bolmer M, van Gemert GJ, Stoter R, King CR, Proellochs NI, Bousema T, Takashima E, Tsuboi T, Jore MM. Pfs230 Domain 7 is targeted by a potent malaria transmission-blocking monoclonal antibody. *NPJ Vaccines* (2023).

**Inklaar MR**, Barillas-Mury C, Jore MM. Deceiving and escaping complement - the evasive journey of the malaria parasite. *Trends in Parasitology* (2022). (Literature review)

Arias-Orozco P, **Inklaar MR**, Lanooij J, Cebrián R, Kuipers OP. Functional Expression and Characterization of the Highly Promiscuous Lanthipeptide Synthetase SyncM, Enabling the Production of Lanthipeptides with a Broad Range of Ring Topologies. *ACS Synthetic Biology* (2021).

(* these authors contributed equally)

# Acknowledgements

My PhD trajectory started officially on the 1st of January 2020, and by the time I have defended my thesis, we have entered 2026. It is thus safe to say that it took some time, and demanded a lot of perseverance in the end. While writing this I am incredibly happy that the end of my PhD is nearly there. Looking back at these years, I have learned a lot and had an immense amount of fun as well. For both the accomplishments and all the other memorable experiences there is a long list of people to thank, so here it goes.

**Matthijs**, ik wil je ontzettend bedanken voor je constante steun in de afgelopen jaren. Ik wist dat ik altijd ik op je kon rekenen, wat misschien wel het meest waardevolle is dat een supervisor kan geven. Je nam de tijd om alles te bespreken wat nodig was, en vaker wel dan niet was de conclusie dat er nog een negatieve controle (of twee) kon worden toegevoegd… Eerlijk waar, je hebt me geholpen een betere onderzoeker te worden, dus dank je wel voor het onderwijzen en uitdagen. Het traject heeft wat langer geduurd dan gepland, wat bij mij natuurlijk voor de nodige frustratie zorgde, maar weet dat ik jouw begrip hiervoor ontzettend waardeer.

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Of course, most of my PhD was set in the laboratory, so in order to keep that up for several years, you need a good vibe. Without a doubt, that is what you, **Roos, Renate, Amanda, Carolina, Karina, Ezra (and yes Matthijs you too)** created. Time flies when you're chatting, making jokes and laughing all the while pipetting. All your support in the lab is dearly appreciated. And **Renate**, thank you for being

unapologetically yourself, you were an amazing companion in the lab and hopefully we continue to keep in touch and host each other for dinner parties.

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When talking about fun times in the lab and around the office, I should not forget the **Virology lab**. You are all great to have around and set a great example of how involved and dynamic a research group can be! I specifically want to thank **Jurgen** for all the times you listened to whatever I was either happy or disappointed about. And **Oscar**, thank you for inviting me for all your spontaneous plans and my apologies for disappointing you when my Dutch calendar would not allow it. Will never forget carrying that damn table all the way to your house…



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Now I want to say a few words about the "office" that was formed after we moved to a sort of haunted house section of the Radboudumc. This was during a time I started writing more and thus spending more time behind my desk. **Sara, Merel, Silvia** and **Marianna**, together you created a wonderful cosy space, with lots of good chats, coffee, tea and chocolate. You made this time much more bearable and want to thank you for that!

**Carolina** and **Felix,** if you would have told me a while ago that I would put you two together in the acknowledgements of my thesis, I would have given you a weird look. But now, it makes a lot of sense. You are both incredibly sweet and wonderful people, and great company for coffee, dinners, parties, concerts, you name it. You made the last years of my PhD and finishing up so much better, simply by just

understanding and having a beer at Aesculaaf. You will be great parents but maybe wait a bit until you take her to Aesculaaf for a beer.

**Sara**, I am so very happy that we connected over the Lowlands project. When you pitched that you wanted to set up a project together, I was immediately convinced. That it would be such a success and that we would end up on a tower backstage overlooking the Alpha while De Jeugd van Tegenwoordig went crazy, I never would have guessed. Thank you for making Lowlands the best experience of my PhD! Saar, you have been a great colleague and friend over the past years. The way you are always genuinely interested in whatever is going on, is very much appreciated. And **Merel**, I can actually say the same about you. At some point during our PhDs we connected over coffee at Blommers and then went on to have a great time at Down The Rabbit Hole and at Drum & Bass, I will never forget! You are so fun to be around, you are incredibly genuine, sweet, and sometimes an unexpected rebel. I hope we continue to be in touch.

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Nu moet ik het nog hebben over de mensen die mijn PhD vanaf de eerste dag een vrijblijvend success hebben gemaakt. **Julie**, niet vaak kom je iemand tegen die zo snel voelt als een goede vriendin. Maar na de woorden "ik ben hier ook net begonnen aan een PhD" was het al snel duidelijk, dit gaan we samen doen. We hebben ontelbaar vaak bij de Aesculaaf gestaan, op terrasjes gezeten en op een gegeven moment de overstap gemaakt naar de Nijmeegse techno feesten. Je doorzettingsvermogen is inspirerend en ik waardeer alle steun die je me hebt gegeven over de jaren enorm! En **Cas**, jij en Julie zijn misschien een package deal, maar ik kan me geen betere deal wensen. Het staat buiten kijf dat jij een van de meest lieve, begripvolle mensen bent die ik ken. Kwaliteiten die mij absoluut door dit hele traject hebben geholpen. En als we het hebben over mensen die mij hier doorheen hebben geholpen, dan sta jij, **Roos,** met stip bovenaan. We hebben intensief samen gewerkt, iets wat altijd moeiteloos ging. Ik kan altijd, maar dan ook echt altijd bij je terecht voor goede raad en daar ben ik je eindeloos dankbaar voor.

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# About the author

Maartje Robin Inklaar was born on the 27th of October 1994 in Westervoort, the Netherlands. She lived there till the age of 18 and attended high school in Duiven (Candea College). After finishing her high school education Maartje moved to Groningen to start the bachelor of Life Science and Technology, choosing the major of Molecular Biology, since studying such small aspects of life interested her most. She continued her education at the University of Groningen with the research master Molecular Biology and Biotechnology. The internships during this time shaped her career. Her first master internship was in the lab of Prof. Oscar Kuipers, under the supervision of Dr. Patricia Arias-Orozco, to express and characterise lanthipeptides originally produced by marine cyanobacteria, to search for new lantibiotics. This internship sparked a motivation for working in the field of microbiology and provided the realisation that working a lab can be fun and rewarding. Maartje's second research internship brought her to the lab of Prof. José Penades, under the supervision of Dr. Andreas Haag. This time abroad in Glasgow, Scotland, taught her a lot about the hypermobility of the *Staphylococcus aureus* genome, phages, pints, whiskey and the beauty of the Scottish landscape. The internship at the Penades lab confirmed it, searching for a PhD position was the next step. The vacancy posted by Dr. Matthijs Jore at the Medical Microbiology department of Radboudumc seemed a good match, and the following years Maartje's research focussed on the malaria parasite, transmission blocking vaccines and the complement system. After completing the PhD trajectory, Maartje switched from academia to working for a Clinical Research Organisation, first at PPD Thermofisher and currently at CR2O. She hopes to help bring medical solutions to a point where they can actually be used by the people who they were designed for.

# PhD portfolio of Maartje Inklaar

Department: **Medical Microbiology**
PhD period: **01/01/2020 – 30/04/2024**
PhD Supervisor(s): **Prof. J.T. Bousema**
PhD Co-supervisor(s): **Dr. Ir. M.M. Jore / Dr. F.J.H. Hol**

| Training activities | Hours |
|---|---|
| **Courses** | |
| • Radboudumc - Introduction day (2020) | 6.00 |
| • RIMLS PhD course (2020) | 21.00 |
| • RIMLS - Introduction course "In the lead of my PhD" (2021) | 15.00 |
| • IMM - The Art of Presenting Science (2021) | 33.00 |
| • RU - Kinderen enthousiasmeren voor Wetenschap (2021) | 28.00 |
| • Radboudumc - Scientific integrity (2021) | 20.00 |
| • RU - Poster Pitching (2021) | 28.00 |
| • RU - Writing Scientific Articles (2022) | 96.00 |
| • RU - Projectmanagement for PhD candidates (2022) | 52.00 |
| • RU - Een PhD en dan? Solliciteren, netwerken en je loopbaan onder de loep (2024) | 20.00 |
| **Seminars** | |
| • Dr. Rajagopal Murugan (2021) | 1.00 |
| • Dr. Blandine Franke-Fayard (2021) | 1.00 |
| • Managing interns: blessing or curse (2022) | 2.50 |
| • Felix Hol seminar (2022) | 1.50 |
| • Seminar Gates foundation (2022) | 3.00 |
| • Aura Timen (2022) | 1.00 |
| • Optimalvax meeting (2022) | 16.00 |
| • Mikha Gabriela (2022) | 1.00 |
| • Cees Dekker seminar (2022) | 1.50 |
| • Suzuki talk (2022) | 2.00 |
| • Entrepreneur track 2nd session (2023) | 2.00 |
| **Conferences** | |
| • PhD retreat (2020) | 8.00 |
| • PhD retreat (2021) | 7.00 |
| • PhD retreat (poster) (2021) | 7.00 |
| • New frontiers (poster) (2021) | 14.00 |
| • ICW (Poster) (2021) | 14.00 |
| • ICW (2021) | 36.00 |
| • Science day (2022) | 8.00 |
| • BioMalPar (Poster) (2022) | 24.00 |
| • Hycult complement symposium (Poster) (2022) | 16.00 |
| • PhD retreat (Poster) (2022) | 24.00 |
| • Conference EMCHD (Oral presentation) (2022) | 46.00 |
| • PhD Retreat RIMLS (Oral presentation) (2023) | 24.00 |
| • BioMalPar (Poster) (2023) | 32.00 |
| • Dutch Complement Symposium (Oral presentation) (2023) | 16.00 |
| • Dutch Malaria day (Oral presentation) (2023) | 8.00 |
| **Other** | |
| • Christmas committee (2021) | 14.00 |
| • Journal club (2021) | 36.00 |
| • Journal club (2022) | 36.00 |
| • Outreach activity Lowlands Science (2024) | 56.00 |

| **Teaching activities** | |
|---|---|
| **Lecturing** | |
| • Teach practical for minor Global Health students (2021) | 14.00 |
| • Teach practical for minor Global Health students (2022) | 8.00 |
| • Teach practical for minor Global Health students (2023) | 8.00 |
| **Supervision of internships / other** | |
| • Supervising master student internship (6 months) (2022) | 56.00 |
| • Supervising master student internship (6 months) (2023) | 45.00 |
| **Total** | **908.50** |


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