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REVIEW

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Plasmodium falciparum merozoite surface protein 3 as a vaccine candidate: a brief review

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ABSTRACT

Despite the many efforts of researchers around the world, there is currently no effective vaccine for malaria. Numerous studies have been developed to find vaccine antigens that are immunogenic and safe. Among antigen candidates, *Plasmodium falciparum* merozoite surface protein 3 (MSP3) has stood out in a number of these studies for its ability to induce a consistent and protective immune response, also being safe for use in humans. This review presents the main studies that explored MSP3 as a vaccine candidate over the last few decades. MSP3 formulations were tested in animals and humans and the most advanced candidate formulations are MSP3-LSP, a combination of MSP3 and LSP1, and GMZ2 (a vaccine based on the recombinant protein fusion GLURP and MSP3) which is currently being tested in phase II clinical studies. This brief review highlights the history and the main formulations of MSP3-based vaccines approaches against *P. falciparum*.

KEYWORDS: Malaria. MSP3. *Plasmodium falciparum.* Vaccine. Immunity.

INTRODUCTION

Malaria is still considered a major public health problem in several tropical and subtropical regions of the world. It is an infection caused by parasites of the genus *Plasmodium*, and is transmitted to humans by mosquitoes of the *Anopheles* genus during blood meals. According to WHO[1] data, in 2019, 228 million cases of the disease were detected worldwide, resulting in 405,000 deaths, with *Plasmodium falciparum* being the agent of most infections[1].

Despite the availability of antimalarial drugs, the development of an effective vaccine is of utmost importance since in many parts of the world problems concerning drug resistance have been evidenced, such as the resistance to artemisinin observed in the Greater Mekong subregion and to mefloquine, observed at the border of Thailand and Myanmar[2,3]. More recently, resistance to dihydroartemisinin-piperaquine against infections caused by *Plasmodium falciparum*, has been detected in individuals living in Cambodia, Thailand and Vietnam[4].

Together with antimalarial drugs, control methods based on the distribution and use of impregnated bed nets, indoor spraying and extinction of mosquito breeding sites have also been successfully applied. However, these methods present only temporary results[1].

Clinical symptoms of malaria arise during the blood phase of the infection, when the burst of infected red blood cells leads to the release of pro-inflammatory factors. The overshooting of the resulting release of cytokines may trigger severe forms of the disease[5-7].

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The development of an efficient vaccine is challenging for several reasons. Firstly, the parasite expresses different antigens at different stages of the infection. Secondly, the parasite employs antigenic variation, displaying timely changing adhesins when they are inside red blood cells[8,9]. Thirdly, the parasite has a vast repertoire of antigenic variability exposed on the surface of merozoites[8]. Furthermore, the parasite is able to dampen the immune response by releasing Macrophage inhibiting factor (MIF), or by the exposure of polymorphic antigens which interact with immune cells such as RIFINs[10,11]. In this way, it is a safe and effective vaccine that requires a combination of humoral and cellular response: the humoral response is capable of inducing opsonization of sporozoites, blocking the invasion of red blood cells and eliminating infected cells, either directly or through ADCI (antibody-dependent cellular inhibition). The cellular response is important for the production of inflammatory cytokines, the stimulation of antibody production by B cells, and the elimination of infected hepatocytes by T CD4+ (helper) or T CD8+ (cytotoxic) cells. Additionally, a vaccine should produce long-lasting memory cells[5].

The most commonly studied vaccines for malaria have the following antigens: circumsporozoite protein (CSP); apical membrane antigen (AMA1), in some studies forming a complex with the RON2L antigen; *P. falciparum* reticulocyte binding-like homologue protein (PfRH5) and merozoite surface protein (MSP). Except for CSP, which belongs to the pre-erythrocytic stage of the parasite, the other antigens are part of the parasite's erythrocytic stage[12-14]. CSP composes the Mosquirix™ vaccine (trade name of RTS,S), which is already recommended for use by WHO, and is indicated for application in children in endemic regions. Even though RTS,S vaccine has only 40% effectiveness and reduces severe malaria cases by 30%, these are still the best results ever achieved for a malaria vaccine. However, studies that seek a more effective vaccine are still necessary[1,15,16].

Vaccine antigens from the erythrocytic stage may help to decrease the parasite burden. Ideally, recognition of these antigens by antibodies should block or delay merozoite invasion into red blood cells and/or lead to quick phagocytosis of merozoites[17]. One of these antigens, merozoite surface protein 3 (MSP3), has been the object of a number of studies. The MSP3 vaccine has been mainly studied through a complex combination with other proteins, such as GLURP (glutamate-rich protein) or LSP (Long Synthetic Peptide). For these reasons, this review focuses on MSP3-based vaccines, tested in both, animal models and in human clinical trials.

Structural characteristics of MSP3

Plasmodium falciparum merozoite surface protein 3 (MSP3) was identified in 1994 by Oeuvray *et al.*[18]. Sera reactive to MSP3 has been identified in immune individuals living in Papua New Guinea, and since then, it has been called secreted polymorphic antigen associated with merozoite (SPAM), however, it is more widely referred to as MSP3[19].

MSP3 is a soluble protein with a molecular weight of approximately 48 kDa. It is present on the surface of merozoites where it forms a protein complex with MSP1, MSP6 and MSP7 through a protein-protein interaction, and this complex is more specifically associated with the p83/p30 and p38/p42 MSP1 complexes, which in turn, are linked to the surface of merozoites through a glycosylphosphatidylinositol anchor directly linked to MSP1[18-20]. The function of this protein is not well understood, however, it is believed that it is bound to receptors during the invasion of erythrocytic cells and extended oligomeric forms[21]. Some important structural characteristics of this protein include the presence of an N-terminal signal sequence; a domain composed of three blocks, each one consisting of four repeated sequences of seven alanine amino acids disposed in tandem; a domain that is rich in glutamic acid and a leucine zipper motif in its C-terminal portion (Figure 1)[20].

Some studies have demonstrated that the response of cytophilic antibodies against MSP3 can generate protective immunity[22]. Additionally, numerous researchers have confirmed MSP3 to be a promising vaccine candidate, especially when several samples isolated from Africa and Asia were analyzed and elucidated that the C-terminal domain of this protein is highly conserved[19,23]. Even tough, other studies conducted in some endemic regions such as Thailand and Burkina Faso studied isolates of the parasite and warned on a diversity of predominant MSP3 haplotypes in different regions, which is a very important issue concerning the vaccine design[24,25].

Animal model studies

Murine model studies

In 1994, a study was carried out by Oeuvray *et al.*[18] to evaluate the protective capacity of the merozoite surface protein (MSP3), which had been recently discovered (Figure 2)[18]. The authors observed that antibodies against protective epitopes of MSP3 can be produced in mice, as well as in humans, in response to a single contact with the parasite through a natural exposure, therefore demonstrating that the epitopes marked by protective antibodies are immunogenic when presented by the parasite.

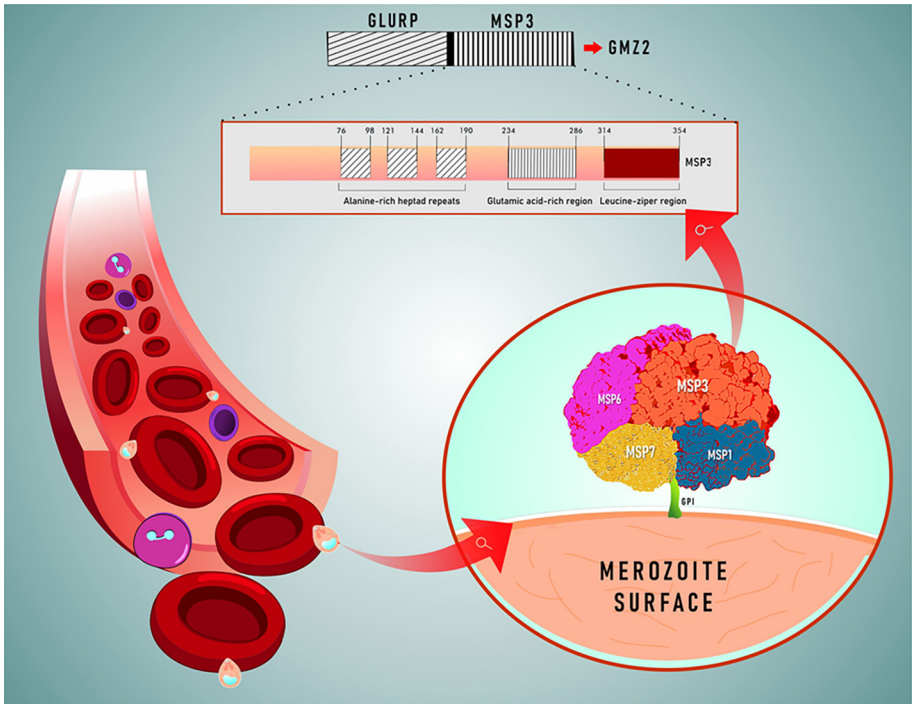

Figure 1 - Scheme of the complete MSP3 structure and the sequence of the GMZ2 vaccine. During *Plasmodium falciparum* infections, bloodstream merozoites invade red blood cells (A) by the interaction of surface proteins to erythrocyte receptors. Among the proteins that participate in this process is the protein complex composed of MSP1, MSP3, MSP6 and MSP7, attached to the merozoite membrane using a glycosylphosphatidylinositol (GPI) anchor (B). The MSP3 sequence in this complex (C) has regions of alanine heptad repeats (diagonal stripes); a glutamic acid-rich region (vertical stripes); and a leucine zipper motif (red). The protein structure in the GMZ2 vaccine corresponds to the association of the GLURP antigen and the MSP3 (D).

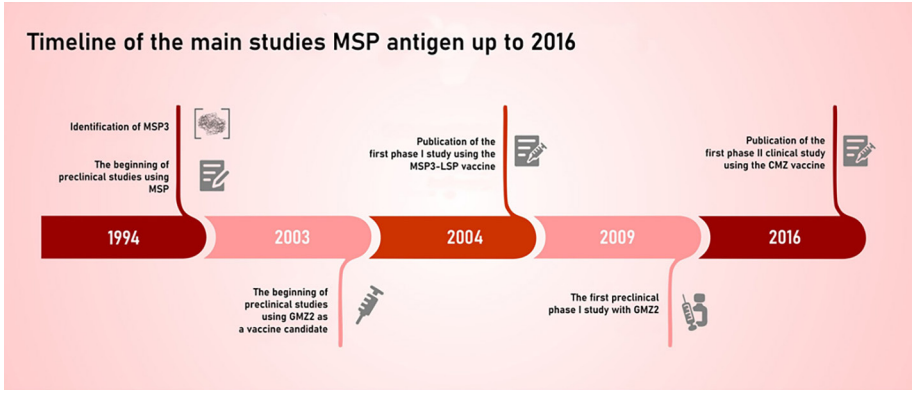

Figure 2 - Timeline of the main studies using the MSP3 antigen up to 2016.

As a result, it was concluded that the correlation between the isotypes of antibodies produced against the 48 kDa epitope, in addition to clinical protection and the ability of anti-MSP3 antibodies to inhibit the parasite's schizogony, indicate that this molecule may be an important target against *P. falciparum*[18]. In adult humans, the mechanism of protection against malaria involves antibody-dependent cell inhibition mediated by blood monocytes. In addition, cytophilic IgG3 and IgG1 antibodies were the main isotypes produced in individuals protected from malaria infection[26].

Given its potential for inducing protection, one study carried out in 2003 by Theisen *et al.*[27] used fusion between MSP3 and another *P. falciparum* antigen, GLURP (glutamate-rich protein) (Figure 2). The authors observed that the antibodies produced against the hybrid antigen showed a strong reactivity against native parasite proteins, which were capable of recognizing both, the parasite's native GLURP and MSP3 proteins when tested by indirect immunofluorescence. The authors concluded that the hybrid molecule can provide superior antigenic presentation compared to the approach with immunization with individual molecules[27].

In 2010, Daher *et al.*[28] carried out a study to analyze the immunogenicity of the C-terminal region of MSP3, and

four different recombinant proteins were produced. It was observed that the combination of six immunogenic regions of the MSP3 family was immunogenic in mice. In addition to inhibiting the growth of the parasite in an ADCI assay, the antibodies produced against this multigenic construct were able to recognize the parasite's native proteins. It is known that such inhibitory antibodies act by inhibiting the processing of surface proteins such as MSP3, blocking the formation of the complex with MSP1 on the surface of merozoites thus affecting its function during invasion[29]. Therefore, they concluded that this new multigenic construct was able to trigger the production of high antibody titers, and can be considered immunogenic in mice[28].

Primate model studies

The protective capacity of MSP3 has already been observed in a study developed in 2002 by Hiaseda *et al*.[30] who used *Aotus nancymaae* monkeys; however, Freund's adjuvant was used, which is not indicated for human immunization. In 2004, a preliminary pre-clinical study, which also used the GLURP protein, was carried out on primates[31]. This study aimed to evaluate the performance of different constructs derived from MSP3 and GLURP antigens by analyzing them in different combinations of adjuvants, as well as their protection capacity after a challenge test with *Plasmodium falciparum* in *Saimiri scireus* primates. Seven different antigen-adjuvant formulations were tested, of which five proved to be immunogenic. Among the combinations that showed high immunogenicity are MSP-3b-IFA (Freund's adjuvant), MSP-$3_{212-380}$-AS02, and MSP-$3_{212-380}$-Montanide ISA720. Regarding the challenge test, some monkeys immunized with MSP-$3_{212-380}$-AS02 or $GLURP_{27-500-}$alum were able to control the level of *P. falciparum* parasitemia in the blood stage of the infection. It is important to highlight that AS02 and alum adjuvants are used in human immunizations. Therefore, these results indicate that GLURP and MSP3 can induce a protective response against an experimental infection with *Plasmodium falciparum*[30,31]. Carvalho *et al*.[32], in 2005, used the recombinant hybrid protein MSP3/GLURP with another combination of adjuvants in *Saimiri sciureus*, aiming at improving the immunogenicity of these proteins. The hybrid protein was combined with the adjuvants Montanide ISA720, alum and Freund's. After immunization, the animals were challenged with *P. falciparum*, with a predominance of the parasite in ring and trophozoite phases. The authors concluded that the hybrid protein MSP3/GLURP can induce protection against malaria infection if antibody titers are proportionally high. In addition, the adjuvant used in combination with the antigen has also a crucial role in response[32].

Clinical trials

The 69 amino acid sequence of this C-terminal region has been shown to have interesting characteristics for the development of a subunit vaccine to be used in several phase I and IIb studies in humans exposed to the disease and people resistant to malaria. Two main candidates for malaria vaccine trials, namely MSP3-LSP and GMZ2, a combination of MSP3 and GLURP[33,34]. Based on promising results observed in animal testing, some studies have focused on evaluating the performance of MSP3 in clinical trials and have used different strategies. In 2004, a phase I clinical trial analyzed the immunogenicity and the safety of a long synthetic peptide derived from a conserved region of MSP3 (MSP3-LSP), which is known to be an important target for antibodies. The volunteers who participated in the research were adults (Figure 2). The groups were analyzed, with each one containing six individuals who received three subcutaneous doses of four different concentrations of peptide in Montanide ISA720, or two concentrations of peptide in aluminum hydroxide as the adjuvant. After immunization, the volunteers were randomized into six treatment groups, divided into two blocks, and the volunteers' immune responses were analyzed. It was observed that the formulation using Montanide ISA720 triggered serious local reactions in the volunteers, so that they were withdrawn from the study, leading to a reduction in the number of doses in the four groups. Nevertheless, no serious adverse reactions were found throughout the study. Regarding the response of the anti-MSP3-LSP antibodies, 23 out of the 30 individuals showed a specific response to the antigen. In addition, recognition of antibodies produced against native *Plasmodium falciparum* MSP3 was detected in 19 out of the 30 individuals, and specific T-cell with interferon-gamma production were also observed. As a result, the presence of memory T-lymphocytes was long-lasting, up to 12 months, especially in the group immunized with aluminum hydroxide adjuvant. It was concluded that, the potential use of this vaccine candidate is supported by a strong induction of cytophilic response. Despite the fact that there were not enough final volunteers for a statistical analysis by which it would be possible to compare the groups of different adjuvants, the one immunized with aluminum hydroxide and MSP3-LSP presented a better result in terms of immunogenicity and tolerance[33].

In 2005, Druilhe *et al*.[34] were the first to develop a clinical study for an MSP3-based vaccine that showed anti-parasitic activity. Again, a humoral and cellular response against MSP3-LSP was observed and the response lasted for up to 12 months. In addition, they found that the antibodies generated were able to inhibit the *in vitro* growth of parasites in the erythrocytic phase in a similar

way of naturally produced antibodies by individuals living in endemic regions. In the *in vivo* passive immunity experiment, where patients' sera were injected into SCID (severe combined immunodeficient) mice infected with *Plasmodium falciparum*, the parasite levels in the animals declined considerably. Such inhibitory effects are related to the reactivity of the antibody to the native protein, which was observed in 60% of the volunteers and lasted for up to 12 months after immunization. The study concluded that, even at low doses of MSP3-LSP immunization, by using Montanide or alum, the antigen was able to induce the production of cytophilic class antibodies, which are specific for highly conserved epitopes of the protein. Moreover, the antigen induced a lasting effect and the antibodies produced against it showed high biological activity against the erythrocytic stage of *P. falciparum*[34].

In 2006, Sirina *et al*.[35] conducted a blind, controlled, phase I study using the MSP3-LSP vaccine, in order to investigate its safety and immunogenicity in adults living in a malaria transmission area in Eastearn Africa. There was no specific response for MSP3-LSP with respect to total IgG, IgG subclasses and IgM, and the response of IFN-γ was stable after vaccination. In conclusion, semi-immune individuals from the endemic region inoculated with the MSP3-LSP vaccine showed good tolerance to the vaccine; however, the it did not stimulate a humoral response, probably due to high rates of pre-existing humoral immunity[35].

Another study was carried out by Lusingu *et al*.[36], in 2009, to evaluate the safety and immunogenicity of a vaccine using MSP3 (Figure 2). For this, the vaccine was tested in children aged 12 to 24 months from Korogwe, Tanzania. Three groups were established: two groups received two doses each of MSP3 emulsified with alum, and the third was a control group (hepatitis B vaccine). The levels of anti-MSP3 antibodies in the study participants were low before the beginning of immunizations and an increase in total IgG anti-MSP3 concentrations was observed after the second and third immunizations in both study groups. Some of the immune responses to MSP3 were higher in both groups when compared to the control one, showing response of specific antibodies belonging to two predominant classes (IgG1 and IgG3). Thus, it was concluded that the MSP3 antigen is safe and is immunogenic in children; however, there is a need for a phase II trial to evaluate the response in children from endemic regions. As both doses used were well tolerated, the authors suggest increasing the concentration of the antigen to better assess the response[36].

The vaccine candidate MSP3-LSP has also been evaluated for its safety and immunogenicity in children who live in Burkina Faso[37]. Children were separated into three groups, in which two groups received different concentrations of MSP3-LSP and the third group received doses of the Engerix B, ahepatitis B vaccine. According to evaluation of sera performed before immunizations, anti-MSP3-LSP levels were similar in all three groups. The total IgG antibody response against MSP3-LSP showed an increase in groups immunized with MSP3-LSP, with no reaction in the control group. In both groups immunized with MSP3-LSP, a predominance response of cytophilic (IgG1 and IgG3) antibodies was observed, which showed an increase after vaccination when compared to the values from beginning of the study. The authors concluded that the vaccine candidate MSP3-LSP is promising in terms of immunogenicity and tolerance[37].

MSP3-GLURP (GMZ2)

GMZ2 belongs to the second class of vaccine candidates for malaria. It consists of a recombinant protein fusion, containing two *Plasmodium falciparum* blood-stage antigens, GLURP and MSP3[38-43]. In 2009, Esen *et al*.[38] published the results of a phase Ia study with GMZ2 using aluminum hydroxide as the adjuvant. European adults were divided into three groups determined by three different doses of GMZ2. The authors observed that all the doses were well-tolerated among the 30 immunized participants. Furthermore, all participants, except for one individual, had a substantial increase in antigen-specific antibodies, with peaks on days 56 or 84 after immunization. Both anti-GLURP and MSP3 antibody levels increased similarly after the third immunization. Regarding the duration of the anti-GMZ2 response, antibodies were still detectable after one year at significantly high levels in all groups, although antibody titers were considerably lower when compared to the ones from days 56 and 84. Nonetheless, the duration of the response indicates that memory B-cells may contribute to the long-term response to the vaccine. As such, the authors concluded that GMZ2 is safe and immunogenic and should be further evaluated as a vaccine[38].

In a study in 2010, Mordmüller *et al*.[39] carried out a survey with 40 adults from the region of Lambarene, Gabon, to investigate the safety and immunogenicity of GMZ2 in adults exposed to malaria. Participants were randomly assigned to receive either a dose of GMZ2 or the rabies vaccine, which was used as a control. Blood samples from immunized participants were followed-up for one year. After the three immunizations with GMZ2, the subjects showed a significant increase in anti-GMZ2 antibody levels, when compared to the control group. On day 365 (one year after vaccination), no significant difference was detected between subjects vaccinated with GMZ2 or with rabies

vaccine (control). They also observed a significant increase of IgG1 and IgG3 subclasses after vaccination with GMZ2, which was not observed for IgG2 and IgG4 subclasses. As for the duration of the response, on day 365, 26 of the 39 (66.7%) subjects still had specific antibodies to the antigen studied. The correlation between vaccine-specific antibodies and memory B cells was not significant. As a result, they concluded that the vaccine is well-tolerated, safe, immunogenic, has no adverse effects, which is encouraging for the continuation of clinical studies[39].

The following year, a randomized phase Ib trial was carried out with the vaccine candidate GMZ2 to observe the immunogenicity and safety of the vaccine in children aged 1 to 5 years from Gabon, in Africa. Children received three doses of GMZ2 at concentrations of 30 µg or 100 µg, or the control vaccine (anti-rabies). When the groups were observed individually, in the GMZ2 group (30 µg), all the subjects showed an increase in anti-GMZ2 antibodies on day 84, while in the other immunized group (100 µg) one individual did not produced antibodies against the vaccine. One year after the first vaccination, the concentration of antibodies for all antigens were similar in the control and in the GMZ2 group (100 µg) to the point that the GMZ group (30 µg) presented slightly higher levels of anti-GMZ2 antibodies when compared to the control group. There was an increase in the memory B-cell response after GMZ2 vaccination; however, no increase in anti-GMZ2 B-cells was observed on day 84 in four children in the group immunized with 30 µg and in three children in the 100 µg group. They concluded that both administered doses of GMZ2 (30 µg and 100 µg) were shown to be immunogenic and safe for children exposed to malaria in the studied region[40].

A phase IIb study was conducted in 2016 to assess the effectiveness of GMZ2 in children, in Africa. In this study, children aged 12 to 60 months were randomized to receive three doses of GMZ2 at a concentration of 100 µg or the control vaccine (anti-rabies) (Figure 2). Children were observed for six months so that they could measure the incidence of malaria among immunized children. In the group of 868 children who received the three doses of GMZ2/aluminum hydroxide, there were 641 cases of malaria, while in the control group, 867 children received doses of the rabies vaccine, therefore there was an incidence of 720 cases of malaria. When statistically analyzed by the ATP protocol, the vaccine efficacy was 14% adjusted for age and location. In an age-adjusted ITT analysis, the vaccine efficacy was 11.3%. The average anti-GMZ2 antibodies increased by up to 8-fold in children who received three doses of the GMZ2/alum vaccine, and this increase was greater in children in the age group of 1-2 years than in children aged 3-4 years. No evidence of decline in the vaccine efficacy was found in the six-month period; however, there is a need for long-term follow-up to better estimate the duration of protection. Thus, despite inducing a substantial immune response, a sufficiently protective response was not shown, and it is necessary to evaluate a more improved formulation or the use of adjuvants that increase the immunogenicity of the vaccine and the duration of protection[41].

Recently, the same group carried out a 2-year follow-up in children aged 12 to 60 months immunized with GMZ2/alum (100 µg), in a randomized, double-blind phase 2b study, for which they used the rabies vaccine as the control. They observed that the vaccine continued to be well tolerated, without major adverse effects. As for the results, the effectiveness of the vaccine showed variations in accordance with the children's age, and there were slightly less malaria episodes in older children. As for the duration of the response, the vaccine did not show evidence of protection in the second year after vaccination, which indicates a decrease in antibody levels against the vaccine, a fact already observed with other malaria vaccine candidates. However, the authors argue that blood phase antigen vaccines are achievable, for this, the formulations of these vaccines need to be improved, including the choice of antigen and adjuvant to be used[42].

In 2019, Dejon-Agobe *et al.*[43] investigated the effectiveness of the GMZ2 vaccine against *Plasmodium falciparum*. The effectiveness was tested in people immunized with the vaccine and then infected with *Plasmodium falciparum* sporozoites, using a protocol for controlled human malaria infection (CHMI). It resulted in 85% of the volunteers presenting *Plasmodium falciparum* parasitemia and 44% with malaria (parasitemia and symptoms). Regarding the humoral response, the levels of anti-GMZ2 antibodies were high in all three groups immunized with GMZ2 when compared to the control group four weeks after the last immunization. Thus, the basal concentrations of specific antibodies produced during immunization were associated with protection against malaria. The authors concluded that GMZ2 is well tolerated and immunogenic in adults naturally exposed to *Plasmodium falciparum*; however, against CHMI, it did not show sufficient protection, even though baseline levels of vaccine-specific antibodies have been associated with protection[43].

Some studies have investigated the relationship between malaria and diseases caused by helminths, as these infections are coendemic in most regions of sub-Saharan Africa. There is an interaction in the immune responses against both pathogens, which may directly affect the effectiveness of the vaccine[41]. Amoani *et al.*[44] observed the influence of hookworm disease (*Necator americanus*)

and antithelmintic treatment on the response of naturally acquired anti-GMZ2 antibodies and constituent antigens. It was reported that individuals who had coinfections with *Plasmodium falciparum* and hookworm had a significant increase in IgG3 levels against GMZ, compared with the group infected only with *P. falciparum* and the control group. On the other hand, after treatment with the antithelmintic albendazole, there was a reduction in the levels of IgG3 anti-GMZ and anti-GLURP. In line with this result, when analyzed individually, IgM and IgG1 anti-MSP3 levels decreased after deworming. The study did not identify a significant association between coinfections and the increase in specific antibodies to MSP3 in an individual manner[44].

Evaluating the influence of helminth infections on the response to GMZ2 vaccine, Nouatin *et al*.[45] observed the effect of *Schistosoma haematobium* and soil-transmitted helminths (STH) on the occurrence of malaria after CHMI (controlled human malaria infection). Anti-GMZ IgG levels were higher in individuals infected with *S. haematobium* than in those infected with *S. stercoralis*. Taking these results into account, the study demonstrated that, depending on the species of helminth, there is a difference in the influence on a specific response after the application of the malaria vaccine candidate. Thus, the results suggest that helminth infections affect the immunogenicity and effectiveness of malaria vaccines[45].

CONCLUSION

Based on the data collected, MSP3-based vaccines, despite being immunogenic, still have major challenges to overcome, i.e., their low protective capacity. Despite successful *in vitro* experiments which demonstrated a potential of MSP3 as an anti-blood stage vaccine constituent, a suitable method to trigger a protective immune response in humans remains to be found. Another aspect to be considered for a future MSP3 vaccine is the concentration of antigens as these concentrations have been observed to influence results in clinical tests. This critical aspect needs to be established for each formulation developed, and would allow a high and effective humoral response against the antigen, without adverse reactions in humans.

New approaches can be employed to improve immunogenicity and efficacy of vaccines containing MSP3, such as the use of viral vectors, DNA/RNA, or liposomal formulations which are currently in use against SARS-CoV-2 infections. Promising new vaccine/adjuvant vehicles such as liposomes and *Bacillus subtilis* spores, and the evaluation of new immunization pathways, such as the nasal mucosal can also bring perspectives for new studies.

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