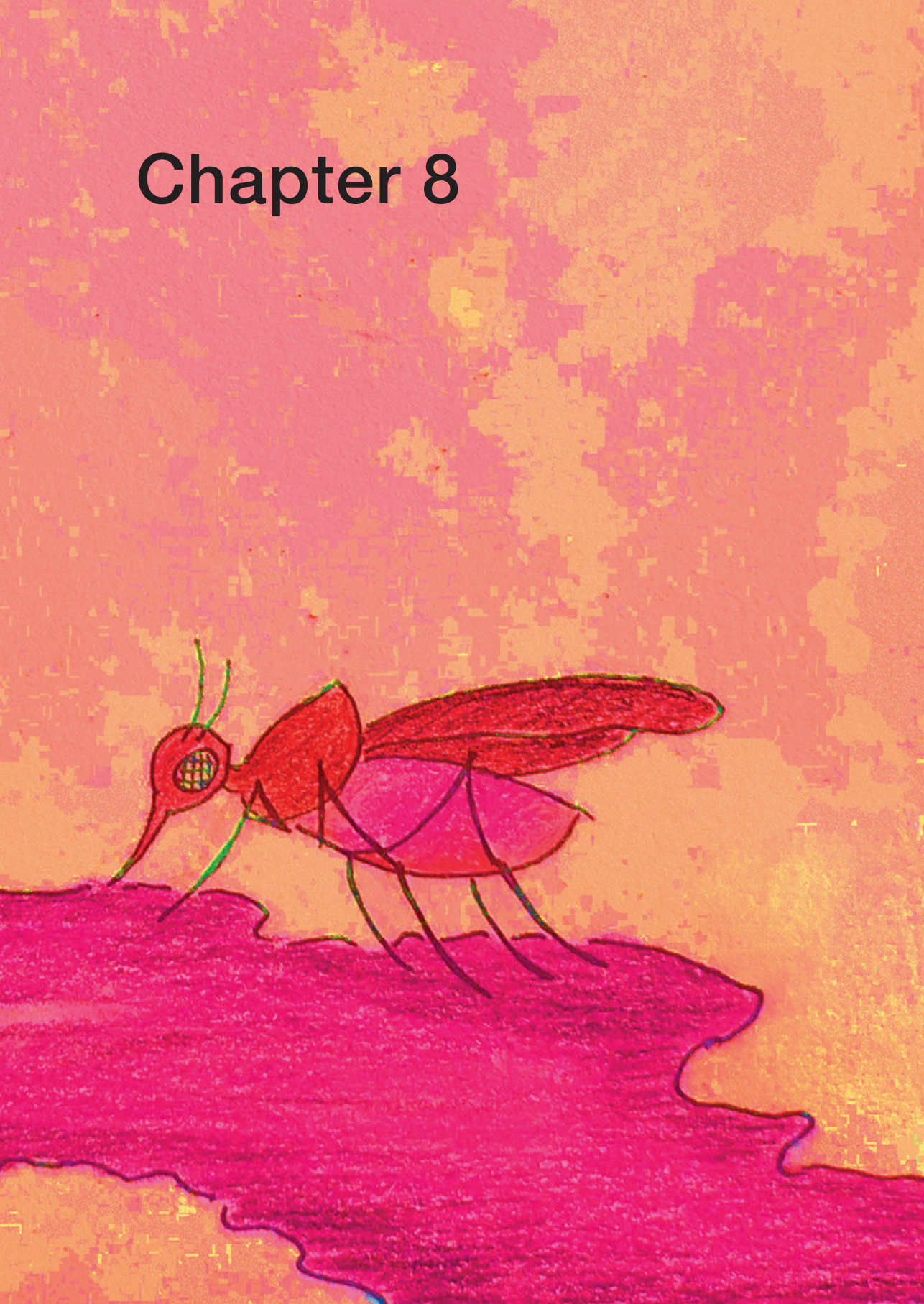
Malaria vaccinology: from the development of a new malaria vaccine to the ethics of clinical trial participation

Lamers, O.A.C.

Chapter 8



Discussion

This thesis describes the development and clinical testing of the late arresting genetically attenuated malaria parasite (GAP) GA2: after confirming its attenuation phenotype in humans, the preliminary protective efficacy of the GAP was assessed against controlled human malaria infection (CHMI) with the homologous parasite strain *Pf3D7*. Additionally, the factors influencing the motivation and decision-making process of medical volunteers taking part in early drug and vaccine studies were analysed, specifically focussing on SARS-CoV-2 vaccine trial participants and analysing the differences between phase 1 clinical trials and controlled human infection (CHI) studies.

GAPs that arrest late in the liver stage of malaria have the potential to increase immunogenicity and protective efficacy. GA2, the first late-arresting GAP to be developed and tested in humans, has shown to be both safe and well-tolerated with high rates of protection in malaria-naïve individuals. While further research is needed to confirm these preliminary findings, GA2 could represent a new tool in the fight against malaria.

The investigation of malaria vaccine candidates relies on clinical testing and hence on the participants who volunteer their time to help the advancement of science. The ethical implications of research involving humans are far-reaching and have been extensively discussed. One of the concerns is that the monetary compensation phase 1 participants receive, may cause them to overlook the risks and dangers of research. However, as outlined in this thesis, medical volunteers have a variety of motivations for participating in clinical trials, which are not all financial in nature. Rather than providing a definitive answer to this complex ethical debate, we would like to highlight the importance of being aware and respecting the value of clinical trial participants' commitment to science.

In this final chapter, we will summarize answers to the following questions:

- Are late-arresting whole sporozoites (WSp) the new frontier of malaria vaccinology?
- What is the importance of the liver stage for the development of immunity against malaria?
- How can a genetically attenuated malaria vaccine be implemented?
- Can the motivations and decision-making process of clinical trial participants be predicted?
- How do controlled human infection studies differ from other clinical research and what are the ethical implications hereof?

Are late-arresting whole sporozoites (WSp) the new frontier of malaria vaccinology?

The quest to develop an effective vaccine against malaria has been ongoing for more than half a century. While whole sporozoite (WSp) vaccines and, more specifically, radiation attenuated sporozoites (RAS), were the first immunization strategy against malaria tested in humans with some success[1,2], the long awaited breakthrough has not yet materialized. Significant advances have been made in the field, including the approval of two subunit vaccines (RTS,S and R21)[3,4] and the development of several alternate WSp vaccine strategies[5-7]. Live sporozoites administered in combination with chemoprophylaxis (CPS) have yielded excellent results, with up to 100% protection of malaria-naïve individuals at low doses[5,8]. The superior protection of CPS compared to RAS highlights the importance of a prolonged development of the attenuated parasite in the host. However, efficacy in endemic regions is variable and logistic challenges complicate the widespread administration of both subunit and WSp vaccines[9]. More specifically, protection by subunit vaccines is closely related to antibody titres and therefore wanes over times as titres decrease as well[4]. In contrast, CPS has shown to induce a more durable immune response[10]. However, CPS also bears the intrinsic risk of exposing healthy individuals to unattenuated parasites[10] and is therefore unsuitable for large-scale administration.

GAPs offer a new hope as they can be made to arrest at various timepoints in the developmental cycle, thereby mimicking the CPS phenotype, without sacrificing safety. To allow for sufficient exposure to the immune system, but avoid disease symptoms, genetic manipulation must occur in the pre-erythrocytic stages of development. Furthermore, parasites need to be attenuated without losing their capacity for sporozoite production and infectivity[11]. Whole genome sequencing of *Plasmodium* allowed for structured investigation and selection of genes[12,13] and for the generation of the first rodent GAPs[12], soon followed by equivalent *Pf* knock-out (KO) parasites[11]. Initially GAPs were early-arresting and, akin to RAS, interrupted development in the first 24-48 hours after administration: despite their safety, their efficacy was lower than desired[6,14-16]. The advent of late-arresting GAPs marks a new generation of WSp vaccine candidates with potentially increased potency, but without the safety concerns of CPS, such as the risks related to the administration of live sporozoites to healthy individuals.

GA2 (*PfΔmei2*), presented in **Chapter 1 and Chapter 5**, is one such late-arresting GAP lacking the *meiosis inhibited 2* (*mei2*) gene, which codes for an RNA binding protein expressed exclusively during the liver stage and involved in DNA segregation. *Mei2* mutants develop normally until the end of the liver stage (6-7 days), but fail to produce functional merozoites[17,18].

As outlined in **Chapter 2**, the development of GAPs for testing in humans was long and at times complicated. Prior to testing in humans, the safety of GA2 was extensively evaluated in pre-clinical models. In cultured human hepatocytes GA2 showed a similar development to wild-type (WT) parasites up until day 9. The GAP was then administered to humanised FRG huHep mice injected with human erythrocytes to assess safety. qPCR analysis on blood taken on days 7 and 9 showed the presence of small amounts of parasitic genetic material, yet in much lower quantities compared to wild type (WT). When blood samples were cultured for 28 days, no parasitic growth was detected. The very small amount of DNA and the lack of growth in culture suggests that the positive qPCR signal was due to the release of aberrant, non-viable merozoites from liver schizonts and not to a blood-stage infection[18].

The murine analogue *PyΔmei2* also induced high protection: immunization of BALB/cJ mice with 1 x 10^4 *PyΔmei2* parasites and subsequent challenge 46 days later with 1 x 10^4 WT parasite resulted in 100% protection, which was maintained when mice were challenged again 56 day later[17] (also see **Chapter 5).**

Given the promising results of the rodent model, safety and preliminary protective efficacy of GA2 were investigated in humans and compared to that of its predecessor GA1 (**Chapter 1**). Even though efficacy of GA1 had already been tested in one previous clinical trial and was known to be limited[6], the set-up of the clinical trial allowed for a direct comparison of an early- (GA1) and late-arresting (GA2) GAP. After safety testing of escalating doses of 15 and 50 mosquito bites in five and the 15 participants, protection by GA2 was assessed against homologous controlled human malaria infection (CHMI). For this purpose, participants were immunized three times, at 28-day intervals, with 50 GA2-or GA1-infected or uninfected mosquito bites. Protection induced by GA2 was 89% and by far exceeded that of its comparators (13% for GA1 and 0% for placebo) and that of previously tested GAPs, which ranged between 0% and 50%[6,16,19].

Subsequent research, described in **Chapter 6**, confirmed the safety of GA2 and investigated its efficacy when administered at lower doses. Ten healthy participants were exposed to a single immunization of 50 GA2-infected mosquito bites and then challenged with five unattenuated mosquito bites six week later. None of the exposed individuals experienced a breakthrough infection and 90% of them were protected against challenge.

These findings unequivocally confirm that late-arresting parasites induce higher protection at much a smaller dose than early-arresting ones. We have shown that the efficacy of WSp vaccines can be improved by prolonging their permanence in the host: extensive exposure to the immune system, wider antigenic variability and increased biomass of late-

arresting parasites are likely the cause of their enhanced immunogenicity. Considering that the duration of the hepatic stage is the most significant difference between early and late-arresting GAPs, our results highlights the importance of the liver for sterile protective immunity against malaria. Going forward, research on WSp vaccines should focus on candidates with a prolonged development, which induce higher protection rates without sacrificing safety and tolerability.

What is the importance of the liver stage for the development of immunity against malaria?

The high protective efficacy of GA2 (89%) described in **Chapter 1** and confirmed in **Chapter 6** provides hope for the future, yet also raises the question of which immunological mechanisms underlie these results. As mentioned previously, the superior efficacy of CPS and late-arresting GA2[5,8], compared to RAS[20] and early-arresting GAPs[6], highlights the importance of the liver stage in achieving immunity against malaria. Indeed, the liver has long been the focus of malaria research because it represents a population bottleneck: sporozoites are thought to spend a short time in the skin and it is only after the release of merozoites into the circulation that parasite replication becomes exponential[21]. Hence, it is not farfetched to assume that an immunological intervention would be most effective in the pre-erythrocytic stage and specifically in the hepatic stage of the *Plasmodium* life-cycle. The first indications that liver stage immunity may be involved in protection against malaria came from mouse experiments, which showed that the adoptive transfer of hepatic mononuclear cells from protected to unprotected animals also transferred protection[22]. Moreover, RAS-immunized non-human primates display high frequencies of $CD4^+$ and $CD8^+$ T cells in liver tissue, even when responses in peripheral blood remain undetectable[23]. Since then, much research has attempted to unravel the complex and often interrelated pathways that define hepatic immunity against malaria. In the following, we will summarize what is known on the topic and attempt to explain which immunological mechanisms could underlie GA2's protective efficacy.

Cellular immunity

T cells have received much attention in the field of malaria research. In **Chapter 1** we described our findings concerning the immunological response of participants immunized with early- and late-arresting GAPs. After stimulating peripheral blood nuclear cells (PBMCs) of participants with infected erythrocytes, we found an increase in *Pf*-specific $CD4^+$ T cells and $\gamma\delta$ T cells expressing the $V\delta2$ chain in GA2- but not GA1-immunized participants. On a single cell level, both $CD4^+$ and $V\delta2^+$ $\gamma\delta$ T cells had an effector-memory phenotype,

producing multiple type 1 cytokines: $CD4^+$ T cells preferentially expressed tumor necrosis factor-α (TNF−α) and interlukin-2 (IL−2), whereas $V\delta2^+$ γδ T cells expressed interferon-γ (IFN−γ) and TNF−α. $CD8^+$, $V\delta2^-$ γδ and natural killer (NK) T cell compartments did not display any *Pf*-specific responses. These findings indicate that GA2 induces memory T cells with a Th1 type phenotype and were largely confirmed in **Chapter 6** as well.

$CD8^+$ T cells

$CD8^+$ T cells have long been hailed as key players in achieving protection against malaria and are thought to detect and eliminate infected hepatocytes[24].

The first experiments identifying a role for $CD8^+$ T cells against malaria date back to the eighties of the previous century: the administration of monoclonal antibodies to block $CD8^+$ T cell in protected mice, abolished such protection, yet this was not true for $CD4^+$ T cells[25]. Besides specificity, the number of $CD8^+$ T cells also matters, as, below a certain threshold, they are unable to confer protection[26].

A recent study found an association between protection in RAS-immunized individuals and a previously unidentified sub-population of $CCR6^+$ $CD8^+$ T cells[27]. Production of IFN-γ and TNFα and the cytotoxic mediators granzyme A and perforin mark these cells early effector memory rather that effector or central memory cells[28]. Similarly, a correlation between activated $CD8^+$ T cells producing granzyme B and protection has been reported after CPS immunization[29,30].

However, these results were difficult to replicate and there are many instances in which protection did not correlate with an increase in $CD8^+$ T cells[5,20,31]. As described in **Chapter 1**, we also did not detect changes in the $CD8^+$ T cell compartment after immunization with GA2. Thus, the idea arose that, upon malaria infection and priming by dendritic cells (DC) in the lymph nodes, $CD8^+$ T cells may relocate to the liver, where they act as tissue-resident memory cells. Additional priming of $CD8^+$ T cells is thought to occur in the liver itself through antigen-presentation by infected hepatocytes[32].

In murine models, the fact that $CD8^+$ T cells do not readily recirculate in parabiotic mice and that abolishing the function of liver-resident T cells also abolishes protection[33], suggests that this subset is liver-resident. Similarly, non-human primates (NHP) intravenously vaccinated with RAS displayed high frequencies of sporozoite-specific and INF-γ-producing $CD4^+$ and $CD8^+$ T cells and even when cellular immune responses were not detected in the periphery, they were abundantly present in the liver[23]. In yet another study with RAS-immunized NHP, the frequency of $CD8^+$ T cells was a hundred times higher in

the liver than in the peripheral circulation: 60% of these liver-resident lymphocytes with an IFN-γ recall response were $CD8^+$ T cells[34].

Given the invasiveness of biopsy procedures, few studies have attempted to directly study the immunological processes occurring in the human liver after malaria vaccination or infection. However, there has been one instance of fine needle aspirates collected from the livers of participants immunized three times with chimpanzee adenovirus 63 (ChAd63) and modified vaccinia virus Ankara (MVA) encoding a multi-epitope string, followed by the pre-erythrocytic thrombospondin related adhesion protein (ME-TRAP), before undergoing CHMI[35]. ChAd63 and MVA encoding ME-TRAP are subunit vaccines designed to activate and attract T cells to the liver by means a prime-boost approach[36]. The researchers identified a population of $CD8^+$ memory T cells that was present in high frequency in hepatic tissue and in low frequency in the peripheral circulation. On the one hand, these results confirmed the liver-residency of malaria-specific $CD8^+$ T cells, on the other they suggested that this subset should also be detectable in the periphery[35]. Why the detection of $CD8^+$ T cells in blood has proven difficult[6,23,37,38] in practice is difficult to say, but could be related to insufficient sensitivity and specificity of the implemented assays. Expanding flow cytometry panels could be one method to increase the chances of detecting more specific cell subtypes.

In conclusion, the lack of $CD8^+$ responses in our samples is in line with findings from previous literature[5,20,31], supporting the idea of a compartmental shift of this cell subset from the circulation to the liver, where they recognize and kill infected hepatocytes. In the future, it would be advisable to use more sensitive techniques to detect $CD8^+$ T cells responses in the peripheral circulation.

$CD4^+$ T cells

$CD4^+$ T cells are thought to play an important role in protection against malaria by acting as direct cytotoxic effector cells, but also as helpers to $CD8^+$ and B cells[39].

Priming and activation probably occurs in lymph nodes upon antigen presentation by DCs, as there is no direct evidence for priming in the liver[40]. Cytokine production by DCs then determines the developmental fate of naïve $CD4^+$ T cells, resulting in a Th1, Th2 or T follicular helper (Tfh) phenotype.

Production of IL-12 by DCs induces Tbet expression by $CD4^+$ T cells and their subsequent development into Th1 cells: pro-inflammatory cytokines, such as IFN-γ, TNF-α and IL-2 have been shown to have anti-parasitic functions. $CD4^+$ T cells of the Th1 subtype may also directly act as effector cells through the production of cytotoxic granules[39].

The role of Th2 cells seems to be limited in malaria, but they are induced by IL-4 production and, in murine models, have been shown to be necessary for the sustained proliferation of $CD8^+$ T cells[41].

Tfh cells are another important subset: induced by IL-6, they are pivotal for the formation of germinal centers and the development of functional B cells. In humans, Tfh cell production is impaired in malaria and polarized towards a Th1 phenotype: T-bet induces atypical memory B cells, which are less efficient at producing antibodies[39,42].

The first studies conducted with RAS looked at cytokine production by PBMCs in general and did not distinguish between lymphocyte subsets. Proliferative responses were observed after incubation of PBMCs with recombinant CSP[43]. Since then, these results have been widely replicated: PBMCs isolated from immunized individuals and incubated with *Plasmodium* sporozoites or asexual blood stages were found to produce proinflammatory cytokines, such as IFN-γ, TNFα and IL-2[38,44,45].When PBMCs were incubated with immortalised cells pulsed with CSP, >95% of cytotoxic cells reacted with OKT4 antibody and were therefore recognized as $CD4^+$ T cells[46]. This was the first indication that $CD4^+$ T cells may play an important role in protection against malaria.

Subsequent studies isolating and focusing on $CD4^+$ T cells specifically found that when presented with unattenuated sporozoites after immunization with WSp, the recall responses of this lymphocyte subset were mostly pro-inflammatory and marked by IFN-γ production[14,23,34,37,47,48]. A recent paper investigating gene expression patterns across RAS-immunized protected and unprotected participants, found that protected participants showed a polarization towards Th1 cells, whereas non protected participants had increased Th2 cells responses[49]. Similar results have been replicated in another study, which found that TNF-α/IFN-γ secreting $CD4^+$ cells were associated with protection in RAS-immunized participants[27].

$CD4^+$ T cells with an effector memory phenotype, expressing IFN-γ, IL-2, or TNF-α, have previously been found to increase after immunization of participants with chemo-attenuated parasites (CPS)[5] and to strongly associate with protection[8,27]. In contrast, unprotected CPS-immunized participants more frequently display $CD4^+$ T cells producing a single cytokine[8,14]. $CD4^+$ T cells expressing the marker of cytotoxic degranulation CD107a with an effector memory phenotype and cytotoxic properties were correlated with protection in yet another CPS immunization study[29].

However, there are also studies that failed to establish a relationship between $CD4^+$ T cells and protection upon vaccination with WSp[6,14]. What accounts for the discrepancy between results is uncertain, but small sample size and low protection rates may have played a role.

Our results align with literature and largely confirm previous findings. The increase of $CD4^+$ T cells with a Th1 phenotype is associated with protection against the disease and thought have anti-parasitic properties. Hence it is likely that at least part of the GA2-induced production is mediated by $CD4^+$ T cells, although it is difficult to say whether that activity is due to the cytotoxic effector functioning or mediated by cytokine production and $CD8^+$ T cell stimulation.

γδ T cells

Another cell subset that has received much attention are γδ T cells. The distinctive feature of γδ T cells is that they have a bridging function between the innate and adaptive immune response, expressing professional antigen-presenting cell markers and even killing opsonized pathogens[50].

It is not known how and where γδ T cells are primed, but the $\delta 2^+$ subset is thought to interact with phosphoantigens produced by *Plasmodium*[51]. It has been further hypothesized that γδ T cells produce INF-γ, TNF-α and M-CSF, possibly activating $CD8^+$ T cells and inducing phagocytic activity by macrophages[52]. It seems that while liver-resident $CD8^+$ T cells induce protection against malaria by killing infected hepatocytes, γδ T cells are required for the activation of said $CD8^+$ T cells. Indeed, abrogation of γδ T cells in RAS-immunized mice led to a reduced accumulation of $CD8^+$ T cells in the liver and nullified protection. However, γδ T cells alone, where not, in the absence of $CD8^+$ T cells, sufficient for protection[53].

Several studies investigating both CPS and RAS immunization, reported that an increased frequency of $V\delta 2^+$ T cells correlated with protection[8,34,53,54]. In a clinical trial, which studied the difference between CPS immunization with a blood-stage active drug (chloroquine) and a liver-stage active drug (pyrimethamine), the frequency of $V\delta 2^+$ T cells was found to be lower in the only unprotected participant in the chloroquine group and dependent on the sporozoite-dose in the pyrimethamine group. Among the participants who had been administered pyrimethamine, expansion of $V\delta 2^+$ T cells in the immunization phase of the trial also correlated with protection[54].

Endemic studies conducted in children as well as adults, have also failed to find an association between γδ T cells and protection after immunization with PfSPZ Vaccine[37,48,55-57].

As to the explanation of these result, different hypotheses have been put forward, including a relative abundance of T regulatory cells and a more tolerogenic immunological microenvironment in these populations. Individuals in malaria endemic regions may have higher percentages of differentiated T cells, leaving behind a smaller pool of naïve T cells

capable of expanding after immunization. These findings align with studies that found that γδ T cells expand after primary infection with malaria, but contract with subsequent exposures[58].

Our results confirm previous findings, by showing that an increase in Vδ2$^+$ T cells in malaria-naïve individuals is correlated with protection against the disease. While their exact role remains unclear, γδ T cells may be important for the recruitment of liver-resident CD8$^+$ T cells.

Humoral immunity

As described in **Chapter 1**, antibody titers against the circumsporozoite protein (CSP) were significantly higher after three immunizations with GA2 compared to baseline, but did not correlate with protection. Titers of anti-apical merozoite antigen 1 (AMA1) and anti-merozoite surface protein 1 (MSP1) antibodies were not increased upon immunization.

A correlation between CSP-antibody titres and protection against malaria has been historically difficult to establish in an unequivocal manner[59]. Still, the sporozoite-neutralizing potential of antibodies has been confirmed *in vitro* and *in vivo*. Incubation of sporozoites with antibodies from immunized participants inhibits their capacity to invade hepatocytes *in vitro*[37,48,60,61]. Similarly, passive transfer of purified antibodies from immunized participants to naïve humanized mice, protected the latter from infection with *Plasmodium* and reduced liver load[34]. Even more to the point, recent studies with monoclonal antibodies have shown that they can prevent more than 80% of homologous infections in malaria-naïve individuals[62,63].

On the other side, it has been suggested that B cell activity and antibody production may be suboptimal in malaria. Only a small subset of antibodies isolated from RAS-immunized protected individuals living in endemic areas have both high affinity and efficacy against sporozoites[64]. The structure of CSP with its many repeat regions make it an unsuitable target for antibody production. CSP can cross-link multiple B cell receptors at the same time, activating B cells without the need of T cell help[65]. This mechanism impairs long-term memory by generating short-lived plasmablasts[66] instead of long-lived plasma cells. Indeed, anti-CSP antibodies produced in response to subunit malaria vaccines, such as RTS,S and R21, decrease more rapidly than those produced after administration of other subunit vaccines (e.g.: tetanus and diphtheria)[67,68]. Similarly, the ability of CSP to cross-link multiple B cell receptors, resulting in strong antigen recognition, may impair affinity maturation[59]. In yet another study it was shown that anti-CSP antibody responses plateau in RAS-immunized individuals, probably due to a negative feedback mechanism and epitope masking, making it impossible to reach protective levels[69].

While antibodies may contribute and, under specific circumstances (high quantity and affinity) may be sufficient to achieve protection[63,70], they are not a reliable correlate of immunity. The impaired production, affinity maturation and persistence of antibodies as well as the large inter-individual variability[59], make it difficult to predict protection based on serum titres alone.

An integrative summary of putative immune responses after GA2 administration

Protection against malaria is a complex multifactorial process that has been difficult to unravel: even though separate elements are known, the bigger picture still eludes us. Based on our current knowledge, we propose the following immunological mechanisms that lead to protection against malaria in general and may underlie the efficacy of GA2 in the current study (**Fig. 1**).

After administration of GA2, naïve $CD8^+$ and $CD4^+$T cells are primed in the skin-draining lymph nodes and the spleen. From here, $CD8^+$ T cells migrate to the liver under the influence of γδ T cells. Proliferation of $CD8^+$ T cells and their differentiation into tissue-resident memory cells is stimulated by further antigen presentation in the liver, which may be enhanced by $CD4^+$ T cells. Once established, liver-resident memory $CD8^+$ T cells exert a cytotoxic function, killing infected hepatocytes. $CD4^+$ T cells may have a similar role, also acting as effector cells. Finally, antibodies may contribute to protection against malaria as well, but for them to work, they need to be present in sufficient amounts and have good binding affinity for their target antigens. While GA2 immunization induced anti-CSP antibodies in our studies, the relatively short time that sporozoites spend in the circulation before moving to the liver suggests that it may be difficult to produce sufficient quality and quantity to fend off an infection. In conclusion, GA2 likely acts on all of the above delineated mechanisms, however, its prolonged permanence in the liver is specifically favorable to the activation of cellular immunity.

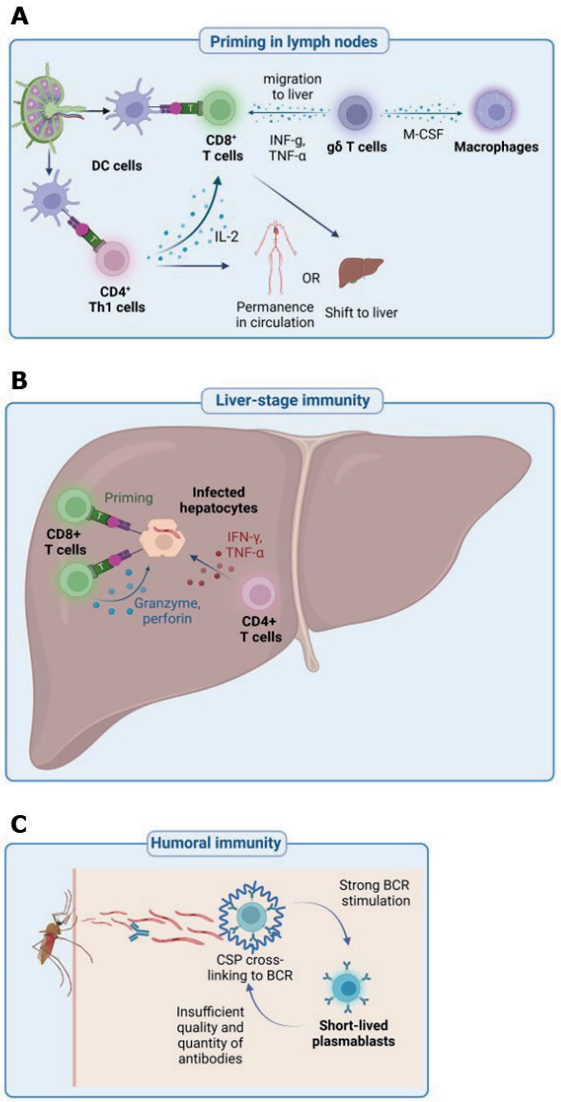


Figure 1. Overview of immunological mechanisms involved in protection against malaria
A. CD8⁺ and CD4⁺ T cells are thought to be primed in skin-draining lymph nodes by DCs. CD4⁺ T cells may act as helper cells to CD8⁺ T cells, producing IL-2 and stimulating proliferation. While it is not clear how and where γδ T cells are primed, they likely have a role in stimulating CD8⁺ T through INF-γ and TNF-α production, also affecting their migration to the liver. γδ T cells may also produce M-CSF, which stimulates macrophages' phagocytic activity .
B. Antigen presentation continues in the liver where infected hepatocytes further prime CD8⁺ T cells. CD8⁺ T cell-dependent killing of infected hepatocytes is perforin- and granzyme- mediated. CD4⁺ T cells are also thought to exert a direct effector function by killing infected hepatocytes.
C. While humoral immunity contributes to protection against malaria, the production of antibodies of sufficient quantity and quality is thought to be impaired. Cross-linking of antibodies instead of binding to target epitopes generates a strong B cell receptor signal which may lead to production of short-lived plasmablasts instead of long-live memory cells.
DC: dendritic cell; IL-2: interleukin 2, INF-γ: interferon-γ; TNF-α: tumor necrosis factor-α, M-CSF: macrophage colony stimulating factor.

How can a genetically attenuated malaria vaccine be implemented?

The good safety profile and relative lack of adverse events reported in **Chapter 1**, warrant the investigation of GA2 as a potential new vaccine candidate.

GA2 efficacy needs to be confirmed with larger numbers: while we did not observe any breakthrough infections of the GAP, it cannot be completely excluded that such an event could occur in a larger sample including more susceptible individuals.

It has been hypothesized that deleting an additional gene could improve GA2's safety: while it could be argued that knocking out more genes is always safer, this strategy does not guarantee success if the deletion targets are poorly chosen or if further genetic manipulation affects immunogenicity. In **Chapter 2** we briefly discuss the late liver-stage arresting replication competent (LARC2), a new GAP that lacks the *liver stage nuclear protein* (*LINUP*) gene in addition to *mei2*. Unlike the murine analogue of GA2 (*PyΔmei2*), administration of 2,5 x 10^5 PyLARC2 sporozoites to highly susceptible BALB/CyJ mice did not cause any breakthrough infections. Protective efficacy of PyLARC2 in mice is also promising and was tested across more conditions than the *PyΔmei2*. Immunization of 10 BALB/cJ mice with 1 x10^4 sporozoites twice at monthly intervals were protected against challenge with 1 x 10^4 WT sporozoites. Intramuscularly immunization of BALB/cJ mice with 2 x 10^4 PyLARC2 sporozoites two times one month apart and subsequent challenge with 1 x 10^4 WT sporozoites resulted in 70% protection and a delay to time to parasitaemia in the unprotected animals. To assess protection over time, BALB/cJ mice were immunized twice, one month apart, with 5 x 10^4 PyLARC2 sporozoites and then challenged six or 12 months with 1 x 10^4 WT sporozoites: protection was 90% in the first group and 60% in the latter[71].

Next the PyLARC2 was tested in outbred mice, which are less responsive to immunization. Twofold immunization at two-month intervals with 5 x10^4 sporozoites and subsequent challenge with 1 x10^3 WT sporozoites two months after the last immunization resulted in 70% protection[71].

Immunization with cryopreserved sporozoites was also attempted: administration of 5 x 10^4 sporozoites at two week intervals followed by challenge with 1 x 10^4 WT sporozoites left all mice protected. Finally, protection of *Py*LARC2 against blood stage infection was tested. After threefold immunization at monthly intervals of BALB/cJ mice with 1 x 10^4 sporozoites, they were injected with lethal *Py* blood stage parasites. All immunized animals were able to completely clear the infection within 12 days[71].

Similar to GA2, when *Pf*LARC2 was tested in FRG HuHep mice injected with human erythrocytes, it was shown to be safe and incapable of producing viable merozoites[71]. Clinical testing will determine its efficacy in humans.

Furthermore, GA2-induced protection needs to hold up in malaria-endemic areas, as almost every WSp vaccine candidate tested to date performed worse in pre-exposed populations[57,72] compared to malaria-naïve ones[5,8,73].

Generally, WSp-induced protection is less robust after heterologous challenge with different *Pf* strains, compared to homologous challenge with the same or a closely related one[5,72]. Genetic variability may allow parasites to evade immunosurveillance[74,75] and accounts for both the slow acquisition of natural immunity and episodes of severe malaria in semi-immune adults after infection with new strains[76].

Another factor influencing vaccine efficacy may be hypo-responsiveness, characterized by pre-existing yet counterproductive immunological mechanisms and chronic inflammation[77].

Pre-existing immunity may be harmful if it causes rapid clearance of the vaccine itself, thereby reducing its immunogenicity[77,78]. Repeated infections with *Plasmodium* or other pathogens may also lead to a state of chronic inflammation which eventually results in immune exhaustion and senescence, skewed immune responses and changes in the lymphoid organs[77]. Exhaustion and senescence refer to the gradual dysfunction and accelerated aging of the immune system, respectively. While these two processes can be mechanistically different, they eventually both result in the decreased reactivity and efficiency of several cell subsets, including NK, B and T cells[77]. In the presence of chronic inflammation, immunological tolerance may develop. While a skewing of the immune response towards a more regulatory type 2 phenotype is necessary to protect from excessive tissue damage, it is less effective at clearing infections[77,79]. Helminth co-infections, which are not uncommon in malaria-endemic regions and preferentially induce a regulatory type 2 immune profile, are negatively associated with vaccine performance[80]. Furthermore, it has been shown that severe malaria infections disrupt normal germinal centre functioning, thereby impairing effective humoral immune responses[81].

If GA2 were indeed to perform worse in pre-exposed populations, one could consider adding adjuvants to sporozoites to make them more immunogenic and boost both innate and adaptive immune responses. One possibility would be co-administer GAPs with compounds that serve as adjuvants and therefore enhance the immune reaction elicited by the vaccine. The drawback is that such substances will most likely be administered independently of the parasites and therefore prompt a systemic, possibly less specific reaction.

Another option would be to genetically modify parasites to express adjuvants on their own: this solution has the potential advantage of a targeted immune response and a more precise delivery of the adjuvant. However, this approach is not easy as it requires the sporozoites to remain viable despite additional manipulations and the immune response to be sufficient for protection yet not so strong as to cause irreversible damage to the host.

The current administration route, namely mosquito bites, represents yet another obstacle: it is not feasible for large-scale implementation and does not comply with the WHO preferred product characteristics[9].

Hence, the production of late-arresting GAPs as an injectable product, as the US-based biotech company Sanaria is currently doing, is a pre-requisite for further vaccine development[82]. Another debate is whether subcutaneous or intramuscular administration of WSp should be preferred over intravenous administration: while intravenous injection of sporozoites is more efficacious[34], subcutaneous and/or intramuscular administration would be more practical[9]. Finally, WSp vaccines require storage in liquid nitrogen at -140°C and careful preparation of the injectable product by trained personnel to avoid contamination and/or infection[83].

Another question that arises is how GA2 would fit in the current panorama of approved malaria-vaccines. Both RTS,S and R21 have been recommended by the WHO for implementation in Africa[84].

RTS,S is a subunit vaccine consisting of CSP expressed with fused and free hepatitis B surface antigen (HBsAg) with the addition of the AS01 adjuvant. In a phase 3 trial, vaccine efficacy at 12 months varied between 55.8% and 31.3% in children (aged 5-12 months) and infants (aged 6-12 weeks), respectively. After three years, vaccine efficacy had decreased to between 28.3%-36.3% in children and to between 25.9% and 18.3% in infants, depending on whether they received a booster vaccine after 20 months or not[4].

R21 is another subunit vaccines that closely resembles RTS,S, but contains less HBsAg and is formulated with the adjuvant Matrix M. The idea is that without the excess HBsAg more CSP would be presented to the immune system[85]. A 2021 study involving 450 children, aged 5-17 months, from a highly endemic region in Burkina Faso were vaccinated three times at four-week intervals followed by a booster 12 months later. All vaccinated children received 5 ug of R21, but two different doses of the adjuvant Matrix M were tested, either 25 ug or 50 ug. Vaccine efficacy was 74% and 77% at six months and 71% and 77% at 12 months in the high and low adjuvant groups, respectively[85]. Results were replicated in a subsequent trial, which enrolled 4644 children, aged five months to

three years, half of them living in regions with seasonal malaria and the other half living in regions with perennial malaria. Immunized participants all received the same dose consisting of 5 µg R21/50 µg Matrix-M and were vaccinated three times at four-week intervals, followed by a booster 12 months later. Vaccine efficacy at 12 months remained high and was 75% at seasonal and 68% at perennial malaria sites[3].

It has been argued that a direct comparison of RTS,S and R21 is difficult and that any judgement about the superiority of one or the other should be suspended. An often criticised point is the dosing: while RTS,S was administered based on child age, R21 was administered just prior to the malaria transmission season, therefore ensuring optimal protection during the most vulnerable period[86]. It should also be noted that only children, aged five months and up, were included in both R21 studies, while the RTS,S phase 3 trial also included infants aged 6-12 weeks. Protective efficacy of RTS,S was lower in the younger age group and the question of R21's efficacy in a similar cohort remains unanswered[87].

Despite the promising results and the enthusiasm with which the WHO-approval of RTS,S and R21 has been greeted, the long-term efficacy and the role of these vaccines in malaria eradication is uncertain[86]. If WSp vaccines were to be approved in the near future, one could hypothesize co-administration with subunit vaccine for optimal activation of humoral and cellular immune responses.

On the other hand, one could also imagine that a population primed by subunit vaccines would have high antibody titres against CSP, which cannot discriminate between WT and attenuated parasites: WSp delivery to the liver could be inhibited or possibly even blocked by high antibody titres. If this were the case, administration of WSp would have to take place instead of or prior to subunit immunization to avoid an antagonistic effect.

Finally, given the success in the fight against SARS-CoV-2 and the possibility of concomitantly presenting multiple antigens, the idea of developing an mRNA vaccine against malaria has recently gained popularity. The question then arises of how mRNA vaccines may compare to WSp vaccines in general and GAPs in particular. While mRNA alone is insufficient to induce $CD8^+$ liver-resident T cells, the formulation of an mRNA lipoplex of the highly conserved PRL6 gene with the modified NK cell-specific agonist α-galactosylceramide$_B$ (αGC_B) showed not only efficient recruitment of $CD8^+$ T cells to the liver but also up to 80% protection in mice. Interestingly, protection was maintained even in pre-exposed mice, signifying that this method can avoid the immune suppression that is often observed in the presence of blood-stage parasitaemia[88]. The complexity of *Plasmodium* favours a vaccination approach that can present multiple antigens and activate several immunological subsets at the same time. The unique possibility of

including many, yet specific, antigens into a single formulation, make mRNA an extremely versatile technology with a distinct advantage over subunit vaccines. The relative ease of production, storage and administration may also favour mRNA vaccines over WSp.

In conclusion, there are two ways to look at GA2 and WSp vaccines in general: either they are considered a viable alternative and addition to currently approved subunit vaccines or they are seen as scientific tools to understand the immune physiology of malaria. While the hope that GA2 may serve to finally strike back against malaria is not unfunded, the many uncertainties and intrinsic drawbacks related to WSp vaccines cannot be omitted either. Hence, the novelty of GA2 lays not only in its excellent protective efficacy but, and maybe more importantly, in its role in proving the importance of the liver stage to achieve protective immunity against malaria.

Can the motivations and decision-making process of clinical trial participants be predicted?

Clinical trials would not be possible without the participation of healthy individuals, who volunteer to be the first on whom new drugs are tested. Since participants of such trials experience no personal benefit except for a financial compensation, some have questioned the undue influence of economical remuneration on the participants' judgement[89,90]. In order to shed light on the matter, we investigated the motivations of participants of SARS-CoV-2 vaccination studies.

As outlined in **Chapter 3**, we found that SARS-CoV-2 participants were mostly motivated by the desire to advance science and help people in The Netherlands. While mixed motivations have been reported in the past[91], the altruistic tendencies were much more pronounced in the current case. The question then arises of what prompted SARS-CoV-2 study participants to behave differently than other medical volunteers. In order to answer this question, we developed a model, which delineates three factors that play a pivotal role in the decision-making process of clinical trial participants, namely rewards, the costs of inaction, and the risks of participation. In the case of SARS-CoV-2 vaccine trials, the rewards were both monetary and personal, as participants were vaccinated ahead of the rest of the population; the cost of inaction was high, because the pandemic affected every member of society; and finally the risks were deemed low by the participants (as assessed in a survey). The favorable combination of these three factors may explain the eagerness of SARS-CoV-2 vaccine trial participants and their relative disregard for financial incentives.

It may also be argued that participants were motivated by true altruism, that is, the genuine desire to help others in the absence of personal gain and even in the face of personal damage. However, the concept of altruism is a complicated one, and it has been shown that most cases of situational altruism are not truly so, as individuals experience some type of advantage from their actions[92]. To test whether the desire to help others and contribute to science is an intrinsic characteristic of SARS-CoV-2 vaccine trial participants, it would be interesting to see whether they maintain their altruistic inclinations in other circumstances as well or whether they were restricted to the pandemic alone.

Altruistic behavior can also be explained in terms of evolutionary advantages if it increases the chances of preserving direct offspring or related individuals[93]. Hence, the pandemic, a situation that threatened the survival of a large number of individuals, may have stimulated evolutionary altruism. This concept is much less applicable to "regular" phase 1 clinical trials, as any studied disease will only ever affect a subset of the population.

The presented model provides a method for assessing the motivations of clinical trial participants and their ethical implications, which may vary depending on the circumstances. The undue influence of financial compensation is an example of how the model could be useful: the importance of economic remuneration for an individual's decision to participate in a clinical trial may depend on what other factors are at play. Hence, while concerns about the undue influence of remuneration may be valid in a scenario where financial compensation is the main reward (i.e.: regular phase 1 clinical trial participants), they may not be applicable in others where participation is associated with other advantages (i.e.: SARS-CoV-2 vaccination trial).

In conclusion, the SARS-CoV-2 pandemic represented an exceptional situation, which made for a very interesting case study. While findings presented here may appear situational, the theoretical model we developed to explain the behavior of clinical trial participants transcends specific circumstances.

How do controlled human infection studies differ from other clinical research and what are the ethical implications hereof?

CHI studies may evoke a negative emotional response and therefore questions surrounding their social value, more so than phase 1 clinical trials[94]. Negatively influencing the perception of CHI studies is the fact that they are generally associated with potentially dangerous pathogens, whereas phase 1 clinical trials are more often referenced in the context of therapeutic drugs. This may lead to the misunderstanding that participants of phase 1 clinical trials may gain direct or indirect benefit from their participation,

whereas CHI study participants may only suffer the negative consequences. However, this therapeutic misconception has been addressed previously: clinical trial participants, whether it be a phase 1 or CHI study, do not by definition stand to gain benefit from their participation[95]. Additionally, the confluence of medical care and research has been debated in the past. Research ethics is distinct from medical ethics, because research has the scope of gaining knowledge rather than benefitting any (patient) population[96].

Finally, some might say that the social value of CHI studies is doubtful, as they are not strictly necessary for the development and approval of new drugs. Standard phase 3 field trials, such as those carried out to test efficacy of SARS-CoV2 vaccines[97], could replace CHI studies. In contrast, phase 1 clinical trials are indispensable for safety assessment of new products[98]. However, given the rising risk of new pandemics, CHI studies and the associated benefit of time- and cost-effectiveness, will become more relevant in the future. Additionally, CHI studies are often the only alternative for studying treatable diseases that cannot reliably be tested in animals first. Another instance in which CHI studies may be preferable to other types of clinical investigation is when disease incidence is too low for regular clinical testing[99].

It has also been argued that CHI studies should be judged by means of a different set of ethical and regulatory criteria than other types of clinical research. Since CHI studies involve the deliberate infection of healthy individuals with pathogens, risk to the participants themselves and to bystanders can be perceived as characteristics that set them apart from other clinical research. However, as delineated in **Chapter 4,** we argue that this is not the case and that CHI studies do not fundamentally differ from regular phase 1 clinical trials.

Phase 1 clinical trials generally involve a remote risk of unknown and potentially serious adverse events[100-102], whereas the risks related to CHI studies are predictable and treatable[103].

Linked to the perceived risk to the participants themselves is the bystander risk, meaning that someone who is not participating in a CHI study could inadvertently be infected as well: while possible in CHI studies, disease spread to third parties is not applicable to phase 1 clinical trials[104].

Hence, we identified risk to bystanders as a factor that may set CHI studies apart from phase 1 clinical trials and therefore requires additional attention. To facilitate the evaluation and assessment of CHI studies in the future, we provide a set of questions that researchers may ask themselves when designing such a trial. While these are not meant

as a replacement for the judgement of a medical ethical committee, they may provide additional guidance in study design.

Concluding remarks

This discussion provides an overview of the current state of GAPs, including the potential immunological mechanisms underlying protection, describing a way forward towards their implementation as malaria vaccines on a larger scale. It also focuses on the motivations of clinical trial participants and on the ethical difference between phase 1 clinical trials and CHI studies.

Late-arresting GAP GA2 combines the advantages of previous immunization methods employing attenuated parasites, namely the safety of RAS and the effectiveness of CPS. GA2's high protective efficacy of 89% warrants further investigation as a potential vaccine candidate. Yet GA2 is also interesting from an immunological perspective as it unequivocally demonstrates the importance of the liver stage to achieve protection against malaria.

None of the research contained in this thesis would have been possible without the participation of medical volunteers. Understanding the motivations of clinical trial participants is important to protect their rights and safeguard the ethical conduct of clinical research. We found that the SARS-CoV-2 pandemic stimulated behavior aimed at helping others and contributing to science, possibly because of the moral proximity of the issue at hand.

In conclusion, this thesis provides an integrated approach to the elimination of infectious diseases, such as malaria, from preclinical research to clinical testing and participant involvement.

References

1 Nussenzweig, R. S., Vanderberg, J., Most, H. & Orton, C. Protective immunity produced by the injection of x-irradiated sporozoites of plasmodium berghei. *Nature* **216**, 160–162 (1967). https://doi.org/10.1038/216160a0

2 Herrington, D. A. *et al.* Human studies with synthetic peptide sporozoite vaccine (NANP)3-TT and immunization with irradiated sporozoites. *Bull World Health Organ* **68 Suppl**, 33–37 (1990).

3 Datoo, M. S. *et al.* Safety and efficacy of malaria vaccine candidate R21/Matrix-M in African children: a multicentre, double-blind, randomised, phase 3 trial. *Lancet* **403**, 533–544 (2024). https://doi.org/10.1016/S0140-6736(23)02511-4

4 Tinto, H. *et al.* Efficacy and safety of RTS,S/AS01 malaria vaccine with or without a booster dose in infants and children in Africa: final results of a phase 3, individually randomised, controlled trial. *Lancet* **386**, 31–45 (2015). https://doi.org/10.1016/S0140-6736(15)60721-8

5 Roestenberg, M. *et al.* Protection against a Malaria Challenge by Sporozoite Inoculation. *New Engl J Med* **361**, 468–477 (2009). https://doi.org/DOI 10.1056/NEJMoa0805832

6 Roestenberg, M. *et al.* A double-blind, placebo-controlled phase 1/2a trial of the genetically attenuated malaria vaccine PfSPZ-GA1. *Sci Transl Med* **12**, 1–9 (2020). https://doi.org/10.1126/scitranslmed.aaz5629

7 Spring, M. *et al.* First-in-human evaluation of genetically attenuated Plasmodium falciparum sporozoites administered by bite of Anopheles mosquitoes to adult volunteers. *Vaccine* **31**, 4975–4983 (2013). https://doi.org/10.1016/j.vaccine.2013.08.007

8 Mordmuller, B. *et al.* Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. *Nature* **542**, 445–449 (2017). https://doi.org/10.1038/nature21060

9 WHO. Malaria vaccines: preferred product characteristics and clinical development considerations. (World Health Organization, Geneva, 2022).

10 Roestenberg, M. *et al.* Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. *Lancet* **377**, 1770–1776 (2011). https://doi.org/10.1016/S0140-6736(11)60360-7

11 Khan, S. M., Janse, C. J., Kappe, S. H. I. & Mikolajczak, S. A. Genetic engineering of attenuated malaria parasites for vaccination. *Curr Opin Biotech* **23**, 908–916 (2012). https://doi.org/10.1016/j.copbio.2012.04.003

12 Mueller, A. K., Labaied, M., Kappe, S. H. I. & Matuschewski, K. Genetically modified parasites as a protective experimental malaria vaccine. *Nature* **433**, 164–167 (2005). https://doi.org/10.1038/nature03188

13 Gardner, M. J. *et al.* Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature* **419**, 498–511 (2002). https://doi.org/10.1038/nature01097

14 Seder, R. A. *et al.* Protection Against Malaria by Intravenous Immunization with a Nonreplicating Sporozoite Vaccine. *Science* **341**, 1359–1365 (2013). https://doi.org/10.1126/science.1241800

15 Kublin, J. G. *et al.* Complete attenuation of genetically engineered Plasmodium falciparum sporozoites in human subjects. *Sci Transl Med* **9** (2017). https://doi.org/10.1126/scitranslmed.aad9099

16 Murphy, S. C. *et al.* A genetically engineered Plasmodium falciparum parasite vaccine provides protection from controlled human malaria infection. *Sci Transl Med* **14** (2022). https://doi.org/ARTN eabn970910.1126/scitranslmed.abn9709

17 Dankwa, D. A., Davis, M. J., Kappe, S. H. I. & Vaughan, A. M. A Plasmodium yoelii Mei2-Like RNA Binding Protein Is Essential for Completion of Liver Stage Schizogony.

Infect Immun **84**, 1336–1345 (2016). https://doi.org/10.1128/Iai.01417-15

18 Franke-Fayard, B. *et al.* Creation and preclinical evaluation of genetically attenuated malaria parasites arresting growth late in the liver. *Npj Vaccines* **7**, 1–17 (2022). https://doi.org/ARTN 13910.1038/s41541-022-00558-x

19 Reuling, I. J. *et al.* An open-label phase 1/2a trial of a genetically modified rodent malaria parasite for immunization against Plasmodium falciparum malaria. *Sci Transl Med* **12** (2020). https://doi.org/10.1126/scitranslmed.aay2

20 Hoffman, S. L. *et al.* Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites. *J Infect Dis* **185**, 1155–1164 (2002). https://doi.org/10.1086/339409

21 Holz, L. E., Fernandez-Ruiz, D. & Heath, W. R. Protective immunity to liver-stage malaria. *Clin Transl Immunology* **5**, e105 (2016). https://doi.org/10.1038/cti.2016.60

22 Guebre-Xabier, M., Schwenk, R. & Krzych, U. Memory phenotype CD8 T cells persist in livers of mice protected against malaria by immunization with attenuated sporozoites. *Eur J Immunol* **29**, 3978–3986 (1999). https://doi.org/Doi 10.1002/(Sici)1521-4141(199912)29:12<3978::Aid-Immu3978>3.0.Co;2-0

23 Epstein, J. E. *et al.* Live Attenuated Malaria Vaccine Designed to Protect Through Hepatic CD8(+) T Cell Immunity. *Science* **334**, 475–480 (2011). https://doi.org/10.1126/science.1211548

24 Lefebvre, M. N. & Harty, J. T. You Shall Not Pass: Memory CD8 T Cells in Liver-Stage Malaria. *Trends Parasitol* **36**, 147–157 (2020). https://doi.org/10.1016/j.pt.2019.11.004

25 Weiss, W. R., Sedegah, M., Beaudoin, R. L., Miller, L. H. & Good, M. F. Cd8+ T-Cells (Cytotoxic/Suppressors) Are Required for Protection in Mice Immunized with Malaria Sporozoites. *P Natl Acad Sci USA* **85**, 573–576 (1988). https://doi.org/DOI 10.1073/pnas.85.2.573

26 Schmidt, N. W. *et al.* Memory CD8 T cell responses exceeding a large but definable threshold provide long-term immunity to malaria. *Proc Natl Acad Sci U S A* **105**, 14017–14022 (2008). https://doi.org/10.1073/pnas.0805452105

27 Mura, M. *et al.* Immunoprofiling Identifies Functional B and T Cell Subsets Induced by an Attenuated Whole Parasite Malaria Vaccine as Correlates of Sterile Immunity. *Vaccines (Basel)* **10** (2022). https://doi.org/10.3390/vaccines10010124

28 Kondo, T., Takata, H. & Takiguchi, M. Functional expression of chemokine receptor CCR6 on human effector memory CD8+ T cells. *Eur J Immunol* **37**, 54–65 (2007). https://doi.org/10.1002/eji.200636251

29 Bijker, E. M. *et al.* Cytotoxic Markers Associate With Protection Against Malaria in Human Volunteers Immunized With Plasmodium falciparum Sporozoites. *Journal of Infectious Diseases* **210**, 1605–1615 (2014). https://doi.org/10.1093/infdis/jiu293

30 Murphy, S. C. *et al.* PfSPZ-CVac efficacy against malaria increases from 0% to 75% when administered in the absence of erythrocyte stage parasitemia: A randomized, placebo-controlled trial with controlled human malaria infection. *PLoS Pathog* **17**, e1009594 (2021). https://doi.org/10.1371/journal.ppat.1009594

31 Hoffman, S. L. & Doolan, D. L. Malaria vaccines-targeting infected hepatocytes. *Nat Med* **6**, 1218–1219 (2000). https://doi.org/10.1038/81315

32 Cockburn, I. A. & Zavala, F. Dendritic cell function and antigen presentation in malaria. *Curr Opin Immunol* **40**, 1–6 (2016). https://doi.org/10.1016/j.coi.2016.01.010

33 Fernandez-Ruiz, D. *et al.* Liver-Resident Memory CD8(+) T Cells Form a Front-Line Defense against Malaria Liver-Stage Infection. *Immunity* **45**, 889–902 (2016). https://doi.org/10.1016/j.immuni.2016.08.011

34 Ishizuka, A. S. *et al.* Protection against malaria at 1 year and immune correlates following PfSPZ vaccination. *Nat Med* **22**, 614–623 (2016). https://doi.org/10.1038/nm.4110

35 Noe, A. *et al.* Deep Immune Phenotyping and Single-Cell Transcriptomics Allow Identification of Circulating TRM-Like Cells Which Correlate With Liver-Stage Immunity and Vaccine-Induced Protection From Malaria. *Front Immunol* **13** (2022). https://doi.org/ARTN 79546310.3389/fimmu.2022.795463

36 Hodgson, S. H. *et al.* Evaluation of the efficacy of ChAd63-MVA vectored vaccines expressing circumsporozoite protein and ME-TRAP against controlled human malaria infection in malaria-naive individuals. *J Infect Dis* **211**, 1076–1086 (2015). https://doi.org/10.1093/infdis/jiu579

37 Jongo, S. A. *et al.* Safety, Immunogenicity, and Protective Efficacy against Controlled Human Malaria Infection of Plasmodium falciparum Sporozoite Vaccine in Tanzanian Adults. *Am J Trop Med Hyg* **99**, 338–349 (2018). https://doi.org/10.4269/ajtmh.17-1014

38 Epstein, J. E. *et al.* Protection against Plasmodium falciparum malaria by PfSPZ Vaccine. *JCI Insight* **2**, 1–14 (2017). https://doi.org/10.1172/jci.insight.89154

39 Perez-Mazliah, D. & Langhorne, J. CD4T-cell subsets in malaria: TH1/TH2 revisited. *Front Immunol* **5**, 1–8 (2015). https://doi.org/ARTN 67110.3389/fimmu.2014.00671

40 Kumar, R., Loughland, J. R., Ng, S. S., Boyle, M. J. & Engwerda, C. R. The regulation of CD4(+) T cells during malaria. *Immunol Rev* **293**, 70–87 (2020). https://doi.org/10.1111/imr.12804

41 Carvalho, L. H. *et al.* IL-4-secreting CD4+ T cells are crucial to the development of CD8+ T-cell responses against malaria liver stages. *Nat Med* **8**, 166–170 (2002). https://doi.org/10.1038/nm0202-166

42 Partey, F. D. *et al.* Atypical memory B cell frequency correlates with antibody breadth and function in malaria immune adults. *Sci Rep* **14**, 4888 (2024). https://doi.org/10.1038/s41598-024-55206-2

43 Herrington, D. *et al.* Successful immunization of humans with irradiated malaria sporozoites: humoral and cellular responses of the protected individuals. *Am J Trop Med Hyg* **45**, 539–547 (1991). https://doi.org/10.4269/ajtmh.1991.45.539

44 Lyke, K. E. *et al.* Multidose Priming and Delayed Boosting Improve PfSPZ Vaccine Efficacy against Heterologous P. falciparum Controlled Human Malaria Infection. *Clin Infect Dis* (2020). https://doi.org/10.1093/cid/ciaa1294

45 Hickey, B. *et al.* IMRAS-A clinical trial of mosquito-bite immunization with live, radiation-attenuated P. falciparum sporozoites: Impact of immunization parameters on protective efficacy and generation of a repository of immunologic reagents. *PLoS ONE* **15**, e0233840 (2020). https://doi.org/http://dx.doi.org/10.1371/journal.pone.0233840

46 Moreno, A. *et al.* Cytotoxic CD4+ T cells from a sporozoite-immunized volunteer recognize the Plasmodium falciparum CS protein. *Int Immunol* **3**, 997–1003 (1991). https://doi.org/10.1093/intimm/3.10.997

47 Lyke, K. E. *et al.* Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection. *P Natl Acad Sci USA* **114**, 2711–2716 (2017). https://doi.org/10.1073/pnas.1615324114

48 Jongo, S. A. *et al.* Increase of dose associated with decrease in protection against controlled human malaria infection by PfSPZ Vaccine in Tanzanian adults. *Clin Infect Dis* **71**, 2849–2857 (2019). https://doi.org/10.1093/cid/ciz1152

49 Du, Y. *et al.* Systems analysis of immune responses to attenuated P. falciparum malaria sporozoite vaccination reveals excessive inflammatory signatures correlating with impaired immunity. *PLoS Pathog* **18**, e1010282 (2022). https://doi.org/10.1371/journal.ppat.1010282

50 Junqueira, C. *et al.* gammadelta T cells suppress Plasmodium falciparum blood-stage infection by direct killing and phagocytosis. *Nat Immunol* **22**, 347–357 (2021). https://doi.org/10.1038/s41590-020-00847-4

51 Deroost, K. & Langhorne, J. Gamma/Delta T Cells and Their Role in Protection Against Malaria. *Front Immunol* **9** (2018). https://doi.org/10.3389/fimmu.2018.02973

52 Minkah, N. K. & Kappe, S. H. I. Malaria Immunity: The Education of an Unnatural Response. *Cell Host & Microbe* **25**, 479–481 (2019). https://doi.org/10.1016/j.chom.2019.03.018

53 Zaidi, I. *et al.* gammadelta T Cells Are Required for the Induction of Sterile Immunity during Irradiated Sporozoite Vaccinations. *J Immunol* **199**, 3781–3788 (2017). https://doi.org/10.4049/jimmunol.1700314

54 Mwakingwe-Omari, A. *et al.* Two chemoattenuated PfSPZ malaria vaccines induce sterile hepatic immunity. *Nature* **595**, 289–294 (2021). https://doi.org/10.1038/s41586-021-03684-z

55 Oneko, M. *et al.* Safety, immunogenicity and efficacy of PfSPZ Vaccine against malaria in infants in western Kenya: a double-blind, randomized, placebo-controlled phase 2 trial. *Nat Med* **27**, 1636–1645 (2021). https://doi.org/10.1038/s41591-021-01470-y

56 Sissoko, M. S. *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* **17**, 498–509 (2017). https://doi.org/10.1016/S1473-3099(17)30104-4

57 Sissoko, M. S. *et al.* Safety and efficacy of a three-dose regimen of Plasmodium falciparum sporozoite vaccine in adults during an intense malaria transmission season in Mali: a randomised, controlled phase 1 trial. *Lancet Infect Dis* **22**, 377–389 (2022). https://doi.org/10.1016/S1473-3099(21)00332-7

58 Howard, J. *et al.* Human Vgamma9Vdelta2 T Lymphocytes in the Immune Response to P. falciparum Infection. *Front Immunol* **9**, 2760 (2018). https://doi.org/10.3389/fimmu.2018.02760

59 Cockburn, I. A. & Seder, R. A. Malaria prevention: from immunological concepts to effective vaccines and protective antibodies. *Nature Immunology* **19**, 1199–1211 (2018). https://doi.org/10.1038/s41590-018-0228-6

60 Behet, M. C. *et al.* The Complement System Contributes to Functional Antibody Mediated Responses Induced by Immunization with Plasmodium falciparum Malaria Sporozoites. *Infect Immun* **86** (2018). https://doi.org/ARTNe0092010.1128/IAI.00920-17

61 Walk, J. *et al.* Modest heterologous protection after Plasmodium falciparum sporozoite immunization: a double-blind randomized controlled clinical trial. *Bmc Med* **15** (2017). https://doi.org/ARTN16810.1186/s12916-017-0923-4

62 Lyke, K. E. *et al.* Low-dose intravenous and subcutaneous CIS43LS monoclonal antibody for protection against malaria (VRC 612 Part C): a phase 1, adaptive trial. *Lancet Infect Dis* **23**, 578–588 (2023). https://doi.org/10.1016/S1473-3099(22)00793-9

63 Wu, R. L. *et al.* Low-Dose Subcutaneous or Intravenous Monoclonal Antibody to Prevent Malaria. *N Engl J Med* **387**, 397–407 (2022). https://doi.org/10.1056/NEJMoa2203067

64 Tan, J. *et al.* A public antibody lineage that potently inhibits malaria infection through dual binding to the circumsporozoite protein. *Nat Med* **24**, 401–+ (2018). https://doi.org/10.1038/nm.4513

65 Fisher, C. R. *et al.* T-dependent B cell responses to Plasmodium induce antibodies that form a high-avidity multivalent complex with the circumsporozoite protein. *PLoS Pathog* **13**, e1006469 (2017). https://doi.org/10.1371/journal.ppat.1006469

66 Yam-Puc, J. C. *et al.* Enhanced BCR signaling inflicts early plasmablast and germinal center B cell death. *Iscience* **24**, 102038 (2021). https://doi.org/10.1016/j.isci.2021.102038

67 Hammarlund, E. *et al.* Durability of Vaccine-Induced Immunity Against Tetanus and Diphtheria Toxins: A Cross-sectional Analysis. *Clin Infect Dis* **62**, 1111–1118 (2016). https://doi.org/10.1093/cid/ciw066

68 White, M. T. *et al.* A combined analysis of immunogenicity, antibody kinetics and vaccine efficacy from phase 2 trials of the RTS,S malaria vaccine. *Bmc Med* **12**, 117 (2014). https://doi.org/10.1186/s12916-014-0117-2

69 McNamara, H. A. *et al.* Antibody Feedback Limits the Expansion of B Cell Responses to Malaria Vaccination but Drives Diversification of the Humoral Response. *Cell Host & Microbe* **28**, 572–+ (2020). https://doi.org/10.1016/j.chom.2020.07.001

70 Kayentao, K. *et al.* Subcutaneous Administration of a Monoclonal Antibody to Prevent Malaria. *N Engl J Med* **390**, 1549–1559 (2024). https://doi.org/10.1056/NEJMoa2312775

71 Goswami, D. *et al.* A replication competent Plasmodium falciparum parasite completely attenuated by dual gene deletion. *EMBO Mol Med* **16**, 723–754 (2024). https://doi.org/10.1038/s44321-024-00057-7

72 Coulibaly, D. *et al.* PfSPZ-CVac malaria vaccine demonstrates safety among malaria-experienced adults: A randomized, controlled phase 1 trial. *Eclinicalmedicine* **52** (2022). https://doi.org/ARTN 10157910.1016/j.eclinm.2022.101579

73 Mordmuller, B. *et al.* A PfSPZ vaccine immunization regimen equally protective against homologous and heterologous controlled human malaria infection. *Npj Vaccines* **7**, 100 (2022). https://doi.org/10.1038/s41541-022-00510-z

74 Kidgell, C. *et al.* A systematic map of genetic variation in Plasmodium falciparum. *PLoS Pathog* **2**, e57 (2006). https://doi.org/10.1371/journal.ppat.0020057

75 Sondo, P. *et al.* Genetically diverse Plasmodium falciparum infections, within-host competition and symptomatic malaria in humans. *Sci Rep* **9**, 127 (2019). https://doi.org/10.1038/s41598-018-36493-y

76 Doolan, D. L., Dobano, C. & Baird, J. K. Acquired immunity to malaria. *Clin Microbiol Rev* **22**, 13–36, Table of Contents (2009). https://doi.org/10.1128/CMR.00025-08

77 van Dorst, M. *et al.* Immunological factors linked to geographical variation in vaccine responses. *Nat Rev Immunol* (2023). https://doi.org/10.1038/s41577-023-00941-2

78 Dobano, C. *et al.* Concentration and avidity of antibodies to different circumsporozoite epitopes correlate with RTS,S/ASO1E malaria vaccine efficacy. *Nature Communications* **10** (2019). https://doi.org/ARTN 217410.1038/s41467-019-10195-z

79 Moncunill, G. *et al.* Distinct Helper T Cell Type 1 and 2 Responses Associated With Malaria Protection and Risk in RTS,S/AS01E Vaccinees. *Clin Infect Dis* **65**, 746–755 (2017). https://doi.org/10.1093/cid/cix429

80 Nouatin, O. *et al.* Exploratory analysis of the effect of helminth infection on the immunogenicity and efficacy of the asexual blood-stage malaria vaccine candidate GMZ2. *PLoS Negl Trop Dis* **15**, e0009361 (2021). https://doi.org/10.1371/journal.pntd.0009361

81 Ryg-Cornejo, V. *et al.* Severe Malaria Infections Impair Germinal Center Responses by Inhibiting T Follicular Helper Cell Differentiation. *Cell Rep* **14**, 68–81 (2016). https://doi.org/10.1016/j.celrep.2015.12.006

82 Richie, T. L. *et al.* Sporozoite immunization: Innovative Translational Science to Support the Fight against malaria. *Expert Review of Vaccines* **22**, 964–1007 (2023). https://doi.org/10.1080/14760584.2023.2245890

83 Garcia, C. R., Manzi, F., Tediosi, F., Hoffman, S. L. & James, E. R. Comparative cost models of a liquid nitrogen vapor phase (LNVP) cold chain-distributed

cryopreserved malaria vaccine vs. a conventional vaccine. *Vaccine* **31**, 380–386 (2013). https://doi.org/10.1016/j.vaccine.2012.10.109

84 WHO. *WHO recommends R21/Matrix-M vaccine for malaria prevention in updated advice on immunization*, <https://www.who.int/news/item/02-10-2023-who-recommends-r21-matrix-m-vaccine-for-malaria-prevention-in-updated-advice-on-immunization> (2023).

85 Datoo, M. S. *et al.* Efficacy of a low-dose candidate malaria vaccine, R21 in adjuvant Matrix-M, with seasonal administration to children in Burkina Faso: a randomised controlled trial. *Lancet* **397**, 1809–1818 (2021). https://doi.org/10.1016/S0140-6736(21)00943-0

86 Birkett, A., Miller, R. S. & Soisson, L. A. The Importance of Exercising Caution When Comparing Results from Malaria Vaccines Administered on the EPI Schedule and on a Seasonal Schedule. *Am J Trop Med Hyg* **107**, 1356–1356 (2022). https://doi.org/10.4269/ajtmh.22-0544a

87 Rts, S. C. T. P. Efficacy and safety of the RTS,S/AS01 malaria vaccine during 18 months after vaccination: a phase 3 randomized, controlled trial in children and young infants at 11 African sites. *PLoS Med* **11**, e1001685 (2014). https://doi.org/10.1371/journal.pmed.1001685

88 Ganley, M. *et al.* mRNA vaccine against malaria tailored for liver-resident memory T cells. *Nat Immunol* **24**, 1487–1498 (2023). https://doi.org/10.1038/s41590-023-01562-6

89 Grady, C., Bedarida, G., Sinaii, N., Gregorio, M. A. & Emanuel, E. J. Motivations, enrollment decisions, and socio-demographic characteristics of healthy volunteers in phase 1 research. *Clin Trials* **14**, 526–536 (2017). https://doi.org/10.1177/1740774517722130

90 Stunkel, L. & Grady, C. More than the money: A review of the literature examining healthy volunteer motivations. *Contemp Clin Trials* **32**, 342–352 (2011). https://doi.org/10.1016/j.cct.2010.12.003

91 Hoogerwerf, M. A., de Vries, M. & Roestenberg, M. Money-oriented risk-takers or deliberate decision-makers: a cross-sectional survey study of participants in controlled human infection trials. *BMJ Open* **10**, 1–8 (2020). https://doi.org/10.1136/bmjopen-2019-033796

92 Fehr, E. & Fischbacher, U. The nature of human altruism. *Nature* **425**, 785–791 (2003). https://doi.org/10.1038/nature02043

93 West, S. A. & Gardner, A. Altruism, spite, and greenbeards. *Science* **327**, 1341–1344 (2010). https://doi.org/10.1126/science.1178332

94 Rid, A. & Roestenberg, M. Judging the social value of controlled human infection studies. *Bioethics* **34**, 749–763 (2020). https://doi.org/10.1111/bioe.12794

95 Miller, F. G. & Joffe, S. Equipoise and the dilemma of randomized clinical trials. *N Engl J Med* **364**, 476–480 (2011). https://doi.org/10.1056/NEJMsb1011301

96 Joffe, S. & Miller, F. G. Bench to bedside - Mapping the moral terrain of clinical research. *Hastings Center Report* **38**, 30–42 (2008). https://doi.org/DOI 10.1353/hcr.2008.0019

97 Falsey, A. R. *et al.* Phase 3 Safety and Efficacy of AZD1222 (ChAdOx1 nCoV-19) Covid-19 Vaccine. *New Engl J Med* **385**, 2348–2360 (2021). https://doi.org/10.1056/NEJMoa2105290

98 Umscheid, C. A., Margolis, D. J. & Grossman, C. E. Key Concepts of Clinical Trials: A Narrative Review. *Postgrad Med* **123**, 194–204 (2011). https://doi.org/10.3810/pgm.2011.09.2475

99 WHO. WHO guidance on the ethical conduct of controlled human infection studies. (Geneva, 2021).

100 Sibille, M., Deigat, N., Janin, A., Kirkesseli, S. & Durand, D. V. Adverse events in phase-I studies: a report in 1015 healthy volunteers. *Eur J Clin Pharmacol* **54**, 13–20 (1998). https://doi.org/10.1007/s002280050413

101 Attarwala, H. TGN1412: From Discovery to Disaster. *J Young Pharm* **2**, 332–336 (2010). https://doi.org/10.4103/0975-1483.66810

102 Brosen, K., Funck-Brentano, C., Kroemer, H. K., Pirmohamed, M. & Schwab, M. Open letter on access to the BIA 10-2474 clinical trial data. *Lancet* **389**, 156–156 (2017). https://doi.org/10.1016/S0140-6736(16)32515-6

103 Roestenberg, M., Hoogerwerf, M. A., Ferreira, D. M., Mordmuller, B. & Yazdanbakhsh, M. Experimental infection of human volunteers. *Lancet Infect Dis* **18**, e312–e322 (2018). https://doi.org/10.1016/S1473-3099(18)30177-4

104 Kimmelman, J. Medical research, risk, and bystanders. *IRB* **27**, 1–6 (2005).