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Original Article

Malaria vaccines: Current developments and immunological insights

Akanksha Verma [a], Ritesh Ranjha [b,c], Kuldeep Singh [b,c], Vinod Yadav [d], Ashima Bhaskar [a], Ved Prakash Dwivedi [a,*], Mradul Mohan [b,c,**]

[a] Immunobiology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India
[b] ICMR-National Institute of Malaria Research, New Delhi, India
[c] Academy of Scientific and Industrial Research, Ghaziabad, India
[d] Department of Microbiology, Central University of Haryana, Mahendragarh, Haryana, India

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ABSTRACT

Malaria is a parasitic disease of public health concern affecting nearly 263 million people globally. Majorly, poor and developing countries are prone to malaria. Children under 5 years are most susceptible to malaria morbidity and mortality. The emergence of drug-resistant parasites is posing a threat to the malaria control and elimination goals. There is a need of the hour to develop new *anti*-malarials along with novel malaria vaccines. The genetic complexity of the parasite and multiple life stages make it challenging to develop malaria vaccines. So far, the WHO has approved only two malaria vaccines. This review discusses the prospects of these two malaria vaccines and the future vaccine candidates targeting different life stages of *Plasmodium*. It also highlights the recent development in identifying the host's immune responses against malaria, novel vaccine candidates, and the ideal vaccine requirement.

1. Introduction

Malaria is a vector-borne disease of global health concern affecting nearly 263 million cases in 2023 [1]. The Sub-Saharan Africa region is predominantly affected by malaria, and it contributes to about 95 % of malaria-related deaths worldwide, among which children below the age of 5 years are more susceptible to severe malaria [2]. Malaria is a parasitic disease caused by the protozoan *Plasmodium*. The genus *Plasmodium* comprises more than 200 species [3], out of which five are known to cause human infection. *Plasmodium falciparum* (*P. falciparum*) is the deadliest species, which causes the most severe form of malaria and is the dominant plasmodium infection in the sub-Saharan region [1]. *Plasmodium vivax* (*P. vivax*) is the dominant parasite in countries outside Africa. *P. vivax is* the most widespread species of *Plasmodium*. It infects reticulocytes and causes malaria, which may progress to a severe form in some cases [4,5].

Artemisinin (ART) and artemisinin-based combination therapy (ACT) are the first-line treatment for malaria [1]. However, there are reports of ART resistance in various regions worldwide [6,7]. With the development of resistance to the approved treatment therapy, it becomes imperative to generate prophylactic and therapeutic vaccines to control the impact of the disease. WHO has introduced an enterprising goal to control malaria incidence cases by at least 90 % by 2030 in all malaria-endemic countries [1]. To meet the introduced needs, various vaccine candidates have been introduced in phase I clinical trials [8].

Despite the efforts from the global scientific community, an ideal malaria vaccine that covers all parasite stages with high efficacy among all age groups (children, adults, and the elderly) is still awaited. WHO has approved using RTS, S/AS01, and R21/Matrix-M vaccines in children below 5 years of age in malaria-endemic countries [9]. RTS, S/AS01 vaccine has shown a moderate 36.3 % efficacy against clinical malaria after 48 months of follow-up in the 5–17 months age group, administered with four doses [10,11]. The vaccine efficacy against severe malaria was 32.2 % over four years of follow-up in the children 5–17 months age group immunised with four doses of RTS, S/AS01 [11]. However, it has several disadvantages, including targeting only one Plasmodium species and age-specific protection [9]. With these drawbacks, researchers are improvising the RTS, S/AS01 vaccine by

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* Corresponding author.
** Corresponding author. Parasite- Host Biology Group ICMR-National Institute of Malaria Research (NIMR) Ministry of Health & Family Welfare, Sector 8, Dwarka, 110077, Delhi, India.
E-mail addresses: vedprakashbt@gmail.com (V.P. Dwivedi), mradul_mohan@yahoo.com (M. Mohan).

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developing different platforms. Such platforms include nanoparticles, and mRNA, carrying similar antigenic determinants as that of RTS, S. WHO has approved another malaria vaccine R21/Matrix M, a nanoparticle-based vaccine [12] R21/Matrix-M has been shown to confer higher protection with an efficacy of 75 %–79 % against clinical malaria in children aged 5–17 months [13] during one year following three doses and one booster [14]. Nevertheless, this is also a pre-erythrocytic vaccine, which has high efficacy (75 %) against clinical malaria as per the WHO goal for malaria vaccine [9]. The manufacturers of RTS, S/AS01 can produce 25 million doses per year, while R21/Matrix-M can produce 250 million doses per year. The cost of one dose of RTS, S/AS01 is approximately 9.3 Euro, while it is 3.9 USD for R21/Matrix-M. This gives R21/Matrix-M an operational edge over RTS, S/AS01 [15].

Other potential vaccine candidates have reached the early phases of clinical trials. These include pre-erythrocytic vaccines such as ME-TRAP, PfSPZ, blood stage vaccines like AMA-1, MSP3, pfEBA175, and transmission-blocking vaccines including Pfs25, Pfs230 [16]. The efficacy and immunogenicity of these candidates are yet to be determined. In this review, we are trying to shed light on the recent advances in developing novel vaccine candidates against malaria.

2. Life cycle of *Plasmodium falciparum*

Pf is a unicellular protozoan with a complex life cycle in two natural hosts-the female *Anopheles* mosquito and the vertebrate hosts [17–19]. Briefly, during the blood feed, the mosquito injects the sporozoites into the dermis of the vertebrate host [20]. The Thrombospondin-related anonymous protein (TRAP) enables the exit of sporozoites from the dermis [21]. The sporozoites glide to reach and penetrate the blood vessels [22]. The sporozoites then enter the bloodstream and get their first site of replication-the liver. These sporozoites infect the hepatocytes and replicate within them to form invasive merozoites. This phase is known as the **pre-erythrocytic stage**, a clinically silent stage. The sporozoites exhibit this function of traversal to hepatocytes via various proteins that include SPECT (sporozoite microneme protein essential for traversal) [23], CelTOS (cell traversal protein for ookinetes and sporozoites) [24], phospholipase (PL), gamete egress and sporozoite traversal protein (GEST) [25]. The other three dominant proteins involved in hepatocyte invasion are Circumsporozoite surface protein (CSP), thrombospondin-related anonymous protein (TRAP), and apical membrane antigen-1 (AMA-1). CSP possesses repeated regions interacting with highly sulfated proteoglycans (HSPGs) on hepatocytes. During the hepatocyte infection, these sporozoites thrive and replicate for 2–16 days (depending on species) and form thousands of merozoites that enter the central bloodstream [26,27].

. Once released in the blood, the merozoites initiate the blood stage called the **asexual erythrocytic stage**. Merozoites infect erythrocytes in a fast, multi-step process involving three crucial steps-pre-invasion, active invasion, and echinocytosis [28]. The current knowledge suggests that the merozoite surface protein 1 (MSP-1) is the major protein involved in the erythrocytic invasion, along with other proteins such as *Pf* reticulocyte binding protein homolog (PfRh), erythrocyte binding-like protein (EBL), calcineurin, *Pf* casein kinase 2 (PfCK2) [29]. The interaction during pre-invasion between merozoites and erythrocytes results in the deformation of the host cell. The erythrocyte surface proteins mainly involved in merozoite interaction are the basigin and Rhoptry Neck Protein (RON) complex [30]. PfRh5 binds to the basigin protein present at the surface of the erythrocyte. This anchoring of the merozoite on the erythrocyte surface facilitates the apical membrane antigen 1 (AMA1) to interact with the RON2 (a part of the RON complex) [28]. After forming several tight junctions between erythrocytes and merozoites, the active invasion of the merozoites involves the release of contents from specialised secretory organelles called rhoptries. The merozoites enter the erythrocyte via the actomyosin motor. It is then followed by the attainment of the third step of the erythrocytic cycle called echinocytosis [31]. The echinocytosis results in the shrinkage of erythrocytes with spiky surface protrusions. After erythrocyte invasion, the merozoites divide by a process called schizogony that lasts 48 h. The method of schizogony in the erythrocytes involves ring formation, followed by trophozoites and schizonts. These schizonts then mature to form 16–32 merozoites that can egress and infect other erythrocytes to repeat the same cycle. The merozoite egress results in the destabilisation and bursting of erythrocytes and the release of hemozoin into the blood [32].

However, few parasites during schizogony progress towards sexual stage development to form gametocytes. This stage is called intra-erythrocytic gametocyte development. The molecular mechanism behind this process is yet to be elucidated. The environmental cues, such as high parasitemia and drug exposure, provoke merozoite commitment toward the sexual stage [33]. The mosquito sucks up the gametocytes for the sexual cycle that completes in the mosquito's midgut. However, the gametocytes hide within the host's bone marrow to avoid immune clearance [34]. Immature gametocytes sequester into the bone marrow, expressing the PfEMP 1 and subtelomeric variant open reading frame (STEOVAR) genes during immune evasion.

The *Plasmodium* is a complex microorganism that employs many proteins to establish the infection. It maintains its virulence by causing extensive modification to its niche, i.e., erythrocytes. The deformed erythrocytes are incapable of circulating in the blood. The parasite secretes various proteins, like pfalhesin, *P. falciparum*-infected erythrocyte membrane protein-1 (PFEMP-1) and sequestrin, that anchor in the membrane of the parasitised erythrocytes [35]. This allows the cytoadherence of the infected cell to the walls of capillaries, leading to deep accumulation of the parasite. This leads to obstructed blood flow, and thereby, local inflammation arises that leads to severe consequences such as cerebral malaria [36]. This is worth acknowledging that the parasite can evade the immune system and survive within the host, leading to chronic infections due to sequestration in deep tissues [37].

3. Immune system and *Plasmodium falciparum*

The host activates different arms of the immune system to mount protective responses against *Pf*. However, *Pf* evades the host immune system successfully by using various strategies. *Plasmodium* is evolutionarily co-evolved with its human host, harnessing the host's processes to accomplish its survival. **Humoral response:** The skin provides the first line of defence in the vertebrate host. The sporozoites spend up to 2–3 h in the dermis, and less than 50 % of sporozoites leave their first site of injection [38]. The immune system targets the free sporozoites via an antibody-mediated mechanism. Neutralizing or inhibitory antibodies tend to prevent the pre-erythrocytic stage of the parasite by binding to the CSP on sporozoites and subsequently prevents sporozoite traversal and blood-stage infection [39]. However, once it enters the liver, the sporozoites take approximately 2 min to infect the hepatocytes. Usually antibodies do not perturb such a rapid infection, as high titers should be circulating in the blood to neutralize the parasite. [40]. Recent studies showed that non-CSP IgG also have the potential to inhibit the invasion of sporozoites into hepatocytes [41]. Yet, these antibodies and their targets have to be identified. Other reports also suggest that the antibodies against MSP2 and MSP3 antigens protect against clinical malaria infection. Humoral immune responses (especially against Pf EMP1) elicited during the early age of infection protect against severe malaria [42].

Dendritic cells (DCs): These cells are known to bridge the two arms of the immune system-innate and adaptive. DCs are found to be interacting with the parasite at each stage of the life cycle. It has been observed that blood-circulating DCs are more responsive in antigen processing and capable of inducing allogeneic T-cell responses, rather than tissue-resident DCs. For instance, DCs in the dermis or liver activate T-cells with weak reactions against the parasite [43]. Nonetheless, Bamboat et al. have shown that liver-resident DCs induce immunogenic

tolerance. This can be one of the probable reasons for the survival of sporozoites within the hepatocytes [44]. Kordes et al. suggest that *Pf* modulates the DCs to suppress the immune system by downregulating TLR9 on DCs that recognise the DNA of the parasite [45]. Another way of immune suppression is minimal production of effector cytokines such as IL-1β, IL-6, and TNF-α. It has also been observed that *Pf* induces the apoptosis of DCs, thereby reducing T-cell stimulation [46]. Urban et al. have shown that the number of HLA DR + blood circulating DCs is lower in malaria-positive subjects than in their healthy counterparts [47].

Complement system: Numerous studies imply the function of different proteins of the complement system in controlling malaria infection at all life cycle stages [48–51]. These proteins are predominantly C1q and C5a, responsible for forming the Membrane Attack Complex (MAC). Collectively, complement-mediated lysis in conjunction with the antibody targets sporozoites, merozoites, parasitised RBCs, and gametocytes [52]. *Pf* utilises a more innovative strategy to evade the killing by the complement pathway during the development of merozoites from schizonts. Merozoites can infect the RBCs even in the presence of complement proteins. Merozoites recruit host-regulating proteins such as Factor H (FH) and Factor H-like protein 1 (FHL-1) to their surface. These host proteins bind to the merozoite surface protein Pf92 and save them from complement lysis [53]. Components of the alternate pathway of the complement system remain active for a few hours in the mosquitoes' midgut. The Plasmodium gametes recruit the Human FH and FHL-1 proteins from blood in the midgut to their surface and finally evade the complement lysis. FH proteins interact with the gamete surface protein GAP 50, which inactivates the complement protein C3b.

Cell-mediated immunity: The preclinical malaria infection studies have shown the role of $CD4^+$ T-cells, $CD8^+$ T-cells, and γδ T-cells. However, the role of T cells in clinical infections has not been well discussed so far. Irradiated sporozoite v have shown the development of memory cell responses against the liver stage malaria. Hence, production of such a vaccine in large quantities for global demand, along with maintenance of the cold chain, is a challenging issue for the whole-organism irradiated sporozoite malaria vaccine. Zander et al. showed in a pre-clinical study that $CD4^+$ T-cells, when interacting with parasite antigens presented by MHC II, differentiate into T_H1, T follicular helper (T_{FH}) cells, T_H17, IL-27-producing $CD4^+$ T-cells, IL-10 $CD4^+$ T-cells, and IL-9-producing T_H9 cells [54]. DCs secrete IL-12 and IL-6, and present parasite antigens to naïve $CD4^+$ T-cells, which further differentiate into T_H1 and T_{FH} cells. T_H1 cells secrete IFN-γ, which acts as a crucial protector against the parasite. However, meta-analysis data demonstrated that high IFN-γ levels correlate with the severity of the disease [55,56]. As antimalarial responses, $CD4^+$ T cells differentiate into T_{FH} cells that are functionally characterised by $CXCR5^+$ $PD\text{-}1^+$ $CXCR3^-$ $CD4^+$ that stimulate germinal centre reactions to engross B cells in producing antibody-secreting plasma cells [57]. The clinical study employing high-dimensional CyTOF flow cytometry, in endemic regions of Malawi, has revealed terminally effector memory $CD4^+$ T cells specific to *Pf*CSP [58]. However, the immunity against parasites is age-dependent, with older patients having high memory cells and significant Antibody titers [58]. Similarly, $CD8^+$ T-cells, when activated, secrete cytotoxic substances that kill the infected cells, such as hepatocytes or erythrocytes. Interestingly, newly identified T_H9 cells modulate T_H17/T_{reg} cells and are responsible for disease severity [59]. In addition, γδ T-cells are known to suppress parasitic infection by forming immune synapses and lysing infected RBCs. Their action mode is similar to $CD8^+$ T-cells, as they cause direct killing and are cytotoxic. These cells secrete granulysin and granzymes [60]. It has been reported that TNF-α provides significant protection against the pre-erythrocytic stage of the parasite [61].

Immune evasion by the parasite, *P. falciparum,* has evolved multiple strategies to evade host immune responses, thus complicating the design of therapeutics. *P. falciparum* is devious in promoting anti-inflammatory Th2 response and apoptosis of Kupffer cells during the liver stage of infection, thereby reducing the MHC-I expression [62]. This allows T-cell tolerance to sustain the stringent environment of the liver [63]. Regardless of downregulating Th2 responses, *P. falciparum* modulates host epigenetic regulation, hampering the metabolic status of immune cells. Metabolic reprogramming is the crucial cellular process within immune cells that mediates protection against diseases. Detailed studies have shown that adrenal hormones and glutamine metabolism are altered during malaria [64]. However, their therapeutic interventions were less explored. Subsequently, research in the murine model of experimental cerebral malaria showed altered regulation of the master regulator of metabolic pathways, i.e., Sirtuin 1. In severe malaria, activation of Sirtuin 1 mediates disease tolerance, protecting against cerebral malaria [65]. Yet, it is worth acknowledging that *P. falciparum* hinders the epigenetic regulation of immune cells, which leads to tissue damage and the severity of the disease. Enormous efforts are needed to study the host immune evasion during malaria, which involves epigenetic regulation of key cellular pathways in immune cells.

P. falciparum expresses RIFIN on the surface of the iRBCs that further interact with inhibitory receptors such as LILRB1 on B cells and NK cells [66]. This study was further corroborated by the severity of the disease, revealing that RIFINs downregulate humoral responses and NK cells mediated cytotoxicity [66]. The host RBCs act as a shield for the parasite and help in evading immune responses. *P. falciparum* forces the internalisation of host vitronectin that later interacts with serine repeat antigen 5 (SERA5). This allows the escape of parasites from the immune system [67]. Nevertheless, the variability among the host immune responses against *P. falciparum* has always been astonishing when designing therapeutics against malaria. Genome-wide association studies (GWAS) and gene expression studies in the African population enlightened us on a few loci that might have played a role in the variability of host immune responses [68–71]. Nonetheless, *P. falciparum* regulates post-transcriptional mechanisms and miRNAs to evade host immune responses. *P. falciparum* promotes its survival by altering the regulation of specific miRNAs, including miR15-a5p, miR16-5p, and miR181c-5p [72]. Whole blood analysis showed downregulation of more than 40 miRNAs in symptomatic patients. Further integrative miRNA-mRNA studies revealed that dysregulation of miRNAs hampers T-cell development, further leading to programmed cell death of immune cells [72].

P. falciparum is artful in avoiding immune clearance by altering its antigens on infected RBCs. *P. falciparum* possesses specific polymorphic proteins, such as PfEMP and MSPs, that contain several *var* domains [73]. The antigenic variation in these *var* regions confers masking to the parasite. The parasite enables the nuclear redox sensor, *P. falciparum* thioredoxin peroxidase-1, that associates with antisense-long non--coding RNA to efficiently transcribe selective *var* genes [74]. *P. falciparum* employs a transcriptional switch to provide biased *var* gene expression in a chronic infection, allowing the structural variation on the surface antigen [75].

4. Malaria control strategies and caveats

Malaria is a global burden, and recent statistics suggest that the incidence and morbidity have worsened during the COVID-19 pandemic [76]. Malaria control depends mainly on antimalarial drugs and vector control [77].

The treatment guidelines for malaria are based on combination therapy, which may consist of two or more effective anti-malarial medicines with different modes of action. Artemisinin-based combination therapy (ACT), which contains artesunate, sulfadoxine, and pyrimethamine, is an accepted malaria treatment. However, this treatment regimen fails in endemic regions where artemisinin resistance is growing [78]. The artemisinin is a pro-drug that undergoes chemical cleavage in the parasite-infected erythrocytes. The parasite digests the host's haemoglobin to obtain nutrients, and thereby, it results in the release of redox-active heme and free ferrous ions. These redox molecules are known to react with artemisinin to activate it chemically. This

further alkylates the parasitic proteins, and the accumulation of ROS within these infected erythrocytes leads to the death of the parasite [79]. The use of phytomolecules, quinine derivatives, and repurposed medications to treat malaria infection is the focus of ongoing research in numerous labs [80–82].

Numerous transmission-blocking drugs prevent the transmission of mature gametocytes from host to mosquito. Primaquine is an *anti*-hypnozoite drug. It was the first anti-malarial drug that completely removed the mature gametocytes from the blood [83]. However, the use of this drug is cautioned due to its side effects in glucose-6-phosphate dehydrogenase-deficient patients [84,85]. The preclinical study indicated that tadalafil inhibited the circulation of gametocyte-affected erythrocytes in blood. These erythrocytes were arrested in the spleen of humanised mice. Hence, it was suggested that this inhibitor could be a novel drug to inhibit transmission [86]. It is important to remember that *P. falciparum* is a complex microorganism with an incompletely characterised proteome. This creates a challenge in developing anti-malarial medications for severe and complex malaria. The two primary interventions used to prevent malaria are long-lasting insecticidal bed nets (LLINs) and indoor residual insecticide spraying (IRS) [87]. The primary insecticide used in LLINs, pyrethroids, is widely resistant to the Anopheles vector, posing a threat to the efficacy of IRS [88]. This calls for developing efficient, secure, and environmentally friendly products that directly attack the vector and obstruct transmission. Due to emerging drug resistance in the parasite and the anti-malarial resistance in the vector, there is a need to focus on alternative malaria control methods, with the vaccine being the most promising.

Vaccines: The most effective method for containing the infection is thought to be the vaccine. Since the 1930s, numerous initiatives have been made to reduce malaria infection. Inactivated sporozoites have been investigated as potential vaccine candidates. Therefore, researchers studied different types of vaccines targeting different stages of the malaria life cycle (see Fig. 1). These included irradiated sporozoites, recombinant vaccines with and without formulations, stage-specific vaccines, and transmission-blocking vaccines. This section focuses on the stage-specific vaccine in terms of its efficacy, advantages, disadvantages, and impact on the host immune system.

a. **Pre-erythrocytic vaccine (PEV):** The sporozoites traverse from the dermis to the hepatocytes and replicate to form invasive merozoites [27]. This allows for the various morphological and genetic changes in the parasite. Various PEVs have been introduced and tested in murine models and adults. The major categories of PEVs include subunit vaccines and whole sporozoite vaccines.

i. **Subunit vaccine:** The sporozoite surface protein, CSP, is the vital protein that enables the interaction between sporozoites and hepatocytes. Consequently, this protein became the potential target for preventing sporozoite invasion in hepatocytes [89]. The C-terminal of CSP carries tetrapeptide repeats of Asn-Ala-Asn-Pro (NANP), including immunodominant $CD4^+$ $CD8^+$ T-cell epitopes and epitopes for B-cell receptors. This C-terminal is fused genetically with the N-terminal region of the Hepatitis B surface antigen and formulated with the adjuvants such as + 3-O-desacyl-4'-monophosphoryl lipid A (MPL). This construct has many repeated regions conferring enhanced presentation to the immune system [90]. Phase II and III clinical trials in various areas such as Ghana, Kenya, Mozambique, and African regions suggested the high titers of anti-CSP antibodies in RTS, S/AS01 vaccinated children [91,92]. It has been suggested that these antibodies are directed against NANP regions, conferring higher protection against malaria. Since this vaccine contains $CD4^+$ and $CD8^+$ T-cell epitopes, flow cytometric analysis of CSP-specific $CD4^+$ T-cells showed increased $CD4^+$ T-cells as they were exposed to booster doses [93]. Nonetheless, these vaccinated individuals did not observe CSP-specific $CD8^+$ T-cells. Phase III clinical trial in pediatric Africans led to the identification of central and effector memory and polyfunctional T-cells [94]. The study was conducted on 105 vaccinated individuals and showed more CSP-specific-HBsAg-specific T-cells producing IL-2, TNF-α, and CD40L than the control group [94]. These $CD4^+$ T-cells were polyfunctional with T_H1 and T_{FH} phenotypes [94]. The antibodies against CSP become ineffective after hepatocyte invasion of sporozoites, which lasts 10–15 min after infection; thus, no recall responses are generated upon RTS, and S vaccination is in later stages [10]. WHO approved a four-dose (three doses and one booster dose) vaccine for children living in malaria moderate to high endemic areas. In October 2021, the WHO certified the 'wide-use' CSP-based vaccine called **RTS, S/AS01** in high-endemic countries. However, the

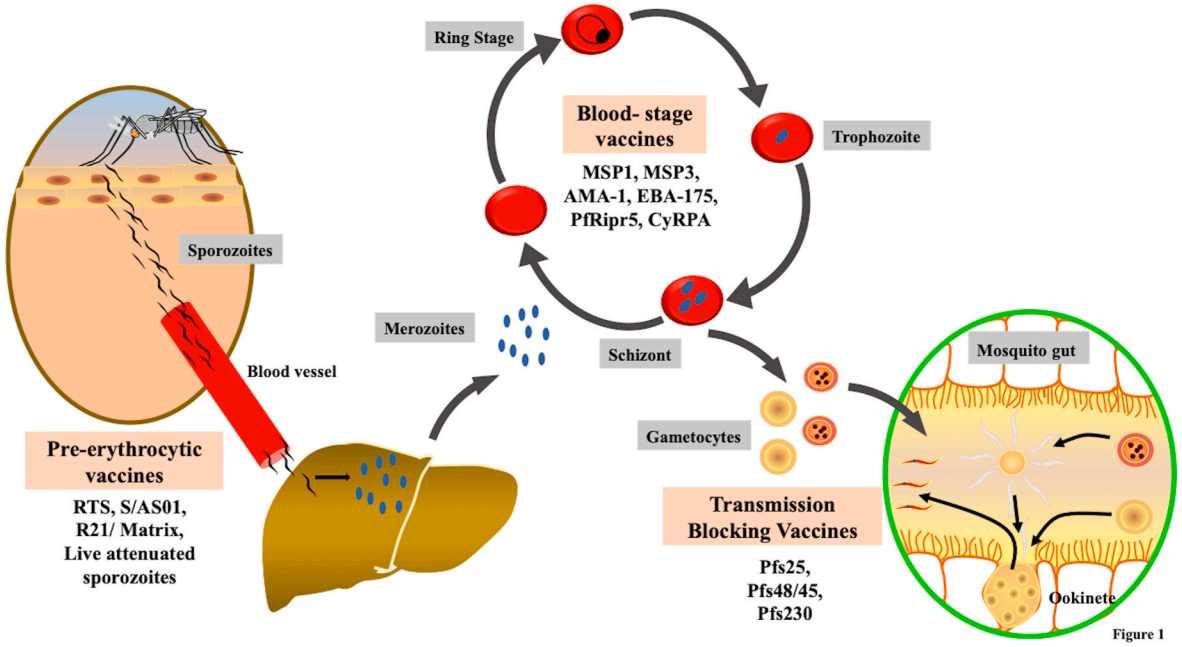

Fig. 1. Targeting different life cycle stages of *Plasmodium falciparum*: Multi-stage development of the parasite is governed by its different antigens which are being targeted for developing vaccines.

disadvantage of this vaccine is its modest efficacy of ~36.3 % and 25.9 % against clinical malaria in children and infants, respectively, and the evoked immunity wanes due to the decrease in antibody titers by 18 months of vaccination [11,95]. The primary case definition of clinical malaria during the trial was the axillary temperature ≥37·5 °C and *Plasmodium falciparum* asexual parasitaemia >5000 parasite/μl. RTS, S/AS01 efficacy against severe malaria was 32.2 % among children (aged 5–17 months) and 17.3 % among infants (aged 6–12 weeks). The endpoint vaccine efficacy was the febrile clinical malaria with parasitemia >5000/μl. The gender specific high mortality (2.4 %) was reported among vaccinated girls in comparison to the control group (1.3 %) in all age groups. The safety signals like meningitis were reported more among vaccinated children than their control counterparts during the phase III clinical trial [95]. However, at least one fatal serious adverse event within 30 days post vaccination was 0.3 % among children (aged 5–17 months) and 0.2 % among the control group. It was found to be higher, 0.6 % among the vaccinated infants (aged 6–12 weeks) in comparison to their control counterparts, 0.3 % [95]. Therefore, these issues must be handled during the post-marketing phase IV clinical trial.

Another successful WHO-approved vaccine, *R21/Matrix-M,* shows more than 75 % protective efficacy against clinical malaria in Phase I/IIb clinical trials [14]. R21/Matrix -M is also a subunit vaccine, prepared by using the repeated region of CSP and hepatitis B surface antigen. The amount of CSP in this fusion protein is much higher than in comparison to the RTS, S/AS01. It induces antibodies targeting the central repeat region of CSP, which is the NANP repeats. Antibodies to NANP repeats have already been established as a correlate of protection in RTS, S/AS01. Pre-clinical study induces increased CSP-specific IgG antibody titers, many $CD8^+$ T-cells, and enhanced B-cell activation [96]. A recent animal model study showed that adeno-associated virus serotype 8 (AAV8) containing CSP can be used as a potential booster to enhance the immune response generated by human adenovirus type 5 (AdHu5). An increase in the frequency of resident memory $CD8^+$ T-cells in the murine liver was observed [97]. Nevertheless, the mechanism by which such immune responses are operated needs further evaluation.

R21/Matrix-M showed the high efficacy (75 %-79 %) against clinical malaria after four doses (three doses and one booster) of vaccine containing high dose (50 μg) of Matrix M adjuvant among children aged 5–17 months at seasonal and standard sites [13]. The case definition of clinical malaria during the R21/Matrix-M vaccine clinical trial was the same as with RTS, S/AS01 [11,13]. However, no end-to-end comparison study has been done for R21/Matrix-M and RTS, S/AS01 vaccines. However, based on currently available data, it seems that R21/Matrix-M is more efficacious than RTS, S/AS01. Safety studies showed that R21/Matrix-M is a well-tolerated vaccine. No serious adverse side effects have been reported so far [13].

ii. **Whole sporozoite vaccine:** In randomised clinical trials, attenuated sporozoites have been evaluated for their vaccine efficacy. The sporozoites are isolated from aseptic mosquitoes and attenuated by different methods, such as X-radiation, chemical means such as antimalarial drugs, or genetic attenuation of specific genes, such as p52, p36, sporozoite asparagine-rich protein-1 (SAP-1) [98]. Roozen et al. have shown the 90 % efficacy during immunisation with late liver stage genetically attenuated (mei2 single knockout) PfSPZ, which was administered by mosquito bite in a placebo-controlled randomised trial [99]. The single inoculation of these genetically attenuated PfSPZ provides sterile immunity against the homologous controlled human malaria infection (CHMI). In another study, Walk et al. have shown the 100 % protective efficacy of chemoprohylaxis and sporozoites (CPS-PfSPZ) immunisation (three doses by mosquito bite) against homologous NF53 *Plasmodium falciparum* strain during a double blind randomised clinical trial [100]. CPS-PfSPZ induces sterile immunity against all homologous infections. However, it mounts modest immunity against the heterologous *Plasmodium falciparum* infections [100].

The whole sporozoites vaccine is known to induce a repertoire of antibodies against many parasite proteins such as CSP, AMA-1, TRAP and EBA,. *P. falciparum*-specific γδ T-cells and $CD4^+$ T-cells are also reported to impart protection against the parasite [101]. In addition, a randomized, controlled Phase I clinical trial has reported that a three-dose regimen of PfSPZ is tolerable with an efficacy of 51 % against natural *P. falciparum* transmission [102]. PfSPZ immunisation provides sterile immunity against homologous infections, because it offers the benefit of antigens to our immune system. Due to this, it has an edge over subunit vaccines. However, a significant hurdle with PfSPZ immunisation is that it confers minimal immunity against heterologous strains [100].

iii. Other proteins: *P. falciparum* harbours some multigene families, such as *var, stevor,* and *rifin* that code for export proteins [103,104]. These export proteins, such as PfEMP1, have been extensively studied and are found to be expressed on the surface of iRBC, enabling the interaction with another host cell, such as the endothelium, resulting in the sequestration of iRBCs within tissues and capillaries [105]. Nonetheless, studies in rodent malaria parasites have uncovered other large multi-gene families, such as *fam* and *pir,* that also code for export proteins. These export proteins are expressed during the intra-hepatic stage and transferred through PVs to establish blood-stage infection. Fam proteins have a START domain that sequesters phosphatidylcholine from the host for membrane biogenesis during the hepatic stage. These proteins are then expressed during the blood stage within the cytoplasm or on the surface of iRBCs [106]. Similarly, a few *pir* proteins are expressed on the edges of merozoites, enabling RBC invasion. These proteins are selectively expressed during the asexual blood stage and contain the potential to evade host immune responses [107]. In line with this, other proteins necessary for invasion into hepatocytes during the intra-hepatic stage, other than the blood stage, are identified. Although this protein PTEX Pore Component EXP2 is known for nutrient transport across the parasitophorous vacuole, it is proven to provide nutrition during parasite development within hepatocytes [108]. Though many studies are necessary to identify its role, EXP2 serves as a promising vaccine candidate that can target dual stages during parasitic development.

b. **Intra-erythrocytic vaccine:** Also known as blood-stage vaccines (BSV), these vaccines target the asexual stage of parasite multiplication, preventing the formation of merozoites and thereby the clinical disease. Various merozoite proteins, such as MSP1, AMA1, EBA-175, and MSP3, are targeted to evaluate their ability to elicit immune responses against the asexual, clinical blood stage [28]. Many IEV or BSV have been enrolled in clinical trials since 2000. However, multiple disadvantages, such as redundant invasion pathways and antigen polymorphism, provided disappointments of lower efficacy. Novel targets have been identified that can combat these blood stages of *Plasmodium*. Such a novel candidate, known as recombinant Vesicular stomatitis virus-based vaccine (rVSV), has been assessed in pre-clinical studies. This vaccine candidate constitutes various merozoite-specific peptides such as AMA1 (residues 98–445 aa), Rh5ΔNL (residues 140–526 aa, but lacking 248–296 aa), and RON2sp (C-terminal residues 2020–2059 aa). Administration of rVSV vaccine candidate induced high IgG titers, $CD4^+$ T-cells, $CD8^+$ T-cells, IFN-γ and IL-2. Also, this candidate successfully inhibited the parasite invasion in the mouse model [109]. However, further efficiency of this vaccine compared to other candidates has yet to be assessed. Recently, a novel vaccine candidate, PfRipr5, was tested to induce inhibitory antibodies against

blood-stage merozoites in ex vivo experiments [110]. However, further validation is required to support its protective efficacy in pre-clinical and clinical models. Merozoites exploit the interplay of specific proteins such as PfRipr5, PfCyRPA and Rh5 to invade the erythrocytes [111]. Rh5 has already been tested for its ability to inhibit parasite growth in mice. However, it does not completely provide sterile protection [112]. Henceforth, truncated PfRipr5, along with FDA-approved adjuvants, showed significant production of inhibitory antibodies against merozoites in a pre-clinical study [110]—similarly, monoclonal antibody targeting administration. In a preclinical animal study, Pf CyRPA is efficient enough to inhibit nearly 90 % of parasitemia [113]. Administration of PfCyRPA induces *Plasmodium*-specific humoral response in small animals and thus shows inhibitory antibodies against the merozoites in *in vitro* assays [114]. It is essential to highlight that the antibodies inhibiting the blood stage are also proven to inhibit the pre-erythrocytic stage [115].

The invasion of red blood cells (RBCs) is a critical determinant of the progression of Plasmodium infection. During invasion, parasite surface proteins are cleaved by a membrane protease known as SUB2 [116]. Genetic depletion studies of SUB2 have demonstrated that its absence results in either the cessation of RBC invasion or impaired development of merozoites, without affecting merozoite egress [117]. Mass spectrometry findings further corroborated this observation, which revealed that genetic depletion of SUB2 resulted in impaired shedding of a broader parasite surface proteome. This encompassed nearly 700 surface proteins, among which MSP 1–7, PTRAMP, AMA-1, MSP-7-like proteins, and Pf92 were more significantly affected [117]. These results highlight that erythrocyte invasion is determined by the collaborative shedding of various proteins, beyond the MSP complex. Consequently, these proteins may serve as potential vaccine candidates when targeted simultaneously. Thus, employing blood-stage antigens can serve as a potent tool for a vaccine against malaria. However, much research is needed to answer the other unaddressed questions.

c. **Transmission-blocking vaccine (TBV):** TBVs are those vaccines that prevent the transmission of disease from one individual to another by inhibiting the pathogen's ability to replicate. These vaccines provide immunity at the community level and not the individual level. TBVs target the parasite's sexual stages (gametocytes, gametes, zygotes or ookinetes), interrupting parasite transmission to the vector. Very few antigens have been targeted to assess their ability to induce protective antibodies against the gametocyte or zygote in the mosquito. Pfs25 is an essential protein and integral part of the ookinete membrane, also protects the ookinete from the proteases of the mosquito's midgut. Pfs25 is the first TBV candidate under clinical trials for its efficiency [118,119]. McLeod et al. have shown that the TBV candidate, such as the Pfs48/45 (pre-fertilisation proteins) duplex candidate, elicits the production of potent inhibitory antibodies to block the transmission in the mouse model [120]. Alkema et al. have shown that the administration of four doses of Pfs48/45-based vaccine induces the production of antibodies to the target proteins Pfs48 and Pfs45 in an open-label clinical trial [121]. The mosquito membrane feeding assay measured the transmission-blocking activity in the serum of study participants. However, these antibodies' concentration was insufficient to block transmission in the malaria naïve population. Similarly, Pfs230 is one of the potential candidates in complex with CSP eliciting immune responses against the pre-erythrocytic and sexual stages of the parasite. The liposomal formulation of these two antigens showed the induction of humoral and cellular immune responses in a pre-clinical animal study [122]. Sagara et al. showed the high activity of gamete-targeting vaccine Pfs230D1-EPA/Alhydrogel compared to zygote-targeting vaccine Pfs25-EPA/Alhydrogel (four doses) during a phase 1 randomised trial. The transmission-reducing activity of Pfs230D1-EPA/Alhydrogel was 73.7 % up to 10 weeks after administration of the fourth dose [123]. All three formulations produced neutralizing antibodies specific to target the proteins Pfs25 and Pfs230D1.

Following a similar path, a bivalent vaccine of Pbg37 and PSOP25 has been tested for efficacy. It was observed that the combination of multi-epitopes as TBV provides better protection against sexual stages of the parasite in the mosquito [124].

5. Challenges and future potentials in the development of a malaria vaccine

Despite significant efforts to develop new vaccines, the interaction between the host immune system and the malaria parasite remains highly intricate. Understanding the proteins involved at different stages of the parasite's life cycle is essential for progress. Several key challenges hinder the development of an ideal malaria vaccine.

A comprehensive understanding of the proteins involved in parasite development is lacking. *Plasmodium falciparum* encodes more than 5000 proteins necessary to grow in mosquito and vertebrate hosts [125].

The high degree of genetic polymorphism among *P. falciparum* antigens reduces the effectiveness of current vaccines. This genetic diversity enables *P. falciparum* to evade the immune response generated by vaccines, as the immune system may not recognise variant forms of the antigen from one strain to another. Consequently, vaccines that offer protection against one parasite strain may be less effective or entirely ineffective against others [126]. Currently approved malaria vaccines cover only the pediatric population; they do not cover all age groups. These vaccines do not offer protection against all dominant *Plasmodium* species and are not as efficacious as other childhood vaccines. The currently approved malaria vaccines do not provide sterile immunity against *P. falciparum* infection. These vaccines do not protect against disease and are ineffective in blocking transmission, a prerequisite for malaria elimination.

The efficacy of the RTS, S/AS01vaccine depends on multiple factors, such as the genetic diversity of the local parasite population. If the *Plasmodium falciparum* population is diverse from the target 3D7 circumsporozoite protein, then it would not be as effective as it is with the 3D7 CSP. The incomplete understanding of the host's immune responses to *P. falciparum* is a significant barrier to developing effective malaria vaccines and therapies. The timing, magnitude, and interplay between pro- and anti-inflammatory cytokines during infection are poorly characterised, making it difficult to predict or modulate immune responses to enhance protection without causing harmful side effects. Additionally, there is limited understanding of the development of memory T and B cells and their longevity, as the antigenic variation affects the efficacy of TCRs and IgG [127].

It is essential to acknowledge that *P. falciparum* is a complex microorganism that hijacks host cells to multiply and spread. Despite existing challenges in vaccine development, there is a need to emphasise ideal vaccine development measures, including high coverage in poor and endemic countries. Hence, the measures for a perfect vaccine include-

1. Determination of conserved antigens among different *Plasmodium* strains that can elicit or divert the host immune system toward protection.
2. Development of a multi-epitope vaccine formulated with human adjuvants that can target each life cycle stage and thus impart protection over subsequent stages.
3. Generation of long-lived, refined, and inhibitory antibodies capable of activating the complement system, antibody-dependent cell-mediated cytotoxicity (ADCC).
4. Induction of B and T-memory cells in response to antigens targeting different life cycle stages.

6. Conclusion (200 words)

Malaria is one of the most devastating diseases till now. The emergence of drug-resistant strains of *Plasmodium* poses a great threat to mankind. Vaccination is the best way to combat any infectious disease. Despite the availability of RTS, S/AS01 and R21/Matrix-M vaccines, new vaccines are required to control this deadly disease. Hence, developing a multistage targeted vaccine with high efficacy is imperative. Various preclinical and clinical trial studies have identified many potent probable vaccine candidates. However, a few volunteers as study subjects led to inconclusive results in many clinical trials. Therefore, there should be extensive clinical trial studies with recruitment of a greater number of volunteers to conduct.

CRediT authorship contribution statement

Akanksha Verma: Writing – original draft. **Ritesh Ranjha:** Writing – review & editing. **Kuldeep Singh:** Writing – review & editing. **Vinod Yadav:** Writing – review & editing. **Ashima Bhaskar:** Writing – review & editing. **Ved Prakash Dwivedi:** Writing – review & editing, Supervision. **Mradul Mohan:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Sources of information

The literature on malaria vaccines and malaria immunity were. were obtained from published literature using PubMed and Google Scholar. The key words used during this work were malaria vaccines, RTSS malaria vaccine, malaria vaccine R21, Sporozoite vaccine, transmission blocking vaccine and malaria immunity.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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