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REVIEW



Transmission-Blocking Vaccines: Harnessing Herd Immunity for Malaria Elimination

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ABSTRACT

Introduction: Transmission-blocking vaccines (TBV) prevent community spread of malaria by targeting mosquito sexual stage parasites, a life-cycle bottleneck, and will be used in elimination programs. TBV rely on herd immunity to reduce mosquito infections and thereby new infections in both vaccine recipients and non-recipients, but do not provide protection once an individual receives an infectious mosquito bite which complicates clinical development.

Areas covered: Here, we describe the concept and biology behind TBV, and we provide an update on clinical development of the leading vaccine candidate antigens. Search terms 'malaria vaccine,' 'sexual stages,' 'transmission blocking vaccine,' 'VIMT' and 'SSM-VIMT' were used for PubMed queries to identify relevant literature.

Expert opinion: Candidates targeting *P. falciparum* zygote surface antigen Pfs25, and its *P. vivax* orthologue Pvs25, induced functional activity in humans that reduced mosquito infection in surrogate assays, but require increased durability to be useful in the field. Candidates targeting gamete surface antigens Pfs230 and Pfs48/45, respectively, are in or nearing clinical trials. Nanoparticle platforms and adjuvants are being explored to enhance immunogenicity. Efficacy trials require special considerations, such as cluster-randomized designs to measure herd immunity that reduces human and mosquito infection rates, while addressing human and mosquito movements as confounding factors.

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1. The Concept of a Malaria Transmission-Blocking Vaccine

Malaria elimination and eradication have received renewed interest and prioritization, but existing tools are insufficient to eliminate from areas of stable transmission [1]. Vaccines played an important role to eliminate or eradicate other infectious diseases of humans or animals, such as smallpox, poliomyelitis, and rinderpest [2,3]. Vaccines are safe, cost-effective, can be administered to large populations in mass campaigns, and provide an extended window of immunological activity against pathogens. While traditionally developed to prevent infection and disease in individuals, the effect of vaccines to prevent onward transmission of the infectious agent is pivotal to elimination and eradication efforts in communities. In this way, vaccines benefit both immunized and non-immunized individuals through herd immunity, thus protecting groups who may not receive the intervention.

Transmission-blocking vaccines (TBV) work by attacking parasites in the mosquito vector to impede their spread through the community (Figure 1) and are needed for malaria elimination programs. TBV have been identified by the Malaria Eradication Research Agenda (MalERA) as a potential tool for malaria eradication, and a priority for development [4]. During elimination campaigns, TBV would be used in conjunction with other approaches, such as vector control, and by

comparison are intrinsically safe for the environment. When added to other malaria vaccines, TBV may reduce the release of escape mutant parasites, with the separate benefit that combined vaccine activities might more effectively interrupt the parasite life cycle and thwart community spread of malaria.

TBV do not provide individual protection if a vaccinee receives an infectious mosquito bite, but instead offer benefits to all community members by reducing the number of infectious mosquitoes and hence the rate of new human infections (Figure 1, center panel). The TBV concept was described in the 1970s using the avian parasite *P. gallinaceum* for proof-of-principle: chickens that were immunized with formalin- or radiation-treated parasites [5] or purified gametes [6] induced antibodies in chickens that immobilized microgametes and prevented parasite transmission to mosquitoes. To mediate their activity, TBV incorporate antigens expressed by gametocytes and developmental stages in the mosquito – gametes, zygotes, and ookinetes – to induce antibodies that attack the parasite as it undergoes sexual stage and sporogonic development in the midgut (Figure 1, right panel). Antibodies can neutralize the parasite by targeting key biological processes (such as male gamete binding red cells during exflagellation), by blocking parasite-mosquito interactions (such as parasite chitinase that digests peritrophic matrix to allow

Article highlights

- TBV prevent community spread of malaria and will be used for elimination campaigns.
- TBV target parasite development in the mosquito and do not directly prevent infection of vaccinees after an infected mosquito bite.
- TBV rely on herd immunity and benefit vaccine recipients and non-recipients alike after infected mosquitoes (and therefore new human infections) decrease in the community.
- Safety is of paramount importance when assessing TBV candidates.
- Clinical TBV development currently focuses on gamete and zygote surface antigens that are known targets of functional monoclonal antibodies.
- Pfs25 and Pvs25 candidates have induced functional activity in malaria-naïve, and for Pfs25, malaria-experienced populations, measured by surrogate mosquito feeding assays with serum or antibodies.
- Current TBV development seeks to increase the degree and durability of functional activity through different antigens, engineered immunogens, vaccine platforms, and adjuvants.
- TBV trials will indicate whether Pfs230 or Pfs48/45 candidates, or new formulations, achieve durable activity that extends across the malaria season in the field.
- Effective TBV will be used with other control tools in elimination campaigns, including vector control, antimalarial drugs, and potentially other malaria vaccines.
- Education of national stakeholders, policy-makers, and the public will key to the success of elimination campaigns that incorporate TBV, a novel and promising malaria vaccine approach.

traversal), or by initiating immune killing effects (such as complement-mediated lysis of gametes by Pfs230 antibodies).

While TBV targets parasites as they attempt to establish infection in mosquitoes, a broader concept termed Vaccine to Interrupt Malaria Transmission (VIMT) coined by the Malaria Eradication Research Agenda (MalERA) encompasses pre-erythrocytic and blood stage vaccines that have an effect on transmission [7] along with TBV. The idea underlying VIMT is that a pre-erythrocytic vaccine that reduced the number of human infections, or a blood stage vaccine that limited infection duration, will also reduce community spread of malaria. Further, mosquito proteins or enzymes [4] such as *Anopheles gambiae* aminopeptidase 1 (AnAPN1), carboxypeptidase and saglin that play a role in parasite development have also been assessed as targets for mosquito stage vaccines, and have also been referred to as transmission-blocking vaccines. MalERA promoted ‘sexual-sporogonic-mosquito stage vaccines to interrupt malaria transmission’ or SSM-VIMT as an acronym to subsume TBV [8], but this terminology has not been widely adopted: a PubMed search of ‘SSM VIMT’ (or ‘SSM-VIMT’) identifies only four publications since 2014 versus more than 60 with a search for ‘malaria TBV’ over the same period.

2. Biology of *Plasmodium* Sexual Stages: Bottleneck in the Malaria Life Cycle

Sexual reproduction is obligatory for *Plasmodium* and occurs in the mosquito midgut, ultimately leading to the sporozoite forms in salivary glands that are injected into the next human host (Figure 1, left panel) – hence the terms

‘sporogony’ or ‘sporogonic development.’ Sexual stages commence in the human host with gametocytogenesis, as a small fraction of merozoites invading red cells are committed to become male or female gametocytes (i.e. pre-gametes) that will subsequently fertilize in and infect mosquitoes. While the pathogenic asexual blood stages of *P. falciparum* can expand to levels of 500,000 parasites/ μ l or more, peripheral blood gametocyte levels are typically far lower. Unlike other human malaria parasite species, in which asexual and sexual forms expand concomitantly in peripheral blood, *P. falciparum* gametocytes remain sequestered during several developmental stages, finally appearing in peripheral blood after 10–12 days as stage V gametocytes, the mature forms that will egress from erythrocytes as gametes in the midgut of blood-fed mosquitoes.

Sexual and sporogonic development offers a significant bottleneck in the *Plasmodium* life cycle, making these stages an attractive target for interventions. After gametocytes are drawn into the mosquito midgut during a bloodmeal, several environmental factors (temperature, pH, xanthurenic acid) stimulate egress from red cells. Each female gametocyte (macrogametocyte) yields a single macrogamete, while male microgametes are activated by intracellular calcium to undergo three rounds of DNA replication followed by endomitosis [9]. Each zygote forms within minutes of mosquito ingestion through fertilization of a single macrogamete by a single microgamete, then progresses through the forms of motile ookinete and finally sessile oocyst on the mosquito midgut. Each infected mosquito midgut will usually harbor fewer than 10–100 oocysts [10], a small number of parasites to target for interventions, albeit many more parasites are ingested in the original bloodmeal.

TBV development has primarily focused on antigens expressed on the surface of gametes, zygotes, and ookinetes (Figure 1C). The first candidates (Pfs230, Pfs48/45, Pfs25, and Pfs28) – which remain the leading candidates more than 3 decades later – were identified using functional monoclonal antibodies generated by vaccinating animals with gametes [11,12] or ookinetes [13]. Initially identified by their orthologues in the avian malaria parasite *P. gallinaceum*, the *P. falciparum* antigens Pfs230 and Pfs48/45 are expressed by gametocytes, appear on the surface of gametes and zygotes, then are shed as zygotes transition to ookinetes [13]. Owing to their stages of expression, antibodies against Pfs230 and Pfs48/45 exert their effects before gamete fertilization, and Pfs230 and Pfs48/45 induce antibodies during human infection [14]. As a consequence, vaccine responses to Pfs230 or Pfs48/45 may be boosted during infections and this could prolong durability of vaccine activity. Clinical trials of Pfs230 or Pfs48/45 vaccines should model antibody decay to understand the impact of intercurrent malaria infections.

Conversely, the *P. falciparum* antigens Pfs25 and even later Pfs28 appear as surface antigens on zygotes as they develop into ookinetes [13]. Pfs25 and Pfs28 are exclusively expressed by parasites in the mosquito vector, and antibodies do not develop against these antigens during human malaria infections. Therefore, Pfs25 and Pfs28 vaccines cannot rely on boosting during natural infections to ensure durability of activity. Pfs25 and Pfs28 antibodies act against the parasite

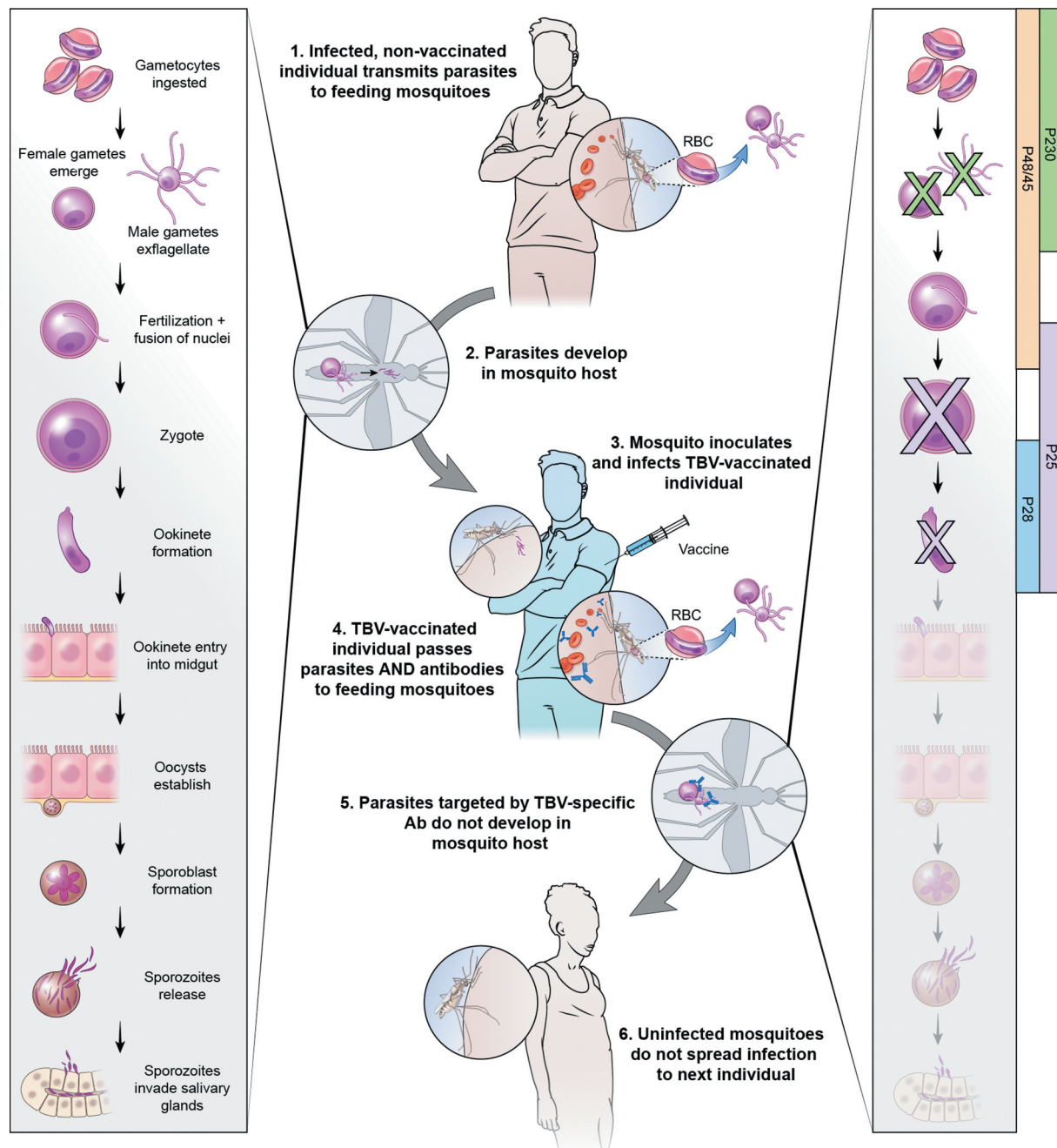


Figure 1. Malaria transmission-blocking vaccines (TBV) target surface antigens of mosquito sexual stage parasites to interrupt the *Plasmodium* life cycle in the mosquito vector and thereby avert new human infections. Mosquitoes that ingest a bloodmeal from an infected individual take up male and female gametocytes that initiate the mosquito sexual stages (left panel) and sporogonic development, ultimately yielding infectious sporozoites in mosquito salivary glands that can be transmitted to another victim. Conversely, when mosquitoes feed on an infected individual who has received TBV immunization, the vaccine-induced antibodies target parasites in a stage-specific fashion that corresponds to surface expression of the target antigen (right panel), averting development of sporozoites and subsequent human infections.

after fertilization. Orthologues of these four leading candidate antigens are expressed by the other human malaria parasite species such as *P. vivax*, and these will likely be prioritized for vaccine development upon evidence of activity with the corresponding *P. falciparum* candidates.

As a general statement, TBV antigens are genetically conserved when compared to other malaria vaccine antigens, presumably owing to limited exposure of the sexual stage parasites to the human immune response, and therefore

limited selective pressure that drives sequence variation and escape mutants. The introduction of TBV might alter this equation, and evidence for vaccine sieving effects on parasite variants should be carefully assessed during clinical trials. The parasite sexual stages represent a key period for the generation of genetic diversity [15] as well as for regulation of virulence during subsequent infection of the mammalian host [16,17]. Whether and how these aspects of sexual stage parasite biology impact vaccine activity and efficacy in

humans, and vice versa, represent intriguing aspects for investigation during TBV clinical trials.

3. TBV Candidate Clinical Development

The four leading TBV candidate antigens are cysteine-rich with multiple 6-cys (Pfs230 and Pfs48/45) or epidermal growth factor (EGF)-like domains (Pfs25 and Pfs28) that have been difficult to express as correctly folded recombinant proteins and in general have proven to be poorly immunogenic. The first to be generated as a recombinant protein was Pfs25 [18]. In preclinical rodent studies, the transmission-blocking activity of Pfs25 immunogens exceeded the activity induced by other TBV antigens or antigen combinations [19,20]. Conversely, the recombinant expression of properly folded 6-cys antigens has proven to be particularly challenging, and only recently achieved for Pfs48/45 [21].

3.1. Pfs25

In light of early success generating recombinant Pfs25 protein along with preclinical evidence for superior functional serum activity [19,20] measured in mosquito feeding assays (see 'Measuring vaccine activity and efficacy' below), Pfs25 (and its orthologue Pvs25) was prioritized for TBV clinical development until recently (Table 1). Pfs25 was included in an attenuated vaccinia virus vector called NYVAC-Pf7, along with several other *P. falciparum* antigens, but immunogenicity was poor for most antigens included in the construct [22]. Perhaps surprisingly, the Pfs25 antigen induced the highest level of antibody, but only marginal transmission-blocking activity was detected in immune sera via laboratory membrane feeding assay (that measures parasite transmission to mosquitoes). Early clinical grade recombinant Pfs25 protein products were prepared in *S. cerevisiae*, but clinical trials were terminated early due to reactogenicity, likely due to unbound antigen in the alum formulation in the first recombinant protein trial (Table 1) [23]. Subsequently, *Pichia pastoris*-expressed Pfs25 was formulated in the more potent adjuvant Montanide ISA 51, a water-in-oil emulsion, in order to further enhance antibody responses. Unfortunately, the trial of Pfs25 (as well as *S. cerevisiae*-expressed Pvs25) in ISA 51 was also halted prematurely owing to significant reactogenicity [24].

Most recently, Pfs25 has been developed as a protein-protein conjugate nanoparticle vaccine. Studies in animals demonstrated functional immunogenicity of Pfs25 was enhanced by chemical conjugation to carriers such as Outer Membrane Protein Complex (OMPC) of *Neisseria meningitidis* [26] or to EPA, a recombinant, detoxified ExoProtein A from *Pseudomonas aeruginosa* [27–29]. In an open-label, dose-escalating Phase 1 trial in the U.S [30], Pfs25-EPA conjugates formulated with Alhydrogel® were generally well tolerated and induced serum transmission-blocking activity. Transmission-blocking activity increased with successive doses, and correlated with antibody titer, and after the final dose, with antibody avidity. In a subsequent trial in malaria-experienced Malian adults [31], Pfs25H-EPA/Alhydrogel® was again well tolerated and induced significant serum transmission-blocking activity. However, statistically significant serum

activity required four doses, and antibody titers decreased rapidly after the fourth dose, suggesting the need for improvements to increase and prolong functional immunogenicity. A plant-derived Pfs25 virus-like particle (VLP) formulated in Alhydrogel also demonstrated modest immunogenicity in human, including weak transmission-blocking activity [32], await to be reported (Table 1).

3.2. Pfs230

TBV functional immunogenicity might be enhanced by targeting antigens other than Pfs25. Pfs230 antibodies lyse *P. falciparum* gametes in the presence of complement [33], which hypothetically could enhance and prolong antibody activity in humans. Pfs230 gene disruption reduces oocyst production by >90%, and disruption of its orthologue in the rodent parasite *P. berghei* impairs male gamete fertility [34,35].

Trials in the U.S. and Mali are examining a Pfs230 domain 1 (D1)-EPA conjugate vaccine candidate (ClinicalTrials.gov ID NCT02942277; NCT03917654). Preclinical studies indicate that Pfs230 immunogens that contain the N terminal region (aa 443–1132) [36], and specifically the first (Domain 1, aa 589–730 [37]) of its 14 6-cys domains [38], can induce functional antibodies. The Pfs230D1 candidate (aa 542–736) currently in clinical trials extends 48 residues upstream of Domain 1, to a site that is apparently cleaved during expression in *Pichia pastoris* [39], and the contribution (if any) of this N terminal fragment to functional epitopes will be of interest. The ongoing trials are evaluating Pfs230D1-EPA alone and in combination with Pfs25-EPA, while also examining different adjuvants, as potential strategies to maximize functional immunogenicity.

3.3. Pfs48/45

Pfs48/45 vaccine development has made recent progress. Of note, Pfs230 and Pfs48/45 are both expressed later in intraerythrocytic gametocyte development and appear on the surface of gametes emerging from red cells shortly after ingestion into the mosquito midgut. Pfs48/45 is a GPI-anchored surface antigen of female and male gametes [40] and a binding partner for Pfs230 [41] which forms a multimeric complex with the *P. falciparum* LCCL domain-containing proteins (PfCCp) that have multiple adhesion domains [42]. Pfs48/45 contains three 6-cys domains and is considerably smaller than Pfs230, but has nevertheless been difficult to prepare as a properly folded recombinant protein, including its C-terminal domain 3 which is known to be a target of potent rodent transmission-blocking mAbs [43].

In an important step forward, properly folded Pfs48/45 domain 3 has been expressed in *Lactococcus lactis* as a fusion with the R0 region of asexual stage Glutamate Rich Protein, resulting in a construct called R0.6 C [44]. Conformation-dependent functional monoclonal antibodies bind R0.6 C (indicating proper folding), and in preclinical animal studies, the candidate induces transmission-blocking antibodies. Subsequently, a fusion protein that incorporates the pro-domain of Pfs230 (i.e. the region upstream of domain 1 included in Pfs230D1) and the C-terminal domain of Pfs48/

Table 1. Past and current TBV clinical trials.

Antigen	Platform (Vaccine name)	Expression System	Adjuvant	Clinical trials. gov ID	Year	Phase	Location	Outcome	Ref
Pfs25	Recombinant subunit (TBV25H)	<i>Saccharomyces cerevisiae</i>	Aluminum hydroxide	Not found	1994	Phase 1	Malaria Vaccine Development Unit, Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, MD, USA	Atypical hypersensitivity reaction. Low antibody titers, 'incomplete transmission-blocking activity'.	[23,25]
Pfs25	Vaccinia virus vector (NYVAC-Pf7) Recombinant subunit (TBV25H)	<i>Saccharomyces cerevisiae</i>	Alum	Not found	1994	Phase 1/2a	Walter Reed Army Institute of Research (WRAIR), Washington, DC, USA	Safe and well-tolerated. No functional activity after NYVAC-Pf7; marginal transmission-reducing activity after TBV25H boost	[22,25]
Pvs25	Subunit protein (Pvs25H)	<i>Saccharomyces cerevisiae</i>	Alhydrogel®	Not found	~2003	Phase 1	Johns Hopkins School of Public Health, Baltimore, MD, USA National Institutes of Health Clinical Center, Bethesda, MD, USA	Safe and well-tolerated; significant transmission-reducing activity, correlated with antibody concentration	[59]
Pfs25 & Pvs25	Subunit protein (Pfs25 and Pvs25)	<i>Saccharomyces cerevisiae</i>	Montanide ISA51	NCT00295581	2005	Phase 1	Johns Hopkins School of Public Health, Baltimore, MD, USA National Institutes of Health Clinical Center, Bethesda, MD, USA	Local reactions including erythema nodosum in two Pvs25 subjects; leukemoid reactions in two subjects; study terminated after low dose. Significant transmission-reducing activity	[24]
Pfs25	Subunit conjugated to EPA, nanoparticle (Pfs25H-EPA)	<i>Pichia pastoris</i>	Alhydrogel®	NCT01434381	2011	Phase 1	Johns Hopkins University, Baltimore, MD, USA	Well-tolerated; >50% transmission-reducing activity in 9/11 subjects after 4 doses	[30]
Pfs25	Subunit conjugated to EPA, nanoparticle (Pfs25H-EPA)	<i>Pichia pastoris</i>	Alhydrogel®	NCT01867463	2013	Phase 1	University of Sciences, Technologies, and Techniques of Bamako (USTTB) Malaria Research and Training Center, Bamako, Mali	Generally well-tolerated; significant transmission-blocking activity after 4 doses	[31]
Pfs25	Plant-derived virus-like particle (Pfs25 VLP-FhCMB)	<i>Nicotiana benthamiana</i>	Alhydrogel®	NCT02013687	2013	Phase 1	Accelovance, Rockville, MD, USA	Acceptable safety and tolerability; weak transmission-reducing activity	[32]
Pfs25	Chimpanzee Adenovirus viral vector (ChAd63 Pfs25-IMX313) Modified Vaccinia Ankara (MVA Pfs25-IMX313)	n/a	n/a	NCT02532049	2015	Phase 1	Center for Clinical Vaccinology and Tropical Medicine (CCVTM), University of Oxford, Churchill Hospital, Oxford, UK NIHR Wellcome Trust Clinical Research Facility, Southampton, UK	Completed, Results not yet reported	
Pfs25	Virus-like particle (Pfs25-IMX313 /Matrix-M1)	<i>Pichia pastoris</i>	Matrix M™	NCT04130282	2019	Phase 1	CCVTM, University of Oxford, Churchill Hospital, Oxford, UK	Ongoing, recruiting	
Pfs25 & Pfs230	Subunit conjugated to EPA, nanoparticle (Pfs25-EPA and Pfs230D1-EPA)	<i>Pichia pastoris</i>	Alhydrogel®	NCT02334462	2015	Phase 1	National Institutes of Health Clinical Center, Bethesda, MD, USA USTTB Malaria Research and Training Center, Bamako, Mali	Completed, Results not yet reported	

(Continued)

Table 1. (Continued).

Antigen	Platform (Vaccine name)	Expression System	Adjuvant	Clinicaltrials.gov ID	Year	Phase	Location	Outcome	Ref
Pfs25 & Pfs230	Subunit conjugated to EPA, nanoparticle (Pfs25-EPA and Pfs230D1-EPA)	<i>Pichia pastoris</i>	AS01	NCT02942277	2016	Phase 1	USTTB Bancoumana Malaria Vaccine Center, Bamako, Mali USTTB Doneguebougou Malaria Research Center, Bamako, Mali Sotuba, Bamako, Mali	Completed, Results not yet reported	
Pfs230	Subunit conjugated to EPA, nanoparticle (Pfs230D1-EPA)	<i>Pichia pastoris</i>	AS01	NCT03917654	2020	Phase 2	USTTB Malaria Research and Training Center, Bamako, Mali	Ongoing, active	

45 was expressed in *L. lactis*, and in preclinical studies, induced significantly higher serum functional activity than did R0.6 C, suggesting a benefit of combining Pfs230 and Pfs48/45 activities [45].

3.4. Novel Antigens

Additional TBV candidates will enter the clinic in future, starting with Pfs48/45. The female gamete surface antigen Pfs47 mediates evasion of the mosquito immune system [46] by interacting with a specific midgut receptor [47] and is thought to thereby determine mosquito receptivity to parasite variants carrying specific Pfs47 alleles. In addition to its role in parasite–mosquito interactions, Pfs47 also induces transmission-blocking antibodies in animals; the specificity of these antibodies has been mapped to a central 52 amino acid (aa) region [48]. The pre-fertilization antigen HAP2/GCS1 (Hapless 2/Generative Cell-Specific 1), which is involved in male fertility, has also been shown to induce transmission-blocking antibodies in preclinical studies [49–52].

Naturally acquired antibody responses to gametocyte proteins have been examined to discover novel vaccine candidates, which hypothetically could act against gametocytes in the human host as well as parasites in the mosquito midgut. When correlated to serum transmission-blocking activity measured in mosquito feeding assays, reactivity of malaria-exposed sera to Pfs48/45, Pfs230, as well as 43 novel gametocyte proteins on a protein microarray, was associated with functional activity [53]. In an alternative approach, differential screening of an expression library with sera from Kenyans who did or did not develop gametocytemia during long-term follow-up, identified eight genes uniquely recognized by individuals who did not develop gametocytemia during infections; among these, PfsEGXP seroreactivity predicted lower gametocytemia across the malaria transmission season [54].

New candidates can be compared or combined with existing candidates to identify the most potent immunogens [for example, ClinicalTrials.gov IDs: NCT02334462; NCT02942277]. Combinations of TBV with other malaria vaccine candidates that target different life-cycle stages will also be assessed for their combined activity to interrupt malaria transmission.

4. Plasmodium vivax TBV Candidates

While traditionally viewed as a ‘benign’ malaria (in comparison to *P. falciparum*) albeit one that causes debilitating illness, increasing evidence has implicated *P. vivax* as an important cause of severe disease and death [55]. *P. vivax* is second to *P. falciparum* as a cause of malaria disease and death, but its toll remains large: approximately 14.3 million malaria episodes each year as the main malaria agent in Asia and Latin America [56]. Despite this, funds for malaria vaccine development have been disproportionately allocated to *P. falciparum*, and studies of *P. vivax* are limited by the inability to grow these parasites in culture. Nevertheless, vaccines may play a particularly important role in *P. vivax* control: *P. vivax* generates dormant liver forms called hypnozoites that are difficult to kill with drugs and cause periodic relapses over months or years even in the absence of new infectious mosquito bites. Relapsing

hypnozoites make *P. vivax* particularly difficult to eliminate and might best be addressed by vaccines that offer extended immunological control.

Progress in the development of *P. falciparum* vaccines will provide a clear path to develop the corresponding products for *P. vivax* using orthologous genes. However, the underlying assumption that vaccine targets efficacious against *P. falciparum* will show similar efficacy against *P. vivax* remains unproven. However, the biology of the two parasite species differs in important ways, and these could impact the efficacy of vaccine interventions. As noted above, *P. falciparum* gametocytes remain sequestered for 10–12 days before appearing in peripheral blood as mature gametocytes, while mature *P. vivax* gametocytes expand in parallel with asexual stages in peripheral blood and undergo rapid turnover [57].

To date, the *P. vivax* vaccine candidates that have entered clinical trials, PvCSP and Pvs25, are the orthologues of those that have advanced furthest as *P. falciparum* pre-erythrocytic (PfCSP) and transmission-blocking (Pfs25) candidates, respectively. As with Pfs25, the first recombinant protein Pvs25 candidate was generated in *S. cerevisiae* [58]. When formulated in Alhydrogel®, *S. cerevisiae*-expressed Pvs25 showed good tolerability, but only modest antibody titers, albeit serum transmission-blocking activity was measurable in mosquito feeding assays and correlated with titers [59]. As noted in the section above, a subsequent trial using Montanide ISA 51 adjuvant to formulate Pvs25 and *P. pastoris*-expressed Pfs25 was stopped early due to systemic reactogenicity in recipients of both vaccines; in particular, two volunteers developed erythema nodosum 18 days after receiving one dose of 20 µg Pvs25/ISA 51 [24].

One strategy to advance *P. vivax* TBV will be to enhance Pvs25 immunogenicity through more potent platforms, such as particle-based platforms that have benefited Pfs25 immunogenicity (see section below). However, results to date with Pfs25 suggest limited activity in humans, and that additional or alternative antigens may be needed for a highly effective and durable TBV. In parallel with the development of the *P. falciparum* candidate Pfs230D1-EPA noted above, manufacturing of Pvs230D1-EPA is ongoing to prepare for clinical trials that may launch in 2021.

Unlike *P. falciparum*, *P. vivax* has not been successfully cultivated in vitro, which complicates development of assays to measure functional activity of antisera raised against vivax candidates. As *P. vivax* TBV advance, investment in assays will also be needed in order to make Go/No-Go decisions to advance products in clinical development. Such assays might include engineered rodent malaria parasites that carry *P. vivax* TBV genes [60], more robust monkey models of *P. vivax* transmission, or controlled human *P. vivax* infections coupled with mosquito feeding assays [61], that can be leveraged to measure TBV activity in vivo before proceeding to field trials.

5. Platforms and Adjuvants to Improve TBV Activity

Pfs25 and Pfs230 recombinant antigens have induced modest immune responses as monomers in preclinical animal studies, and this has been borne out by the poor immunogenicity of recombinant Pfs25 in human trials. Poor immunogenicity is

explained in part by difficulty in generating properly folded recombinant protein: numerous disulfide bonds stabilize the structure of leading TBV antigens and make recombinant expression difficult [37,62,63]. As a consequence, vaccine developers have pursued individual domains or smaller fragments of TBV antigens to prepare immunogens [38,39,44,64–44,64–66], rendering them poorly immunogenic – possibly due to a dearth of strong T cell epitopes as well as difficulty recreating conformational B cell epitopes displayed on native antigen.

One strategy to improve the immunogens is to gather structural information on the antigen and on antigen-antibody complexes to better design domain boundaries or to engineer immunogens that focus the immune response on neutralizing epitopes, and this is discussed in detail elsewhere in this volume (see Patel PN and Tolia NH, Structural vaccinology of malaria transmission-blocking vaccines). In parallel, numerous vaccine delivery technologies and immune modulatory adjuvants are being explored to enhance immunogenicity of existing candidates [26,65,67–70]. All these efforts seek to increase immunogenicity while avoiding undue safety concerns. Safety signals invite special scrutiny for TBV candidates, because vaccine recipients do not derive a direct protective benefit that prevents infection. Instead, vaccine recipients and non-recipients alike benefit after herd immunity lowers transmission and hence infection for the entire community.

Protein-protein conjugation technology is being pursued as a safe, efficient, robust and cost-effective platform to prepare nanoparticles. In this approach, poorly immunogenic subunit protein antigens are chemically conjugated to immunogenic carrier proteins to generate vaccine candidates [27,71]. This approach draws on experiences with polysaccharide-protein conjugate vaccines, which have been spectacularly successful as an approach to vaccinate against deadly bacterial agents such as *Haemophilus influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae* [72]. During the early development of PfCSP malaria vaccines, synthetic peptide [73] or recombinant protein [74] was conjugated to carrier proteins tetanus toxoid (TT) or detoxified Toxin A of *Pseudomonas aeruginosa*, respectively, and tested in humans.

As the first proof-of-concept test of the protein-protein conjugate approach for TBV, Pfs25 was conjugated to the outer membrane protein complex of *Neisseria meningitidis* (OMPC) and studied for immunogenicity in rhesus monkeys [26]. Pfs25-OMPC induced antibody titers that were 1–3 logs greater than the Pfs25 monomer, whether formulated in Montanide or alum adjuvants, and demonstrated strong transmission-blocking activity in mosquito membrane feeding assays. Antibody responses were durable over several months and could be boosted with monomer antigen.

Subsequent work in this area has focused on chemically linking TBV candidate antigens to individual immunogenic carrier proteins [28,75]. The cross-linked multimers generated by chemical conjugation of proteins form nanoparticle structures that induce increased antibody responses [28,75–77]. For example, scanning electron microscopic examination reveals the Pfs25-EPA polymer to be a heterogeneous mix of nanoparticles with an average diameter of 20 nm [27]. The particle

properties of these antigens may intrinsically contribute to improved immunogenicity, and the carrier proteins may also elicit T cell help for antigens that otherwise lack potent T cell epitopes. In addition to conjugates generated by chemical cross-linking, researchers are developing TBV particles through other means like fusion proteins that assemble into virus-like particles (VLPs) or covalent and non-covalent linkages of target antigens displayed on the VLP surface [69,78–81].

As described above, recombinant Pfs25 conjugated to Exoprotein A (Pfs25-EPA) was safe and immunogenic in clinical trials [30,31]. Following the encouraging results with Pfs25-EPA, a second TBV conjugate vaccine candidate has been generated by coupling EPA to Pfs230 domain 1 (D1), a 22 kDa recombinant protein fragment that corresponds to amino acids Ser542-Gly736 and includes domain 1. Pfs230D1 has progressed to clinical testing in both malaria-naïve US subjects and in malaria-experienced individuals in Mali (ClinicalTrials.gov IDs: NCT02334462; NCT02942277).

CRM197 and Tetanus Toxoid (TT) have been extensively studied in polysaccharide conjugate vaccines and are used in approved bacterial vaccines [82]. In a recent study, TBV antigens were assessed as immunogens after conjugation to two commercially available CRM197 products: CRM197 expressed in the periplasm of *Pseudomonas fluorescens*, and EcoCRM® prepared as soluble, intracellular properly folded protein in *E. coli* [83]. This study also examined TT, as well as a 50 kDa recombinant N-terminal fragment of tetanus toxin heavy chain (rTThc) expressed in *E. coli*, as carriers for TBV. In mice, Pfs230 conjugates prepared with CRM197, EcoCRM® and TT all induced greater functional activity compared to conjugates prepared with EPA [84].

Immune-potentiating adjuvants are another important tool to enhance vaccine responses. However, these often raise issues for tolerability, as described above for trials of TBV antigens formulated in alum or Montanide adjuvants. Nevertheless, adjuvants available for clinical studies have increased since the 1980s, in part driven by the quest to enhance immunogenicity of HIV and malaria vaccines [85]. For example, the efficacy achieved by the RTS,S anti-infection malaria vaccine (manufactured by GSK, Inc.) required formulation in a potent adjuvant system (AS), either AS02: a combination of immunostimulants (the saponin fraction QS-21 and MPL [3-deacylated monophosphoryl lipid]) with an oil in water emulsion; or AS01: a combination of immunostimulants QS-21 and MPL with liposomes [86].

RTS,S/AS01 (under the trade name Mosquirix) has completed Phase 3 testing, received a favorable review from the European Medicines Agency, and is currently in implementation programs [87]. The success of RTS,S, and the possibility of combination with an effective TBV to interrupt parasite spread through both anti-infection and transmission-blocking vaccine activities, has prompted trials of Pfs25 and Pfs230 vaccines in AS01 formulations (NCT02942277; NCT03917654). In rodent studies, formulation of Pfs230 conjugates in GLA-LSQ (a combination of QS-21 and GLA (synthetic MPL) with liposomes similar to AS01) induced higher antibody titers and increased functional activity compared to alum adjuvant [84]. Pfs230 conjugates formulated in Alhydrogel® yielded a Th2 type

response while GLA-LSQ formulations induced a mixed Th1/Th2 profile, with the latter being advantageous to Pfs230 responses that depend on complement for gametocidal activity. For Pfs25, the combination of 1) conjugation to the carrier protein EPA and 2) formulation in GLA-LSQ was especially effective at inducing T follicular helper and long-lived plasma cell responses [76].

6. Measuring TBV Activity and Efficacy

The clinical benefits of TBV are mediated through an unorthodox activity: vaccine recipients are not protected from the bite of infectious mosquito, but instead will not transmit their own infection to another biting mosquito. Vaccine recipients and non-recipients alike benefit when herd immunity reduces community spread of malaria infections. Other malaria vaccines confer benefits directly to vaccine recipients by preventing or controlling parasitemia after infectious mosquito bites, hence their efficacy is measured at the individual level as a reduction in risk or severity of infection.

TBV functional activity can be measured at the individual level as a reduction in risk of infection for mosquitoes that feed on vaccinated versus control subjects. However, the clinical benefit of TBV must be measured at the group or community level, as a reduction in the entomologic inoculation rate or the community incidence of malaria infection. Such cluster-randomized controlled trials are expected to be large, complex and costly to execute [88]. Consequently, TBV developers have designed early clinical trials to establish proof-of-principle that vaccines induce functional antibodies in humans that reduce mosquito infections.

6.1. Surrogate Assays

During early phase trials, robust functional assays are needed to provide surrogate measures of TBV efficacy by quantifying subjects' ability to block parasite infectivity to mosquitoes. These assays rely on mosquito feeding on gametocyte-infected blood, and in the most common format, mosquitoes are dissected roughly a week after the infected bloodmeal to detect and count oocysts on mosquito midguts. The results of these assays provide the basis for early Go/No-Go decisions to advance vaccine further in clinical development.

Standard membrane feeding assays (SMFAs) have been the gold standard assay to measure TBV humoral responses *ex vivo*, and have been qualified per International Conference on Harmonization guidelines [50,89]. SMFA are conducted by adding test sera or purified immunoglobulin to gametocytes grown in culture, then feeding the combination to laboratory-reared mosquitoes through an artificial membrane [50,90,91]. By comparing mosquitoes that receive vaccine antisera versus control sera (or IgG), oocyst counts are used to calculate transmission-blocking activity (TBA, the % reduction in the prevalence of mosquito infection) and transmission-reducing activity (TRA, the % reduction in mean oocyst count per mosquito). SMFA specifically measures the functional activity of antibodies against sexual stage parasites, and therefore will not measure the functional activities of pre-erythrocytic or

blood stage vaccines that also may be useful for interrupting malaria transmission.

While TBA and TRA are related endpoints, that relationship depends on the intensity of parasite transmission in each assay which is measured in the control mosquitoes (i.e. fed gametocytes in the presence of nonimmune serum or antibody) and is highly variable between assays. As oocyst count increases in control mosquitoes, TBA decreases, so that at high control oocyst counts TBA may not be detected even in the presence of sera with high TRA. In general, control oocyst counts in SMFA studies exceed the counts observed in wild-caught mosquitoes, hence TBA estimated by SMFA may be too stringent as a predictor of vaccine activity. While TBA holds intrinsic appeal as an endpoint since a single oocyst can render a mosquito infectious to humans, TRA is more robust to variations in transmission intensity between assays and hence is recommended as the preferred endpoint to compare results between assays. Further, TRA can be used to estimate TBA at natural oocyst count levels based on a statistical model [92].

The direct membrane feeding assay (DMFA) employs the same design as the SMFA, with the exception that gametocyte-infected blood freshly collected from infected individuals is used in lieu of gametocytes grown in culture, to feed and infect mosquitoes. When used to test activity of antisera, infected blood samples must be pelleted and washed to remove plasma, which is replaced with test and control sera. Using this format, the DMFA measures the activity of vaccine antisera in the same way as SMFA, except the activity is assessed against heterogeneous parasites and polyclonal infections that better represent the diversity of field populations [93]. Care is required to ascertain whether oocysts that develop are those of *P. falciparum*, versus other malaria parasite species that naturally circulate in a community (and are not the target of *P. falciparum* TBV); a similar approach will be needed to measure the activity of *P. vivax* TBV against *P. vivax* parasites specifically.

Alternatively, DMFA can be performed with fresh whole blood samples used immediately after collection from vaccine trial participants on a systematic and regular basis, in order to quantify the frequency of parasite transmission events. When conducted in this format, DMFA collects similar data to direct skin feeds (DSF, also referred to as direct feeding assays or DFA). For DSF, laboratory-reared mosquitoes in containers are allowed to feed through netting directly on the skin of study participants [94]. DSF and DMFA performed in this format can generate population-level data that is compared between vaccine recipients and controls to confirm a statistically significant effect of the investigational product on the risk of parasite transmission events. The statistical analyses of these data can examine the proportion of individuals who transmit during a season, the time to the first transmission event during follow-up, the frequency of transmission events, the proportion or rate of infected mosquitoes, or the oocyst burden in infected mosquitoes. Again, speciation is required to confirm whether oocysts that develop are those of *P. falciparum* and therefore should be included in statistical analyses.

DMFA and DSF show high concordance when compared head-to-head in studies of the same subjects, based on correlation analyses of the proportion of mosquitoes infected [93].

However, DSF better represent the natural event and reproduce the complex interactions that occur during mosquito blood-feeding in the context of the local immune response and naturally-occurring gametocyte densities [95]. Despite their correlation, DSFs are more efficient than DMFA when measured as the proportion mosquitoes infected, and also less susceptible to human error [93,96,97]. In particular, a decrease in temperature induces gametocyte egress and formation of gametes, and thus errors in sample processing can prematurely activate parasites and reduce infectivity in DMFA [98,99]. The initial goal of field trials using these assays is to show that TBV candidates will significantly reduce the risk that naturally circulating parasites are transmitted to mosquitoes. Subsequently, the goal will be to demonstrate that this activity is durable and persists across an entire transmission season.

Early phase trials offer an opportunity to assess the impact of host and environmental factors on vaccine functional activity. For example, concurrent infections can impact immune responses to vaccines and are common in malaria-endemic areas. Helminths have been observed to impact antibody responses to other vaccines, but preclinical rodent studies did not find an impact of chronic intestinal helminths on responses to Pfs230 vaccine [100], and this should be studied in human trials. Because malaria infection can also impair responses to malaria vaccines [101], the benefit of pretreatment with antimalarials before TBV administration should be examined.

6.2. Efficacy Trials

As trials progress to larger numbers of participants, the goal is to show that these vaccines prevent malaria spread in human populations. To be practical for an elimination campaign, TBV activity should be sufficiently durable to prevent parasite spread across an entire transmission season, with its activity restored for a second season by a single additional dose the following year. TBV may be tested alone or in combination with another malaria vaccine, such as an anti-infection pre-erythrocytic vaccine, albeit both components must have proven activity to justify inclusion in a combination. Further, the vaccine combination may need to demonstrate greater transmission-interruption activity than the anti-infection vaccine alone to justify proceeding with clinical development. DSF (or DMFA using whole blood from study participants) provides a surrogate measure of the activity of either TBV, anti-infection vaccines, or the combination, to reduce mosquito infection events caused by naturally circulating parasites, and to justify advancing the combined product further in clinical development.

Larger trials will be required to show that TBV reduce the risk of new infections within a vaccinated group, reflecting herd immunity that prevents community spread of *P. falciparum*. The functional activity measured by mosquito feeding assays that will be required to eliminate *P. falciparum* infection from a particular community remains unknown, and therefore the criteria to advance TBV candidates to cluster-randomized trials remain a subject of discussion. Based on mathematical models, initial estimates held that TBV should reduce mosquito infections by $\geq 85\%$ to achieve

elimination goals [102], but gaps in understanding limit our ability to use surrogate assays of mosquito infections to predict the degree to which TBV will reduce human infections [103]. According to animal studies, interventions with relatively low levels of transmission-blocking activity can contribute to elimination when their effects are compounded over multiple cycles of transmission and they are most effective in low transmission settings [104].

An expert consultation considered the optimal design for a Phase 3 trial of TBV that could lead to licensure, assuming an indication for 'the reduction in incident infection by *Plasmodium falciparum* in a community' [88]. Cluster-randomized trials, which have been used for other malaria interventions such as bed nets and mass drug administration, were recommended. Cluster randomization is required to measure vaccine indirect effects, such as the herd protection by which a TBV conveys its benefits. Considering the potential for movement of both subjects and mosquitoes between clusters, the experts recommend a sentinel group within a cluster, surrounded by a buffer area that would mitigate the confounding effects of human or mosquito movements. However, a cluster remains undefined, and could be a neighborhood, village, or other population unit. Ultimately, a cluster should be defined as the area in which TBV herd immunity can be effective (and therefore measured), considering the potential for human and mosquito movements as well as other factors that will vary between potential sites.

Family compounds could suffice as clusters to measure herd protection in some settings, if infections circulate at that level. This could occur within individual households in some areas, based on the behavior of female *Anopheles* mosquitoes. Female mosquitoes blood-feed and then oviposit within a few days at the nearest water source, raising the prospect that they might preferentially return to the same family compound. Data support the possibility of household transmission: risk of infection for an individual is related to the infection status of other household members [105]; malaria infections cluster within households [106,107]; single-household clusters can persist for months [108]; and in general, *Anopheles* movement extends less than 2 km [109]. Mosquitoes may limit their movement when sites for oviposition are nearby and readily accessible, and this possibility should be evaluated as a criterion for selecting vaccination clusters.

Ultimately, TBV will be used together with other interventions that contribute to elimination, such as vaccines against other parasite stages, or drugs and vector control measures. Upon evidence for the efficacy of a TBV or its combination with malaria vaccines targeting other stages, and ideally upon licensure for the intended indication, future studies will assess how VIMT are used in conjunction with other tools to achieve the goal of elimination in different countries or regions.

7. Expert Opinion: the Author's Expert view on the Current Status of the Field Under Discussion

TBV have advanced to Phase 2 clinical trials over the past decade in response to a renewed policy emphasis on malaria elimination and eradication. While researchers continue to identify novel antigens that may be included in TBV, clinical

development to date has focused on the leading candidates identified in the 1980s using functional monoclonal antibodies. Trials of candidates based on the zygote surface antigen Pfs25/Pvs25 have firmly established that TBV can induce significant serum functional activity in both malaria-naïve and malaria-experienced populations, however TBV functional immunogenicity must be made more durable to have an impact in the field. Ongoing trials of candidates based on gamete surface antigens Pfs230 and, in the near future, Pfs48/45, will determine whether these antigens, or antigen combinations, will be superior to Pfs25 alone and provide the durable functional activity that will reduce parasite transmission to mosquitoes across a malaria season. In parallel, numerous groups are exploring platforms to enhance TBV immunogenicity, with an emphasis on developing nanoparticles or virus-like particles, spurred in part by the success of Pfs25 protein-protein conjugated nanoparticle vaccines that enhanced functional immunogenicity without increasing reactogenicity or raising safety concerns. Adjuvants have been key to enhance efficacy of malaria pre-erythrocytic vaccines, and encouraging data from preclinical studies support clinical trials that will assess new TBV formulations, particularly using liposome-based adjuvants, to enhance functional immunogenicity including durability.

8. Five-year view: a Speculative Viewpoint on how the Field will Evolve in 5 Years Time

Clinical studies in the next 5 years will establish whether candidates based on the antigens Pfs25, Pfs230 domain 1, and/or Pfs48/45 domain 3, may suffice to induce durable serum transmission-blocking activity and warrant expanded Phase 3 testing, or whether novel antigens will be required. New *P. vivax* TBV candidates will enter the clinic.

Novel candidate antigens, or engineered immunogens informed by structural studies of existing candidate antigens in complex with antibody, will continue evaluation in preclinical studies and could progress to the clinic within 5 years. Existing clinical candidates can serve as a benchmark to assess whether new antigens or engineered immunogens represent a sufficient improvement in activity to warrant clinical development.

Pfs25 candidates will likely advance as particle-based vaccines in some form, while future clinical data will determine whether Pfs230 and Pfs48/45 candidates similarly require generation of nanoparticles for optimal immunogenicity. Clinical development will also include assessment of different formulations that enhance immunogenicity. While the list of adjuvants available for clinical malaria vaccine development is relatively short and has proprietary constraints, preclinical studies indicate that liposomal adjuvants, such as the AS01 adjuvant used in the successful pre-erythrocytic malaria vaccine RTS,S, will likely be prioritized for evaluation in the coming years. New platforms or adjuvants will require particular scrutiny in the clinic to reassure regulators and the public that each TBV candidate maintains an acceptable safety profile to be considered for mass vaccination programs and a clinical benefit that relies on herd immunity.

The status of the field in 5 years depends largely on the results seen in clinical trials; upon clear evidence of durable transmission-blocking activity with the candidates currently entering the clinic, multi-site or multi-country efficacy trials may start in that time window. Direct skin feed (DSF) assays and direct membrane feeding assays (DMFA) are surrogate assays that will demonstrate whether TBV are reducing transmission of naturally circulating parasites in field trials. For the most promising candidates, studies using a cluster-randomized design will be used to measure the efficacy for reducing new human infections, although the designation of a cluster requires further definition, may vary from site to site, and its resulting size will drive the complexity and cost of such late-phase trials.

Ultimately, effective TBV are likely to be used together with malaria vaccines against other parasite stages, such as pre-erythrocytic vaccines that prevent human infection, if the combination is shown to have greater activity to interrupt transmission versus either component alone. Trials of TBV combined with other malaria vaccines advancing in clinical development, such as the RTS,S vaccine currently in implementation studies in African children, can start in the coming years. Initially, combination trials will start with co-administration of two vaccines, and evidence for increased activity of the combination will prompt development of a co-formulated product. Public education will be a critical component of success before implementation of this promising but unorthodox vaccine approach to malaria control and elimination; information-sharing with national stakeholders and policy-makers will be an important investment over the next 5 years.

Declaration of interest

No potential conflict of interest was reported by the author.

References

Papers of special note have been highlighted as either of interest (*) or of considerable interest (**) to readers.

- Galatas B, Sauter F, Marti-Soler H, et al. A multiphase program for malaria elimination in southern Mozambique (the Magude project): a before-after study. *PLoS Med.* 2020;17(8):e1003227.
- Fenner F, Henderson DA, Arita I, et al. Smallpox and its Eradication. Geneva: World Health Organization; 1988. p. 1500.
- Rinderpest ND. Driven to extinction. *Science.* 2008;319(5870):1606–1609.
- Alonso PL, Brown G, Arevalo-Herrera M, et al. A research agenda to underpin malaria eradication. *PLoS Med.* 2011;8(1):e1000406.
- Gwadz RW. Successful immunization against the sexual stages of *Plasmodium gallinaceum*. *Science.* 1976;193(4258):1150–1151.
- One of two seminal papers (see Ref 6) that described vaccination with whole malaria parasites to achieve immunity against the sexual stage transmissible forms
- Carter R, Chen DH. Malaria transmission blocked by immunisation with gametes of the malaria parasite. *Nature.* 1976;263(5572):57–60.
- One of two seminal papers (see Ref 5) that described vaccination with purified gametes induced antibodies that blocked parasite transmission to mosquitoes
- The mal ERACGoV. A research agenda for malaria eradication: vaccines. *PLOS Med.* 2011;8(1):e1000398.
- The mal ERARCPoTfME. malERA: an updated research agenda for diagnostics, drugs, vaccines, and vector control in malaria elimination and eradication. *PLOS Med.* 2017;14(11):e1002455.

9. Billker O, Dechamps S, Tewari R, et al. Calcium and a calcium-dependent protein kinase regulate gamete formation and mosquito transmission in a malaria parasite. *Cell*. 2004;117(4):503–514.
10. Zollner GE, Ponsa N, Garman GW, et al. Population dynamics of sporogony for *Plasmodium vivax* parasites from western Thailand developing within three species of colonized *Anopheles* mosquitoes. *Malaria J*. 2006;5:68.
11. Renner J, Carter R, Rosenberg Y, et al. Anti-gamete monoclonal antibodies synergistically block transmission of malaria by preventing fertilization in the mosquito. *Proc Natl Acad Sci USA*. 1980;77(11):6797–6799.
12. Kaushal DC, Carter R, Renner J, et al. Monoclonal antibodies against surface determinants on gametes of *Plasmodium gallinaceum* block transmission of malaria parasites to mosquitoes. *J Immunol*. 1983;131(5):2557–2562.
13. Carter R, Miller LH, Renner J, et al. Target antigens in malaria transmission blocking immunity. *Philos Trans R Soc Lond B Biol Sci*. 1984;307(1131):201–213.
14. Graves PM, Carter R, Burkot TR, et al. Antibodies to *Plasmodium falciparum* gamete surface antigens in Papua New Guinea sera. *Parasite Immunol*. 1988;10(2):209–218.
15. Childs LM, Prosper OF, Langsley G. Simulating within-vector generation of the malaria parasite diversity. *PLoS One*. 2017;12(5):e0177941.
16. Spence PJ, Jarra W, Levy P, et al. Vector transmission regulates immune control of *Plasmodium* virulence. *Nature*. 2013;498(7453):228–231.
17. Conteh S, Kolasny J, Robbins YL, et al. Dynamics and outcomes of plasmodium infections in *grammomys surdaster* (*Grammomys dolichurus*) thicket rats versus inbred mice. *Am J Trop Med Hyg*. 2020;103. Doi:10.4269/ajtmh.20-0183.
18. Barr PJ, Green KM, Gibson HL, et al. Recombinant Pfs25 protein of *Plasmodium falciparum* elicits malaria transmission-blocking immunity in experimental animals. *J Exp Med*. 1991;174(5):1203–1208.
- **A report of the first recombinant sexual stage antigen, yeast-expressed Pfs25 which elicited serum transmission blocking activity in rodents and nonhuman primates**
19. Menon V, Kapulu MC, Taylor I, et al. Assessment of antibodies induced by multivalent transmission-blocking malaria vaccines. *Front Immunol*. 2017;8:1998.
20. Kapulu MC, Da DF, Miura K, et al. Comparative assessment of transmission-blocking vaccine candidates against *Plasmodium falciparum*. *Sci Rep*. 2015;5:11193.
21. Theisen M, Jore MM, Sauerwein R. Towards clinical development of a Pfs48/45-based transmission blocking malaria vaccine. *Expert Rev Vaccines*. 2017;16(4):329–336.
22. Ockenhouse CF, Sun PF, Lanar DE, et al. Phase I/IIa safety, immunogenicity, and efficacy trial of NYVAC-Pf7, a pox-vectored, multiantigen, multistage vaccine candidate for *Plasmodium falciparum* malaria. *J Infect Dis*. 1998;177(6):1664–1673.
23. Stowers AW, Keister DB, Muratova O, et al. A region of *Plasmodium falciparum* antigen Pfs25 that is the target of highly potent transmission-blocking antibodies. *Infect Immun*. 2000;68(10):5530–5538.
24. Wu Y, Ellis RD, Shaffer D, et al. Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51. *PLoS One*. 2008;3(7):e2636.
25. Kaslow DC. Transmission-blocking vaccines. *Chem Immunol*. 2002;80:287–307.
26. Wu Y, Przysiecki C, Flanagan E, et al. Sustained high-titer antibody responses induced by conjugating a malarial vaccine candidate to outer-membrane protein complex. *Proc Natl Acad Sci USA*. 2006;103(48):18243–18248.
- **This study demonstrated that conjugation to a carrier enhances immunogenicity and durability of a recombinant Pfs25 protein vaccine in monkeys.**
27. Shimp RL, Rowe C, Reiter K, et al. Development of a Pfs25-EPA malaria transmission blocking vaccine as a chemically conjugated nanoparticle. *Vaccine*. 2013;31(28):2954–2962.
28. Qian F, Wu Y, Muratova O, et al. Conjugating recombinant proteins to *Pseudomonas aeruginosa* ExoProtein A: a strategy for enhancing immunogenicity of malaria vaccine candidates. *Vaccine*. 2007;25(20):3923–3933.
29. Qian F, Rausch KM, Muratova O, et al. Addition of CpG ODN to recombinant *Pseudomonas aeruginosa* ExoProtein A conjugates of AMA1 and Pfs25 greatly increases the number of responders. *Vaccine*. 2008;26(20):2521–2527.
30. Talaat KR, Ellis RD, Hurd J, et al. Safety and Immunogenicity of Pfs25-EPA/Alhydrogel(R), a transmission blocking vaccine against *Plasmodium falciparum*: an open label study in malaria naive adults. *PLoS One*. 2016;11(10):e0163144.
31. Sagara I, Healy SA, Assadou MH, et al. Safety and immunogenicity of Pfs25H-EPA/Alhydrogel, a transmission-blocking vaccine against *Plasmodium falciparum*: a randomised, double-blind, comparator-controlled, dose-escalation study in healthy Malian adults. *Lancet Infect Dis*. 2018;18(9):969–982.
- **The first field trial of a TBV demonstrated that Pfs25 conjugate vaccine induces functional antibodies that reduce parasite transmission to mosquitoes.**
32. Chichester JA, Green BJ, Jones RM, et al. Safety and immunogenicity of a plant-produced Pfs25 virus-like particle as a transmission blocking vaccine against malaria: a phase 1 dose-escalation study in healthy adults. *Vaccine*. 2018;36(39):5865–5871.
33. Read D, Lensen AH, Begarnie S, et al. Transmission-blocking antibodies against multiple, non-variant target epitopes of the *Plasmodium falciparum* gamete surface antigen Pfs230 are all complement-fixing. *Parasite Immunol*. 1994;16(10):511–519.
34. van Dijk MR, van Schaijk BC, Khan SM, et al. Three members of the 6-cys protein family of *Plasmodium* play a role in gamete fertility. *PLoS Pathog*. 2010;6(4):e1000853.
35. Eksi S, Czesny B, van Gemert GJ, et al. Malaria transmission-blocking antigen, Pfs230, mediates human red blood cell binding to exflagellating male parasites and oocyst production. *Mol Microbiol*. 2006;61(4):991–998.
36. Bustamante PJ, Woodruff DC, Oh J, et al. Differential ability of specific regions of *Plasmodium falciparum* sexual-stage antigen, Pfs230, to induce malaria transmission-blocking immunity. *Parasite Immunol*. 2000;22(8):373–380.
37. Gerloff DL, Creasey A, Maslau S, et al. Structural models for the protein family characterized by gamete surface protein Pfs230 of *Plasmodium falciparum*. *Proc Natl Acad Sci U S A*. 2005;102(38):13598–13603.
38. Tachibana M, Miura K, Takashima E, et al. Identification of domains within Pfs230 that elicit transmission blocking antibody responses. *Vaccine*. 2019;37(13):1799–1806.
39. MacDonald NJ, Nguyen V, Shimp R, et al. Structural and immunological characterization of recombinant 6-cysteine domains of the *Plasmodium falciparum* sexual stage protein Pfs230. *J Biol Chem*. 2016;291(38):19913–19922.
40. Kocken CH, Jansen J, Kaan AM, et al. Cloning and expression of the gene coding for the transmission blocking target antigen Pfs48/45 of *Plasmodium falciparum*. *Mol Biochem Parasitol*. 1993;61(1):59–68.
41. van Dijk MR, Janse CJ, Thompson J, et al. A central role for P48/45 in malaria parasite male gamete fertility. *Cell*. 2001;104(1):153–164.
42. Simon N, Kuehn A, Williamson KC, et al. Adhesion protein complexes of malaria gametocytes assemble following parasite transmission to the mosquito. *Parasitol Int*. 2016;65(1):27–30.
43. Roeffen W, Teelen K, van As J, et al. *Plasmodium falciparum*: production and characterization of rat monoclonal antibodies specific for the sexual-stage Pfs48/45 antigen. *Exp Parasitol*. 2001;97(1):45–49.
44. Singh SK, Roeffen W, Mistarz UH, et al. Construct design, production, and characterization of *Plasmodium falciparum* 48/45 R0.6C subunit protein produced in *Lactococcus lactis* as candidate vaccine. *Microb Cell Fact*. 2017;16(1):97.
45. Singh SK, Thrane S, Chourasia BK, et al. Pfs230 and Pfs48/45 fusion proteins elicit strong transmission-blocking antibody responses against *Plasmodium falciparum*. *Front Immunol*. 2019;10:1256.

46. Molina-Cruz A, Garver LS, Alabaster A, et al. The human malaria parasite Pfs47 gene mediates evasion of the mosquito immune system. *Science*. 2013;340(6135):984–987.
47. Molina-Cruz A, Canepa GE, Alves ESTL, et al. Plasmodium falciparum evades immunity of anopheline mosquitoes by interacting with a Pfs47 midgut receptor. *Proc Natl Acad Sci U S A*. 2020;117(5):2597–2605.
48. Canepa GE, Molina-Cruz A, Yenkindok-Douti L, et al. Antibody targeting of a specific region of Pfs47 blocks Plasmodium falciparum malaria transmission. *NPJ Vaccines*. 2018;3:26.
49. Mori T, Hirai M, Kuroiwa T, et al. The functional domain of GCS1-based gamete fusion resides in the amino terminus in plant and parasite species. *PLoS One*. 2010;5(12):e15957.
50. Miura K, Deng B, Tullo G, et al. Qualification of standard membrane-feeding assay with Plasmodium falciparum malaria and potential improvements for future assays. *PLoS One*. 2013;8(3):e57909.
51. Blagborough AM, Sinden RE. Plasmodium berghei HAP2 induces strong malaria transmission-blocking immunity in vivo and in vitro. *Vaccine*. 2009;27(38):5187–5194.
52. Angrisano F, Sala KA, Da DF, et al. Targeting the conserved fusion loop of HAP2 inhibits the transmission of Plasmodium berghei and falciparum. *Cell Rep*. 2017;21(10):2868–2878.
53. Stone WJR, Campo JJ, Ouedraogo AL, et al. Unravelling the immune signature of Plasmodium falciparum transmission-reducing immunity. *Nat Commun*. 2018;9(1):558.
54. Nixon CP, Nixon CE, Michelow IC, et al. Antibodies to PfsEGXP, an early gametocyte-enriched phosphoprotein, predict decreased plasmodium falciparum gametocyte density in humans. *J Infect Dis*. 2018;218(11):1792–1801.
55. Baird JK. Evidence and implications of mortality associated with acute Plasmodium vivax malaria. *Clin Microbiol Rev*. 2013;26(1):36–57.
56. Battle KE, Lucas TCD, Nguyen M, et al. Mapping the global endemicity and clinical burden of Plasmodium vivax, 2000–17: a spatial and temporal modelling study. *Lancet*. 2019;394(10195):332–343.
57. Sinden RE, Gilles HM. The malaria parasites. In: editors, Warrell DA, Gilles HM. *Essential Malariology*. 4th ed. London, UK: Hodder Arnold; 2002:8–34.
58. Miles AP, Zhang Y, Saul A, et al. Large-scale purification and characterization of malaria vaccine candidate antigen Pvs25H for use in clinical trials. *Protein Expr Purif*. 2002;25(1):87–96.
59. Malkin EM, Durbin AP, Diemert DJ, et al. Phase 1 vaccine trial of Pvs25H: a transmission blocking vaccine for Plasmodium vivax malaria. *Vaccine*. 2005;23(24):3131–3138.
- **The report of the first human trial of a transmission-blocking vaccine against P. vivax.**
60. Blagborough AM, Yoshida S, Sattabongkot J, et al. Intranasal and intramuscular immunization with Baculovirus dual expression system-based Pvs25 vaccine substantially blocks Plasmodium vivax transmission. *Vaccine*. 2010;28(37):6014–6020.
61. McCarthy JS, Griffin PM, Sekuloski S, et al. Experimentally induced blood-stage Plasmodium vivax infection in healthy volunteers. *J Infect Dis*. 2013;208(10):1688–1694.
62. Outchkourov NS, Roeffen W, Kaan A, et al. Correctly folded Pfs48/45 protein of Plasmodium falciparum elicits malaria transmission-blocking immunity in mice. *Proc Natl Acad Sci U S A*. 2008;105(11):4301–4305.
63. Carter R, Coulson A, Bhatti S, et al. Predicted disulfide-bonded structures for three uniquely related proteins of Plasmodium falciparum, Pfs230, Pfs48/45 and Pf12. *Mol Biochem Parasitol*. 1995;71(2):203–210.
64. Singh SK, Roeffen W, Andersen G, et al. A Plasmodium falciparum 48/45 single epitope R0.6C subunit protein elicits high levels of transmission blocking antibodies. *Vaccine*. 2015;33(16):1981–1986.
65. Roeffen W, Theisen M, van de Vegte-bolmer M, et al. Transmission-blocking activity of antibodies to Plasmodium falciparum GLURP.10C chimeric protein formulated in different adjuvants. *Malaria J*. 2015;14:443.
66. Lee SM, Wu Y, Hickey JM, et al. The Pfs230 N-terminal fragment, Pfs230D1+: expression and characterization of a potential malaria transmission-blocking vaccine candidate. *Malaria J*. 2019;18(1):356.
67. Scaria PV, Rowe CG, Chen BB, et al. Outer membrane protein complex as a carrier for malaria transmission blocking antigen Pfs230. *NPJ Vaccines*. 2019;4:24.
68. Kumar R, Ray PC, Datta D, et al. Nanovaccines for malaria using Plasmodium falciparum antigen Pfs25 attached gold nanoparticles. *Vaccine*. 2015;33(39):5064–5071.
69. Jones RM, Chichester JA, Mett V, et al. A plant-produced Pfs25 VLP malaria vaccine candidate induces persistent transmission blocking antibodies against Plasmodium falciparum in immunized mice. *PLoS One*. 2013;8(11):e79538.
70. Chan JA, Wetzel D, Reiling L, et al. Malaria vaccine candidates displayed on novel virus-like particles are immunogenic and induce transmission-blocking activity. *PLoS One*. 2019;14(9):e0221733.
71. Jones DS, Rowe CG, Chen B, et al. A method for producing protein nanoparticles with applications in vaccines. *PLoS One*. 2016;11(3):e0138761.
72. Berti F, Micoli F. Improving efficacy of glycoconjugate vaccines: from chemical conjugates to next generation constructs. *Curr Opin Immunol*. 2020;65:42–49.
73. Herrington DA, Clyde DF, Losonsky G, et al. Safety and immunogenicity in man of a synthetic peptide malaria vaccine against Plasmodium falciparum sporozoites. *Nature*. 1987;328(6127):257–259.
74. Fries LF, Gordon DM, Schneider I, et al. Safety, immunogenicity, and efficacy of a Plasmodium falciparum vaccine comprising a circumsporozoite protein repeat region peptide conjugated to Pseudomonas aeruginosa toxin A. *Infect Immun*. 1992;60(5):1834–1839.
75. Scaria PV, Chen B, Rowe CG, et al. Protein-protein conjugate nanoparticles for malaria antigen delivery and enhanced immunogenicity. *PLoS One*. 2017;12(12):e0190312.
76. Radtke AJ, Anderson CF, Riteau N, et al. Adjuvant and carrier protein-dependent T-cell priming promotes a robust antibody response against the Plasmodium falciparum Pfs25 vaccine candidate. *Sci Rep*. 2017;7:40312.
77. Qian F, Aebig JA, Reiter K, et al. Enhanced antibody responses to Plasmodium falciparum Pfs28 induced in mice by conjugation to ExoProtein A of Pseudomonas aeruginosa with an improved procedure. *Microbes Infect*. 2009;11(3):408–412.
78. Singh SK, Thrane S, Janitzek CM, et al. Improving the malaria transmission-blocking activity of a Plasmodium falciparum 48/45 based vaccine antigen by SpyTag/SpyCatcher mediated virus-like display. *Vaccine*. 2017;35(30):3726–3732.
79. Marini A, Zhou Y, Li Y, et al. A universal plug-and-display vaccine carrier based on HBsAg VLP to maximize effective antibody response. *Front Immunol*. 2019;10:2931.
80. Leneghan DB, Miura K, Taylor IJ, et al. Nanoassembly routes stimulate conflicting antibody quantity and quality for transmission-blocking malaria vaccines. *Sci Rep*. 2017;7(1):3811.
81. Brune KD, Buldun CM, Li Y, et al. Dual plug-and-display synthetic assembly using orthogonal reactive proteins for twin antigen immunization. *Bioconjug Chem*. 2017;28(5):1544–1551.
82. Pichichero ME. Protein carriers of conjugate vaccines: characteristics, development, and clinical trials. *Hum Vaccin Immunother*. 2013;9(12):2505–2523.
83. Hickey JM, Toprani VM, Kaur K, et al. Analytical comparability assessments of 5 recombinant CRM197 proteins from different manufacturers and expression systems. *J Pharm Sci*. 2018;107(7):1806–1819.
84. Scaria PV, Chen BB, Rowe CG, et al. Comparison of carrier proteins to conjugate malaria transmission blocking vaccine antigens, Pfs25 and Pfs230. *Vaccine*. 2020;38(34):5480–5489.
85. Garcon N, Di Pasquale A. From discovery to licensure, the adjuvant system story. *Hum Vaccin Immunother*. 2017;13(1):19–33.
86. Agnandji ST, Fernandes JF, Bache EB, et al. Clinical development of RTS,S/AS malaria vaccine: a systematic review of clinical phase I–III trials. *Future Microbiol*. 2015;10(10):1553–1578.

87. Duffy PE, Patrick Gorres J. Malaria vaccines since 2000: progress, priorities, products. *NPJ Vaccines*. 2020;5:48.
88. Delrieu I, Leboulleux D, Ivanson K, et al.; Malaria Transmission Blocking Vaccine Technical Consultation G. Design of a phase III cluster randomized trial to assess the efficacy and safety of a malaria transmission blocking vaccine. *Vaccine*. 2015;33(13):1518–1526.
89. Churcher TS, Blagborough AM, Delves M, et al. Measuring the blockade of malaria transmission—an analysis of the standard membrane feeding assay. *Int J Parasitol*. 2012;42(11):1037–1044.
90. van der Kolk M, De Vlas SJ, Saul A, et al. Evaluation of the standard membrane feeding assay (SMFA) for the determination of malaria transmission-reducing activity using empirical data. *Parasitology*. 2005;130(1):13–22.
91. Miura K, Takashima E, Deng B, et al. Functional comparison of *Plasmodium falciparum* transmission-blocking vaccine candidates by the standard membrane-feeding assay. *Infect Immun*. 2013;81(12):4377–4382.
92. Swihart BJ, Fay MP, Miura K. Statistical methods for standard membrane-feeding assays to measure transmission blocking or reducing activity in malaria. *J Am Stat Assoc*. 2018;113(522):534–545.
93. Bousema T, Dinglasan RR, Morlais I, et al. Mosquito feeding assays to determine the infectiousness of naturally infected *Plasmodium falciparum* gametocyte carriers. *PLoS One*. 2012;7(8):e42821.
94. Muirhead-Thomson RC. The malarial infectivity of an African village population to mosquitoes (*Anopheles gambiae*); a random xenodiagnostic survey. *Am J Trop Med Hyg*. 1957;6(6):971–979.
95. Bousema T, Churcher TS, Morlais I, et al. Can field-based mosquito feeding assays be used for evaluating transmission-blocking interventions? *Trends Parasitol*. 2013;29(2):53–59.
96. Graves PM, Burkot TR, Carter R, et al. Measurement of malarial infectivity of human populations to mosquitoes in the Madang area, Papua, New Guinea. *Parasitology*. 1988;96(2):251–263.
97. Diallo M, Toure AM, Traore SF, et al. Evaluation and optimization of membrane feeding compared to direct feeding as an assay for infectivity. *Malaria J*. 2008;7:248.
98. Graves PM. Studies on the use of a membrane feeding technique for infecting *Anopheles gambiae* with *Plasmodium falciparum*. *Trans R Soc Trop Med Hyg*. 1980;74(6):738–742.
99. Billker O, Lindo V, Panico M, et al. Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito. *Nature*. 1998;392(6673):289–292.
100. Coelho CH, Gazzinelli-Guimaraes PH, Howard J, et al. Chronic helminth infection does not impair immune response to malaria transmission blocking vaccine Pf3D7-EP4/Alhydrogel(R) in mice. *Vaccine*. 2019;37(8):1038–1045.
101. Sissoko MS, Healy SA, Katile A, et al. Safety and efficacy of PfSPZ vaccine against *Plasmodium falciparum* via direct venous inoculation in healthy malaria-exposed adults in Mali: a randomised, double-blind phase 1 trial. *Lancet Infect Dis*. 2017;17(5):498–509.
102. Penny MA, Maire N, Studer A, et al. What should vaccine developers ask? Simulation of the effectiveness of malaria vaccines. *Plos One*. 2008;3(9):e3193.
103. Nunes JK, Woods C, Carter T, et al. Development of a transmission-blocking malaria vaccine: progress, challenges, and the path forward. *Vaccine*. 2014;32(43):5531–5539.
104. Blagborough AM, Churcher TS, Upton LM, et al. Transmission-blocking interventions eliminate malaria from laboratory populations. *Nat Commun*. 2013;4:1812.
105. Stresman GH, Kamanga A, Moono P, et al. A method of active case detection to target reservoirs of asymptomatic malaria and gametocyte carriers in a rural area in Southern Province, Zambia. *Malaria J*. 2010;9:265.
106. Smith JL, Auala J, Tambo M, et al. Spatial clustering of patent and sub-patent malaria infections in northern Namibia: implications for surveillance and response strategies for elimination. *PLoS One*. 2017;12(8):e0180845.
107. Bejon P, Williams TN, Nyundo C, et al. A micro-epidemiological analysis of febrile malaria in Coastal Kenya showing hotspots within hotspots. *Elife*. 2014;3:e02130.
108. Gaudart J, Poudiougou B, Dicko A, et al. Space-time clustering of childhood malaria at the household level: a dynamic cohort in a Mali village. *BMC Public Health*. 2006;6:286.
109. Thomas CJ, Cross DE, Bogh C. Landscape movements of *Anopheles gambiae* malaria vector mosquitoes in rural Gambia. *PLoS One*. 2013;8(7):e68679.