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CHAPTER 1

General introduction

The controlled human infection model

Controlled human infection (CHI) models are an important research tool, where healthy volunteers are experimentally infected with a pathogen. So far models have been established for a broad range of viruses, bacteria and parasites. CHIs can be used for a variety of objectives and are, at the moment, mainly used to investigate the efficacy of new vaccines and drugs.[1] Generally CHI trials are performed using a randomised controlled trial (RCT) as study design. In these RCTs the intervention group receives the new vaccine before the controlled infection, or the new drug after the controlled infection (figure 1) and the control group receives a placebo. Besides testing new vaccines or drugs, CHIs also give the opportunity to study the host-pathogen interaction, to profile immune parameters and to test or optimize new diagnostic parameters.

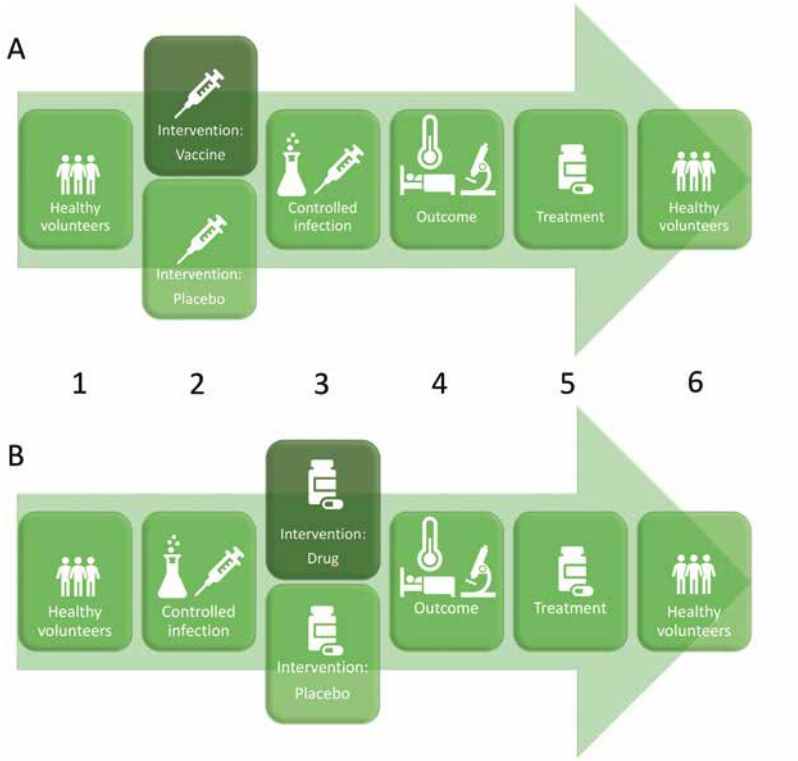

Figure 1. Schematic overview of controlled human infection trials. A. Schematic overview of controlled human infection (CHI) trials for vaccine research or **B.** drug research. Columns represent the different trial stages. **1.** Population, a group of volunteers is being screened to participate in a CHI. **2A.** Intervention, participating volunteers are given a vaccine or placebo. **2B/3A.** Controlled infection, after a predefined period volunteers are deliberately being infected with a pathogen. **B3.** Intervention, participating volunteers are given a drug or a placebo/established vaccine. **4.** Outcome, volunteers are being tested to see whether they developed the disease, historical data can serve as a control group. **5.** Treatment, all volunteers receive pathogen specific treatment.

Over the last decades CHI models are increasingly used.[2] They have accelerated the drug and vaccine development for several pathogens. This led to the development of novel vaccines,[3] but also contributed to stopping the further development of unsuccessful candidates.[4] CHIs are increasingly being used as proof of principle for vaccines before starting phase 2 studies and often act as gatekeeper before moving to large scale phase 2 or 3 field studies in endemic countries.[5]

This proof of principle has several advantages compared to field trials in endemic areas.[5,6] Generally, a low number of volunteers are needed in CHIs to achieve the endpoint, often between 20 and 100, compared to hundreds to 100,000 volunteers in phase II field trials in endemic areas.[2] As a consequence it reduces the unnecessary exposure of potentially vulnerable populations to interventions that might be ineffective. Another advantage is that multiple products can be tested in parallel and the risk of late clinical failure will be minimised (figure 2). Together, this leads to a reduction in the overall costs and a reduced overall risk during development.

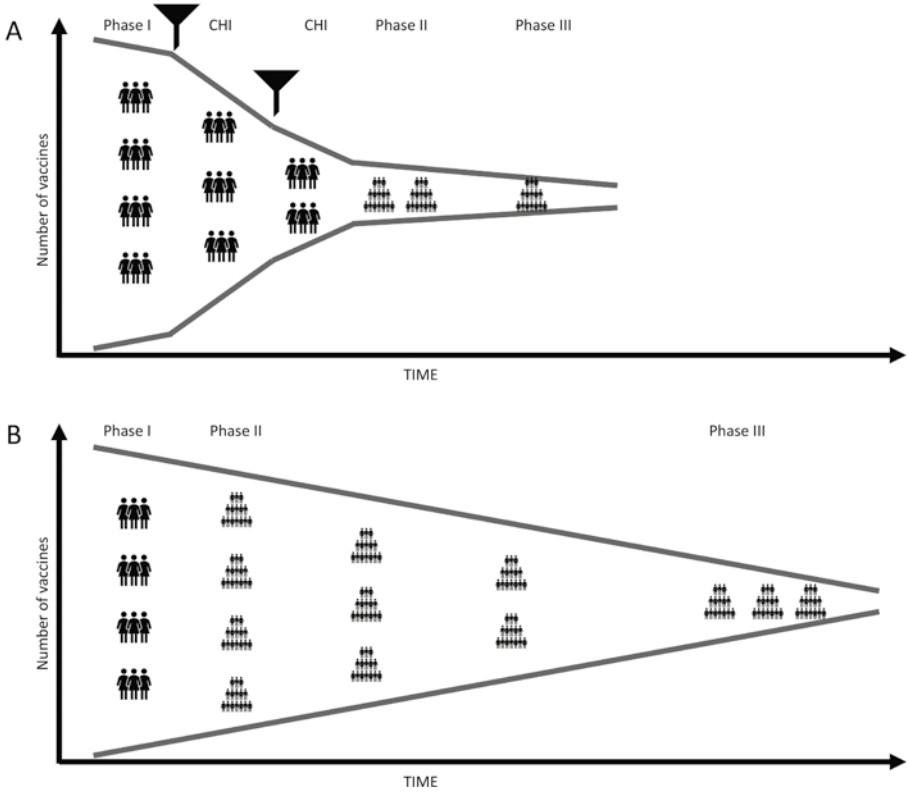

Figure 2. Vaccine development phases. The number of vaccines, the number of volunteers and the time until registration of a vaccine for each phase of clinical development with (**A**) or without (**B**) the use of controlled human infections.

These advantages have led to the development of new CHI models. To be able to design a new model, the specific pathogens should be produced in a controlled process. Preferably this process follows the good manufacturing practice (GMP) guidelines. More important, the expected adverse events in volunteers should be treatable or self-curable and cannot lead to long-term or permanent disabilities. Well-established CHI models can be altered or refined over time, for example when new diagnostic tools to detect the pathogen become available.

Malaria

Malaria is one of the world's most devastating infectious diseases with 219 million cases and over 435,000 deaths in 2017,[7] with most cases and deaths occurring in sub-Saharan Africa. *Plasmodium falciparum* is responsible for most deaths, while the other species, *Plasmodium ovale*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium knowlesi*, mainly cause morbidity. The non-symptomatic liver stage of malaria precedes the symptomatic erythrocytic stage where patients develop symptoms such as fever, malaise, fatigue, headache, myalgia and arthralgia. In severe cases patients can develop neurological complications, severe anemia, coma and eventually they could die.

Effective treatment is available, though it is not always continuous accessible in remote areas. This may lead to anti-malarial drugs being administered too late. In addition, treatment may fail due to resistance of parasites against specific drugs. As a single infection does not induce complete immunity and multiple infections only induce semi-immunity, the need for a vaccine to prevent disease is high. Currently there is one licensed vaccine, RTS,s which is registered as Mosquirix,[8] which has a limited efficacy of 50% in children between 5-17 months old, 14 months after vaccination.[9] A pilot vaccine program with Mosquirix, for children up to 2 years old, started in Malawi, Ghana and Kenya in 2019.[10,11]

Controlled human malaria infections

The infection of volunteers with malaria for various objectives has been performed since the 1940's.[12] Hereafter, the importance of controlled human malaria infection (CHMI) studies was increasingly recognised.

The most common used CHMI study design is the double blind RCT, where both volunteers and research physicians are blinded. In these studies, the administration of several immunisations to healthy volunteers is followed by a controlled infection, the CHMI. During the CHMI sporozoites are generally administered by the bites of *P. falciparum*-infected laboratory reared *Anopheles stephensi* mosquitoes.[13,14] These mosquitoes become infected after the feeding of a blood meal containing *in vitro* cultured *P. falciparum* in erythrocytes.[13,14] However, the CHMI can also be performed by direct venous inoculation of malaria sporozoites.[15]

The use of CHMIs has resulted in several important scientific advances over the past decades in malaria vaccine research. One of the most important is the first proof of efficacy of the first licensed malaria vaccine, Mosquirix.[8] Due to its limited efficacy which wanes over time, it is important to continue vaccine research aiming at a vaccine with a higher efficacy. Preferably a

protective efficacy of more than 75% against clinical malaria and reduction of transmission of the parasite as is proposed in the malaria vaccine technology roadmap.[16]

Whole sporozoite vaccines

In contrast to the vaccine Mosquirix which targets the circumsporozoite protein on sporozoites,[8] new vaccines based on whole sporozoites are being developed. These whole sporozoite vaccines seem to be potent.[17,18] Most of these vaccines are based on the *P. falciparum* strain NF54, originating from a case of airport malaria in the Netherlands,[19] or its daughter strain 3D7.[20]

In 1999, a ground-breaking milestone was accomplished with whole sporozoite immunisation. Full protective immunity in nearly 100% of volunteers was induced after immunisation with radiation attenuated sporozoites (RAS).[17] Another type of whole sporozoite immunisation appeared to be even more potent. Exposure of volunteers to infected mosquito bites under chloroquine chemoprophylaxis (chemoprophylactic sporozoites, CPS) resulted in 100% protection. This potency is probably related to a longer exposure time to parasites.[21] Alongside chemoprophylaxis and RAS-based vaccines, a new type of whole sporozoite vaccines has recently been introduced, based on genetically attenuated parasites (GAPs). With the deletion of parasite genes which are crucial to develop within the liver or crucial to release merozoites into the blood stream, parasite development will arrest in the liver.[22]

Schistosomiasis

Worldwide 252 million people are infected with *Schistosoma*. Infections are mainly caused by *Schistosoma mansoni* and *Schistosoma haematobium*, but can also be caused by *S. japonicum, S. mekongi, S. intercalatum* or hybrid forms. Schistosomiasis is listed by the WHO as one of the main neglected tropical diseases.[23,24] The endemicity of the disease is focal and is determined by the presence of the intermediate host, the fresh water snail. Each of the *Schistosoma* species infects another species of freshwater snails. The infected snails release cercariae, which can penetrate the skin of the definite host. In their host they mature and migrate through several tissues. Adult worms generally reside in the mesenteric venules (*S. mansoni*) or venous plexus of the bladder (*S. haematobium*), where they mate and start producing eggs. The eggs travel through the lumen of the intestine or the bladder of their host and are released with urine of feces. When adult worms accidentally reside in other veins, their eggs will travel through other tissues. When the eggs get trapped in tissue they promote inflammatory responses at their deposition site, which can induce formation of granulomas and fibrosis.[25] Depending on the site this can result in various forms of pathology, such as liver cirrhosis and portal hypertension.[25]

Treatment of schistosomiasis relies on one drug only, praziquantel, of which the reported efficacy varies between 42 and 91% in endemic areas.[26-28] Current control programs rely on mass drug administration (MDA) with praziquantel and are being used to reduce the burden of infection.[29] There is a delay in regular treatment as people develop disease only after years of infections and re-infection. As a consequence of this suboptimal treatment, a vaccine would be an essential tool in the control of schistosomiasis. The WHO suggests that a vaccine should

fully prevent from infection in 40% of people or reduce the worm burden by 40%.[30] However, a more stringent goal of 75% reduction in worm burden has been proposed by the research community.[31] Currently three vaccines against *S. mansoni* are in various stages of (pre-) clinical development: Sm-TSP-2, rSm14/GLA-SE, and Sm-p80.[32-34]

Controlled human *Schistosoma* infections

The development of a controlled human schistosome infection (CoHSI) model would enhance the schistosomiasis vaccine development. It can be used as a tool to select vaccine candidates early in clinical development and consequently prevent late clinical failure.[1,2] Also, this model would allow characterisation of the immune response against schistosomes in the human host. This could give insight in symptoms involved in an acute schistosomiasis, also known as Katayama syndrome, and allows for optimisation of diagnostic assays.

To be able to develop this new infection model it is important that the schistosome life cycle is available. The maintenance of this life cycle and production of cercariae should be regulated in accordance with the principles of good manufacturing practice (GMP). Furthermore, it is important to be able to ensure the safety of volunteers at all times by optimising the infection (cercarial) dose, frequent follow-up control visits, availability of a research physician at all times, adequate diagnostic tools with the possibility to determine whether treatment has been sufficient, and the availability of adequate drug treatment.

Optimizing controlled human infections

The use of healthy volunteers is common in CHI studies. The ethical principles of CHIs are comparable to those of phase 1 trials, where there is a minimal risk, without direct benefit to the volunteers.[6] However, there is a delicate balance between the acceptance of CHI studies by researchers and the community. The central ethical issue seems to lie in the risk of harm to volunteers.[35] It is important to realize that the aim of CHI studies is not to induce harm, but to gain scientific benefit or benefit to society. As a consequence, this model should only be used under strict conditions.[35,36]

An ethical framework, mainly based on the Nuremberg code and declaration of Helsinki,[37,38] has been proposed to evaluate the ethics regarding CHI studies.[5,6,39] In short: 1) clear scientific rationale 2) minimize risks and discomfort to volunteers 3) exclude vulnerable populations 4) clear and thorough informed consent procedure 5) financial compensation for required time and suspected discomfort 6) compensate research-related injury 7) protect the public 8) potential benefit of the research should outweigh harm of the volunteers, and 9) right to withdraw from research.[5,6] It is the ethical obligation of the researcher to ensure the safety of his volunteers.

Scope and outline of this thesis

In this thesis new *P. falciparum* strains were investigated and introduced in CHMI studies. In addition, the CHMI model is used to test the efficacy of a new GAP vaccine, PfSPZ-GA1 vaccine. Besides the use of this well-established CHI model, the design of a new model, the CoHSI model is introduced and the results of the first study performed with this model are presented. Ultimately, we describe how the use of historical controls in CHIs can be used to improve the safety of CHI studies.

The first part of the thesis focusses on CHMI studies.

Chapter 2 provides insight in the liver stage and blood stage development of the new strains, NF135.C8 and NF166.C10, compared to the generally used strain NF54. From previous CMHI studies with NF54 it is known that five infected mosquito bites result in 100% infection rate in non-vaccinated malaria naïve volunteers. In **Chapter 3** the number of infected mosquito bites that are necessary to guarantee a 100% infection rate in non-vaccinated malaria-naïve volunteers after exposure to one of the two new *P. falciparum* strains, NF135.C8 and NF166. C10 are tested. Additionally, in **Chapter 4** the results of the first phase I and CHMI trial with a genetically attenuated parasite (GAP) malaria vaccine administered by direct venous inoculation are shown. At first the safety of the vaccine was tested in a phase I study. When these results were positive a CHMI study followed, where, three immunisations with a GAP vaccine, PfSPZ-GA1 Vaccine, based on NF54 were followed by a homologous, NF54, mosquito bite CHMI.

The second part of the thesis focusses on the development of the CoHSI model

In **Chapter 5** the establishment of male cercarial production for a controlled human schistosome infection model is described. Based on this production the CoHSI model was implemented. In **Chapter 6** we describe the development of Katayama syndrome in two of our volunteers after CoHSI. **Chapter 7** describes the results of the first CoHSI study, including the safety and optimal dose for infection for future studies.

The last part of this thesis focusses on the improvement of implemented CHI models.

In **Chapter 8** we give suggestions to optimize CHI models and to reduce their risk with the use of historical controls.

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I

Possibilities of the controlled human malaria infection model

CHAPTER 2

Infectivity of *Plasmodium falciparum* sporozoites determines emerging parasitemia in infected volunteers.

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Abstract

Malaria sporozoites must first undergo intrahepatic development before a pathogenic blood-stage infection is established. The success of infection depends on host and parasite factors. In healthy human volunteers undergoing controlled human malaria infection (CHMI), we directly compared three clinical *Plasmodium falciparum* isolates for their ability to infect primary human hepatocytes *in vitro* and to drive the production of blood-stage parasites *in vivo*. Our data show a correlation between the efficiency of strain-specific sporozoite invasion of human hepatocytes and the dynamics of patent parasitemia in study subjects, highlighting intrinsic differences in infectivity among *P. falciparum* isolates from distinct geographical locales. The observed heterogeneity in infectivity among strains underscores the value of assessing the protective efficacy of candidate malaria vaccines against heterologous strains in the CHMI model.

Introduction

Malaria infections are initiated by blood-feeding female *Anopheline* mosquitoes injecting *Plasmodium* sporozoites into their mammalian host. Although most of the sporozoites get trapped in the dermis or draining lymph nodes, a small but crucial fraction succeed in penetrating dermal vasculature, whence they are carried into the bloodstream, including eventually the hepatic circulation (1). There they invade the liver parenchyma, traversing numerous cells before a few eventually succeed in invading a permissive hepatocyte where they undergo schizogony (2). Each liver-stage schizont releases an estimated ≥40,000 merozoites (3), which invade erythrocytes, causing blood-stage disease. The respective steps in the preerythrocytic life cycle of the *Plasmodium* parasite initially form a substantial bottleneck and subsequently an enormous multiplication opportunity (4).

However, it is unclear how the success rate of these various preerythrocytic steps ties together to determine the total burden of parasites emerging from the liver and hence the course of pathogenic blood-stage infection. It is also unknown to what extent malaria isolates vary in infectivity with regard to preerythrocytic infection or, conversely, whether humans differ in their intrinsic permissiveness. Addressing the latter issue is central to the rational design of controlled human malaria infection (CHMI) studies for clinical evaluation of candidate malaria vaccines (5). Here, we investigated whether three clinical isolates of *Plasmodium falciparum* differed in their intrinsic ability to accomplish the sequential steps of preerythrocytic development in liver hepatocytes and how this affected the course of blood-stage infection in humans. We studied parasite dynamics both *in vivo* in malaria-naïve volunteers undergoing CHMI and in a recently optimized *in vitro* model using freshly isolated human hepatocytes (6, 7). *In vitro* experiments were performed with sporozoites harvested from exactly the same batches of infected mosquitoes used on the same day for the human infections. Alongside the longstanding laboratory strain NF54 (8) and the recently reported NF135.C10 clone (9), we described the development and use in humans of the *P. falciparum* clone NF166.C8. We showed that intrinsic differences in infectivity existed between *P. falciparum* isolates with regard to their ability to invade and multiply within human hepatocytes *in vitro*. The degree of liver-stage infection correlated directly with the magnitude of the first wave of blood-stage parasites to emerge from the liver *in vivo* and correlated inversely with the prepatent period in CHMI subjects.

Results

Dynamics of parasitemia during CHMI

In total, 23 subjects across two separate CHMI studies were each exposed to sets of five mosquitoes carrying the NF54, NF135.C10, or NF166.C8 isolates of *P. falciparum* (study flowcharts, study subject demographics, and laboratory characteristics of these *P. falciparum* isolates are provided in fig. S1 and tables S1 and S2, respectively). All subjects developed patent

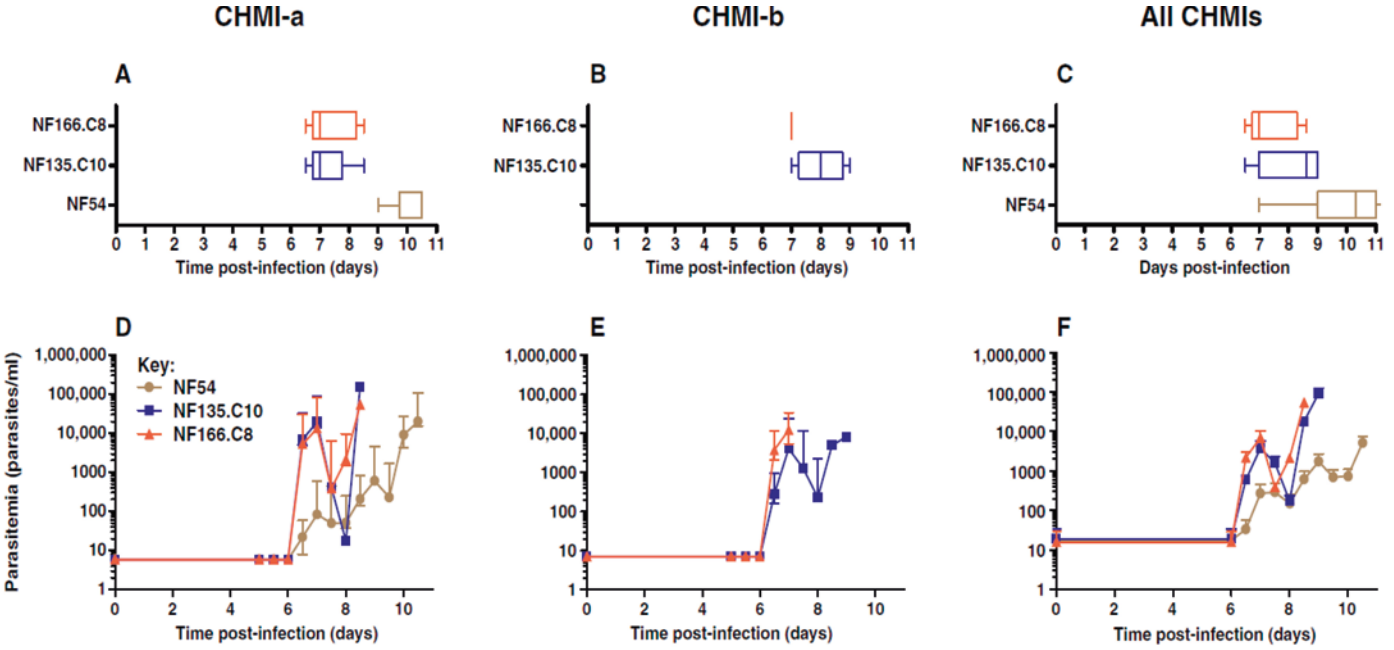

Figure 1. Prepatent period and kinetics of parasitemia in CHMI with three *P. falciparum* strains. (**A**) Prepatent periods in the CHMI-a study (n = 5 per group). (**B**) Prepatent periods in the CHMI-b study (n = 4 per group). All subjects infected with the NF166.C8 strain in the CHMI-b study had prepatent periods of exactly 7.0 days, which is shown as a single line in (B). (**C**) Combined data from all 10 comparable CHMI studies performed at our center using thick smear microscopy as an end point (n = 56 NF54, n = 13 NF135.C10, and n = 5 NF166.C8). Box-and-whisker plots represent median/interquartile range and range per strain. In the CHMI-a study (A) and all studies in (C), prepatent periods were determined by time to positive thick blood smear; in the CHMI-b study (B) and subsequent studies at our center, prepatent periods were determined by time to positive qPCR. (**D** to **F**) Development of submicroscopic parasitemia after infection, as determined retrospectively by qPCR in the CHMI-a study (D) (n = 5 per group), the CHMI-b study (E) (n = 4 per group), and combined data from all 18 CHMI studies at our center using either thick smear microscopy or qPCR as an end point (F) (n = 90 NF54, n = 22 NF135.C10, and n = 14 NF166.C8). Data represent geometric mean ± 95% CI of all pretreatment samples per group per time point. All subjects in the CHMI-a and CHMI-b studies subsequently received a standard course of atovaquone-proguanil treatment and had their parasitemia cleared completely. Brown circles, NF54 strain; blue squares, NF135.C10 strain; red triangles, NF166.C8 strain.

parasitemia and were curatively treated with atovaquone-proguanil once thick smear–positive (first study, CHMI-a) or quantitative real-time fluorescence polymerase chain reaction (qPCR)–positive (second study, CHMI-b). In CHMI-a (Fig. 1A), the prepatent period as determined by thick smear was significantly shorter for subjects infected with either the NF135.C10 clone (mean, 7.2 days; range, 6.5 to 8.5 days) or the NF166.C8 clone (mean, 7.4 days; range, 6.5 to 8.5 days) than for those infected with the NF54 strain of *P. falciparum* (mean, 10.2 days; range, 9.0 to 10.5 days); mean differences were, respectively, 3.0 days [95% confidence interval (CI), 1.7 to 4.3] and 2.8 days (95% CI, 1.5 to 4.1) [$P < 0.001$ for both comparisons, one-way analysis of variance (ANOVA)/Tukey's post hoc test, $n = 5$ per group]. No significant difference existed between NF135.C10 and NF166.C8 (mean difference, 0.2 day; 95% CI, −1.1 to 1.5; $P > 0.05$). In CHMI-b (Fig. 1B), the prepatent period as determined by qPCR criteria did not differ significantly between NF135.C10 (mean, 8.0 days; range, 7.0 to 9.0 days) andNF166.C8 (7.0 days in all four subjects) *P. falciparum* strains (mean difference, 1.0 day; 95% CI, −0.3 to 2.3) ($P = 0.09$, one-sample t test, $n = 4$ per group). The prepatent period observed for NF54 in the CHMI-a study was similar to that of n = 56 malaria-naïve subjects in all 10 of our CHMI studies (mean, 10.3 days; range, 7.0 to 16.0 days), in which treatment was initiated upon positive standardized thick blood smear analysis (table S3). Likewise, the prepatent period for NF135.C10 in CHMI-a was similar to that of $n = 13$ subjects across all 3 of those 10 studies that also included infections with NF135.C10 strain parasites (mean, 8.0 days; range, 6.5 to 9.0 days; table S3). In a meta-analysis of all 10 studies, the prepatent period of NF54 remained significantly ($P < 0.001$) longer than that of either NF135.C10 or NF166.C8 (Fig. 1C).

As determined retrospectively by qPCR in CHMI-a subjects, submicroscopic parasitemia became detectable before thick smear positivity in all subjects. Parasitemia was evident 6.5 days after infection in all NF135.C10-infected and NF166.C8-infected subjects and by day 7.5 (6.5 to 8.5) in the NF54-infected group (mean difference, 1.0 day; 95% CI, 0.1 to 1.9; Fig. 1D and Table 1). The magnitude of the initial wave of parasitemia emerging from the livers of both NF135.C10-infected and NF166.C8-infected subjects was greater compared to that of NF54-infected subjects, although this magnitude did not itself differ significantly between NF135. C10-infected and NF166.C8-infected subjects (Fig. 1D and Table 1). Overall, peak parasitemia per subject before initiation of antimalarial treatment with atovaquone-proguanil did not differ between groups (Table 1).

In CHMI-b, submicroscopic parasitemia was first detected by qPCR on day 6.5 after infection in all four subjects in both the NF135.C10 group and the NF166.C8 group. The magnitude of the initial wave of parasitemia emerging from the liver did not differ between NF135.C10 and NF166.C8 (Fig. 1E and Table 1). Although the initial wave of parasitemia in CHMI-a was slightly lower for the NF54 strain ($P = 0.046$) and somewhat higher for the NF135.C10 strain ($P = 0.0045$), as compared to our other CHMI studies (table S3), the magnitude of the first wave of NF54 parasitemia was around 10-fold lower compared to either NF135.C10 or NF166.C8 parasitemias in a meta-analysis of all 18 of our CHMI studies (Fig. 1F and Table 1).

Table 1. Characteristics of *P. falciparum* parasitaemia in CHMI subjects *in vivo.*

	NF54[1]	NF135.C10[1]	NF166.C8[1]	NF54 vs NF135.C10[2]	NF54 vs NF166.C8[2]	NF135.C10 vs NF166.C8[2]
CHMI-a						
No. of subjects per group	5	5	5			
First detection of parasitemia by qPCR (days after infection)	7.5 (6.5-8.5)	6.5 (6.5-6.5)	6.5 (6.5-6.5)	**1.0 (0.1 to 1.9)***	**1.0 (0.1 to 1.9)***	-
Peak parasitemia (log_{10} para-sites/mL)						
First wave[1]	1.96 (1.23-3.52)	4.28 (3.41-4.79)	3.89 (2.83-4.79)	**-2.32 (-3.69 to -0.96)****	**-1.93 (-3.30 to -0.56)****	0.39 (-0.97 to 1.76)
Overall[2]	4.43 (3.84-5.17)	4.64 (4.24-5.19)	4.22 (2.83-4.79)	-0.20 (-1.19 to 0.78)	0.21 (-0.77 to 1.20)	0.42 (-0.56 to 1.40)
CHMI-b						
No. of subjects per group	-	4	4			
First detection of parasitemia by qPCR (days after infection)	-	6.5 (6.5-6.5)	6.5 (6.5-6.5)	-	-	-
Peak parasitemia (log_{10} para-sites/mL)						
First wave[1]	-	3.61 (2.59-4.19)	4.09 (3.77-4.34)	-	-	-0.48 (-1.63 to 0.66)
Overall[2]	-	3.94 (3.82-4.19)	4.09 (3.77-4.34)			-0.15 (-0.62 to 0.32)
All CHMI studies						
No. of subjects per group[5]	90	22	14			
First detection of parasitemia by qPCR (days post-infection)[5]	7.2 (6.3-10.6)	6.7 (6.5-7.0)	6.5 (6.5-6.5)	0.52 (-0.004 to 1.0)	**0.70 (0.070 to 1.3)***	0.18 (-0.57 to 0.93)

Table 1. ***Continued.***

	NF54[1]	**NF135.C10**[1]	**NF166.C8**[1]	**NF54 vs NF135.C10**[2]	**NF54 vs NF166.C8**[2]	**NF135.C10 vs NF166.C8**[2]
Peak para-sitemia (log_{10} parasites/mL)						
First wave[1,5]	2.72 (1.00-4.67)	3.67 (2.59-4.79)	3.78 (2.78-4.79)	**-0.95 (-1.41 to -0.49)*** **	**-1.06 (-1.61 to -0.51)*** **	-0.11 (-0.79 to 0.58)
Overall[2,6]	4.28 (3.13-5.17)	4.72 (3.35-5.84)	4.22 (2.83-4.97)	**-0.44 (-0.86 to -0.009)***	0.065 (-0.58 to 0.71)	0.50 (-0.23 to 1.23)

[1]Data represent mean per group [range] [2]Data represent mean differences between groups [95% CI of difference]. Bold face indicates statistical significance (*p<0.05, **p<0.01 and ***p<0.001 by 1-way ANOVA/Tukey's post-hoc test). [3]Peak height of parasitaemia during the first wave to emerge from the liver, defined as the highest parasite density measured by qPCR per subject between day 6.5-8.0 post-infection; data represent geometric mean [range] per group. [4]Peak height of parasitaemia per subject at any time post-infection (generally at start of treatment); data represent geometric mean [range] per group. [5]Data included from all 18 comparable CHMI studies performed at our centre using either thick smear microscopy or qPCR as an endpoint (table S3). [6]Data included from only those 10 CHMI studies performed at our centre in which subjects were treated upon positive standardised thick blood smear (n=56 NF54-infected, n=13 NF135.C10-infected and n=5 NF166-infected subjects, respectively).

Clinical course and adverse events in subjects undergoing CHMI

Mild-moderate adverse events were experienced by all CHMI-a subjects after infection (table S4A). Grade 3 adverse events occurred in five subjects, including general malaise/fatigue prohibiting daily activities in four subjects (across all three groups) and fever >39.0°C in two NF166.C8-infected subjects (table S4A). The duration of general fatigue/malaise was greater in NF54-infected subjects [5.0 (3.0 to 7.8) days] compared to NF135.C10-infected [2.7 (1.3 to 4.6) days] or NF166.C8-infected subjects [2.7 (1.6 to 4.0) days]; the mean difference for both comparisons was 2.6 days (95% CI, 0.0 to 5.1) ($P < 0.05$,one-way ANOVA/Tukey's post hoc test, $n = 5$ per group; table S4A). Overall, no other statistically significant difference in frequency,duration, or gradation of adverse events was observed between the three groups (table S4A). The clinical characteristics of the CHMI-b study did not differ markedly from those of the CHMI-a study, and no statistically significant differences were observed between the NF135.C10-infected and NF166.C8-infected groups (table S4B). No serious adverse events (SAEs) occurred in either study.

Genetic characterization of *P. falciparum* isolates

The genetic identity of the three *P. falciparum* isolates used in the CHMI-a and CHMI-b studies was assessed by PCR for the polymorphic repetitive RII region of Glutamate-Rich Protein (GLURP); the K1, MAD20, and R033 allelic variants of block 2 of Merozoite Surface Protein 1 (MSP1); and the IC1 and FC27 variants of block 3 of MSP2 (fig. S2). GLURP bands were observed corresponding to amplified DNA fragment lengths of about 950, 980, and 680 base pairs (bp) for NF54, NF166. C8, and NF135.C10 strains, respectively (fig. S2). MSP1 K1 bands corresponding to fragment lengths of 220 and 210 bp were observed for NF54 and NF166.C8 strains, but these isolates failed to produce an MSP1 MAD20 band (fig. S2). NF135.C10, in contrast, did produce an MSP1 MAD20 band with a fragment length of 180 bp (fig. S2). MSP2 IC1 bands corresponded to fragment lengths of 480, 580, and 560 bp for NF54, NF166.C8, and NF135.C10 strains, respectively (fig. S2). 18S bands were detectable as amplified DNA fragments of around 130 bp in all isolates (fig. S2).

Hepatocyte invasion and schizogony by sporozoites *in vitro*

In parallel, we assessed the infectivity of the three clinical *P. falciparum* isolates NF54, NF135.C10, and NF166.C8 for fresh human hepatocytes *in vitro*, using sporozoites dissected from the same batches of mosquitoes used to infect subjects in the CHMI-a study (experiment A) and derived from independent gametocyte cultures used in the CHMI-b study up to 2 years later (experiments B1 and B2) (Fig. 2). A higher proportion of NF135.C10 and NF166.C8 sporozoites established infection in human hepatocytes compared to the NF54 strain in these three experiments ($P < 0.001$ overall by repeated-measures ANOVA and $P < 0.01$ for NF54 versus NF135 and NF54 versus NF166 individually by Tukey's post hoc test; Fig. 2). In addition, mature NF166.C8 and NF135.C10 schizonts tended to be larger than NF54 schizonts, and concomitantly, the increase in the number of nuclei per schizont from day 2 to day 6 appeared to be greater for NF135.C10 and NF166.C8 compared to NF54 (Table 2). These combined data indicated that both sporozoite invasion and schizont development were more efficient in the NF135.C10 and NF166.C8 strains than in the NF54 strain.

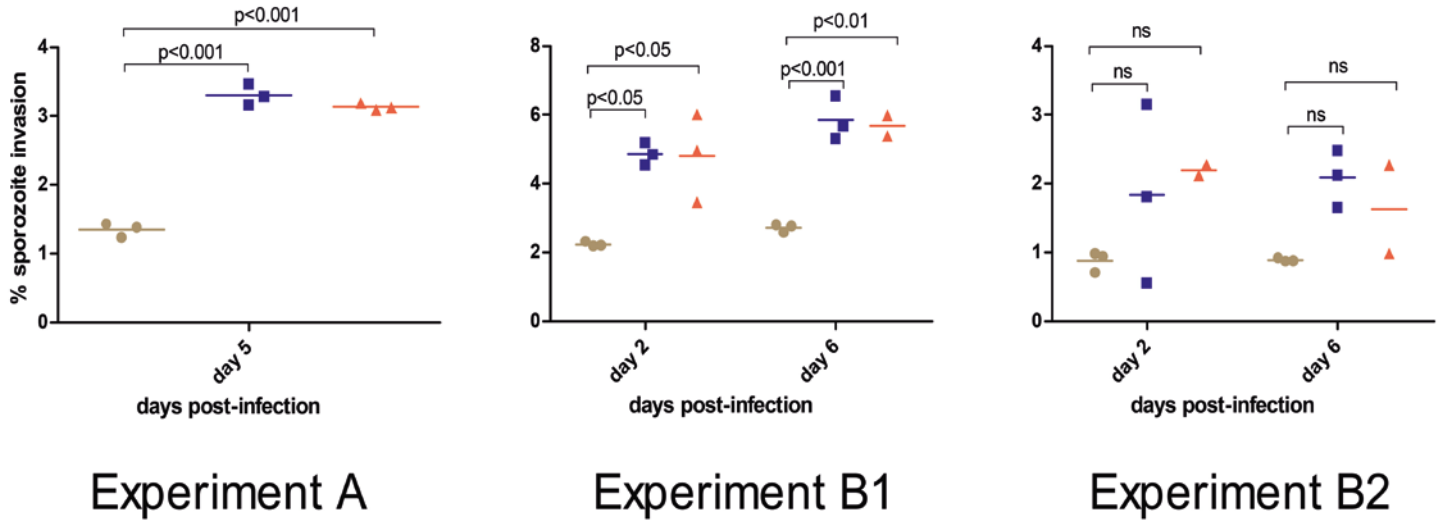

Figure 2. Invasion of human hepatocytes *in vitro* by three *P. falciparum* strains. Freshly isolated human hepatocytes were co-incubated with freshly isolated NF54 sporozoites (brown circles), NF135.C10 sporozoites (blue squares), or NF166.C8 sporozoites (red triangles) *in vitro*. The number of infected hepatocytes per well was counted by immunofluorescence microscopy at the given time points after infection. Shown are the results of three independent experiments. Experiment A was performed with 40,000 sporozoites per well harvested from mosquito batches used to infect subjects in the CHMI-a study and assessed at 5 days after infection. Experiments B1 and B2 were performed with 50,000 sporozoites per well harvested from mosquitoes fed on gametocyte cultures used in the CHMI-b study and assessed at 2 and 6 days after infection. Experiment A was performed at a different institute to experiments B1 and B2, and each of the three experiments was performed with human hepatocytes from a different donor. Data represent the percentage of sporozoites achieving successful invasion in each of three triplicate wells per condition (some NF166.C8 strain wells only in duplicate); horizontal lines represent group means. *P* values by one-way ANOVA with Tukey's posttest. ns, not significant.

Table 2. Characteristics of *P. falciparum* infectivity of human hepatocytes *in vitro*.

			NF54	NF135.C10	NF166.C8
experiment B1	day 2	#schizonts/well[1]	1100 (1,091-1,158)	2420 (2,273-2,595)	2479 (1,727-3,005)
		schizont Ø (µm)[2]	3.0 (2.3-4.1)	3.4 (0.9-4.5)	2.8 (1.8-3.6)
		#nuclei/well[3]	18,533 (15,187-20,077)	20,090 (17,823-26,446)	17,980 (15,731-25,785)
		#nuclei/schizont[4]	16.8	8.3	7.3
	day 6	#schizonts/well[1]	1,383 (1,289-1,398)	2,840 (2,656-3,276)	2,843 (2,692-2,993)
		schizont Ø (µm)[2]	11.2 (9.0 – 13.5)	18.3 (12.5 – 25.0)	16.1 (8.9 – 26.8)
		#nuclei/well[3]	56,181 (52,810-69,893)	216,449 (181,107-313,381)	248,961 (201,453-394,328)
		#nuclei/schizont[4]	40.6	76.2	87.6
		multiplication rate[5]	2.4	9.2	12.1
experiment B2	day 2	#schizonts/well[1]	373 (320-458)	902 (275-1575)	1,098 (1,059-1,137)
		schizont Ø (µm)[2]	1.7 (0.9-3.6)	2.8 (1.8-5.4)	3.4 (1.8-7.1)
		#nuclei/well[3]	8,858 (7,226-9,585)	16,726 (10,228-16,726)	21,968 (20,742-23,195)
		#nuclei/schizont[4]	23.8	18.5	20.0
	day 6	#schizonts/well[1]	438 (436-461)	1,061 (826-124)	813 (492-1134)
		schizont Ø (µm)[2]	11.4 (6.3-16.1)	18.9 (12.5-28.6)	12.5 (8.9-17.0)
		#nuclei/well[3]	95,295 (94,347-11,184)	382,508 (364,489-451,483)	ND
		#nuclei/schizont[4]	218	361	ND
		multiplication rate[5]	11.5	19.4	ND

ND = not determined. [1]Number of liver-stage schizonts per well, counted by immunofluorescence microscopy following staining with anti-HSP70 MAbs, as in Fig 2. Data represent median [range] across triplicate wells. [2]Schizont diameters, as measured by immunofluorescence microscopy and digital image analysis. Data represent mean [range] of *n*=19 to 49 schizonts per sample. NF135.C10 schizonts were larger than NF54 schizonts at day 2 ($P < 0.05$) and day 6 ($P < 0.001$) in both experiments and were also larger than NF166.C8 schizonts ($P < 0.05$ to $P < 0.001$ in all comparisons except experiment B2, day 2); NF166.68 schizonts were larger than NF54 schizonts at day 2 in experiment B2 ($P < 0.001$) and at day 6 in experiment B1 ($P < 0.001$). *P* values by one-way ANOVA with Tukey's posttest. [3]Number of parasite nuclei per well, as determined by qPCR; data represent median [range] of triplicate wells. [4]Number of nuclei per schizont, as calculated by dividing the median #nuclei per well by the median #schizonts per well. [5]Fold-increase in number of nuclei per infected human hepatocyte from day 2 to day 6 after infection, as calculated by dividing the median #nuclei per schizont at day 6 after infection by that at day 2.

Correlations between the *in vitro* human hepatocyte invasion assay and *in vivo* parasite dynamics

Given that identical sporozoite batches were used for both the human hepatocyte invasion assays and human infections in the CHMI-a study, we queried whether sporozoite infectivity *in vitro* might be predictive of blood-stage infection *in vivo*. The magnitude of the first wave of parasitemia emerging from the liver in study subjects inoculated with each strain (Table 1) appeared to correlate with the infectivity of sporozoites of that strain in the human hepatocyte invasion assay *in vitro* (Table 2) (Pearson's $R = 0.994$, $P = 0.068$, $n = 3$ strains). Furthermore, a strong negative association existed between sporozoite infectivity *in vitro* and the prepatent period of that strain *in vivo* ($R = -1.0$, $P = 0.005$).

Discussion

Here, we systematically assessed the dynamics of the obligate liver stage of human malaria infection, from mosquito bite to the emergence of blood-stage parasites, by using three different clinical isolates of *P. falciparum* and directly linking data on sporozoite infectivity in an *in vitro* human hepatocyte invasion model with *in vivo* measurements of parasitemia in subjects undergoing CHMI.

Both *in vitro* and *in vivo* findings support the notion that a substantially greater initial burden of blood-stage parasites is released from the livers of subjects infected with the NF135.C10 or NF166.C8 *P. falciparum* strains than from those infected with the NF54 strain. This in turn has significant effects on the subsequent course of blood-stage infection, with total parasite numbers more quickly reaching levels detectable by thick smear microscopy, shortening the prepatent period by several days. The difference in parasite burden between strains *in vivo* was even greater than the differences in invasion and development observed *in vitro*, a phenomenon that may be partially explained by suboptimal conditions for sporozoite invasion and development *in vitro* as compared to *in vivo*. In support of this explanation, the diameter of the NF54 strain liver-stage schizonts in our human hepatocyte cultures in this study (Table 2), as well as in a previous study (10),was 1.5- to 5-fold smaller than those measured *in vivo* in two humanized mouse models [the fumarylacetoacetate hydrolase–deficient mouse (3) and the severe combined immunodeficient mouse homozygous for the urokinase-type plasminogen activator transgene under the albumin promoter (11)]. Measuring the size of liver-stage schizonts in these mouse models might reveal even bigger differences between *P. falciparum* isolates than those found here *in vitro*. Whereas more than a decade separates the original isolation date of each of these three strains of *P. falciparum* (table S2), the use of specific master cell banks (MCBs) for CHMI studies restricts the cumulative culture period for the generation of gametocytes of each strain. In our opinion, this is unlikely to explain the marked differences in phenotype of the strains *in vivo* with regard to liver-stage burden.

Both sporozoite infectivity (Fig. 2) and the size of mature liver-stage schizonts of strain NF135. C10 in particular (Table 2) appeared to be greater than those for the NF54 strain, suggesting that two distinct mechanisms may be contributing to the greater number of merozoites released *in vivo*. In contrast, the duration of liverstage development was markedly similar between isolates

(Fig. 1, D to F). Although the mean time to qPCR positivity was slightly longer for the NF54 strain, we regularly detected NF54 parasites in blood samples by day 6.5 after infection (Table 1) (12). The small magnitude of the first wave of NF54 parasitemia suggested that the earliest parasites to emerge from the liver may fall below the detection limit of the qPCR. In any case, this short delay in qPCR positivity (0.52 and 0.70 day relative to the NF135.C10 and NF166.C8 strains, respectively, across all studies, Table 1) would appear to form only a minor determinant of the overall delay in prepatent period (2.3 and 2.9 days, respectively; Fig. 1C).

Finally, the variability in both the timing and the magnitude of the first wave of parasitemia among subjects within each study group was relatively limited in comparison to the difference in that magnitude between groups, suggesting that innate host factors may have little influence on either the duration or efficiency of liver-stage development in primary *P. falciparum* infections in our study population.

Although no clear relationship has been found between salivary gland sporozoite load and the number of sporozoites inoculated by probing/feeding mosquitoes (13), higher salivary gland loads have been suggested to enhance the infectivity of sporozoites (14). However, NF54-infected mosquitoes used in the CHMI-a study actually contained higher salivary gland sporozoite loads than did NF166.C8-infected or NF135.C10-infected mosquitoes from the same colony (table S2), a finding that is consistent with batches of mosquitoes used for other CHMIs at our center (table S3). The advent of CHMIs using parenteral sporozoite inoculation allowing better control over the number of administered sporozoites may further help to disentangle this point.

As a direct consequence of their higher liver burden, the time frame between release of the first NF135.C10 and NF166.C8 parasites from the liver and the initiation of antimalarial treatment with atovaquone-proguanil (upon parasitemia attaining the threshold by thick smear microscopy) incorporated only a single erythrocytic cycle. This is insufficient to reliably determine their blood-stage multiplication rate (5).

A possible limitation of this study is that sporozoite infectivity and the development of parasitemia were only assessed in malaria-naïve volunteers from malaria nonendemic countries. The observed infectivity between the parasite strains may be different in endemic populations with variable degrees of semi-immunity. Therefore, it will be of interest to compare these clones in CHMI in endemic settings. A further limitation is that only three *P. falciparum* isolates from different geographical backgrounds were tested for comparison in human hepatocyte cultures and by CHMI. It remains to be seen to what extent the three strains are representative of the overall diversity among the global *P. falciparum* population (15). Increasing the portfolio of *P. falciparum* clones for CHMI studies may provide further support for our findings. Both the NF135.C10 (9) and NF166.C8 strains described here were developed and characterized with the specific intent to increase the currently limited portfolio of *P. falciparum* strains available for heterologous challenge CHMI studies. For NF54 or its derivative clone 3D7, the minimum infecting dose to guarantee a 100% infection rate in malaria-naïve subjects in such CHMI studies is generally held to be five mosquito bites (16), 3200 cryopreserved sporozoites inoculated intravenously (17), or 75,000 cryopreserved sporozoites inoculated intramuscularly (18). Given the higher observed infectivity of NF135.C10 and NF166.C8 sporozoites, the minimum dose to reliably achieve 100% infection must be explored in further studies. Whereas interventions that target hepatocyte invasion aim at one of the two most constrained bottlenecks in the parasite's life cycle (4), our data indicate that relatively limited but relevant heterogeneity does exist among strains with regard to sporozoite infectivity (Fig. 2), which should be taken into account when designing and evaluating such interventions.

A major advantage of CHMI studies is the relatively small number of subjects required for the evaluation of vaccine efficacy, due to the homogeneity of the profile of parasitemia within each group and between homologous groups, as illustrated here by our CHMI-a and CHMI-b studies. Given the clear-cut difference in parasite dynamics between *P. falciparum* isolates, however, homologous challenge studies of malaria vaccines will remain insufficiently representative, and CHMIs with heterologous strains are required to more accurately predict the efficacy of promising vaccine candidates.

Materials and Methods

Study design

Two CHMIs were performed using the well-established *P. falciparum* research strain NF54 and the new clones NF135.C10 and NF166.C8. The primary objective of the first study (CHMI-a) was to compare parasite dynamics of these isolates during infection, and that of the second (CHMI-b) study was to determine the proportion of subjects in each group who developed patent parasitemia, as described below. Secondary objectives of both studies included clinical parameters and further measures of parasite kinetics.

Healthy malaria-naïve adult (age 18 to 35 years) Dutch volunteers were recruited at the Harbour Hospital, Rotterdam, after signing informed consent. Our screening procedures have been described previously (19), and a complete list of inclusion/exclusion criteria is provided in table S5. In the first CHMI study described here (CHMI-a), 15 subjects (9 men and 6 women; table S1) were randomly allocated (ratio 1:1:1) to three parallel groups of $n = 5$, to be infected by bites of five mosquitoes per subject carrying, respectively, the NF54 strain of *P. falciparum*, the NF135.C10 clone, or the new NF166.C8 clone. In the second study (CHMI-b), 24 subjects were randomly allocated (ratio 1:1:1:1:1:1) to six parallel groups of $n = 4$, to bites by one, two, or five mosquitoes carrying, respectively, either NF135.C10 or NF166.C8 strains. For comparison with CHMI-a, only the two CHMI-b groups exposed to the bites of five infectious mosquitoes are reported here (that is, eight subjects in total, of whom three were men, and five were women; table S1). Flow charts for both studies are shown in fig. S1. Sample sizes were chosen pragmatically, based on longstanding experience with NF54 and (limited) NF135.C10 data. The CHMI-a study was powered to detect a 0.9 log (7.9-fold) difference in parasite burden between strains.

At inclusion, subjects received a unique pseudonymized study code. Once the predetermined number of subjects per study was included, two departmental employees not otherwise involved in the study linked a random number generated in Excel to each study code and allocated the required number of study codes to each group, using simple ranking of the associated random numbers. This allocation list was provided only to insectary technicians, who prepared feeding cages containing the required strain and number of *P. falciparum*–infected mosquitoes and labeled these otherwise identical cages with only the respective study code. Thus, study subjects, clinical investigators, and laboratory personnel assessing study end points (thick smear microscopy and qPCR) remained blinded to group allocation throughout each study.

All subjects within each study underwent infection on the same day, and considering the extremely close monitoring of CHMI study subjects for safety reasons, all subjects were expected

to complete follow-up. Data from all subjects were included in the study, and there were no specific rules for data exclusion, outliers, or premature cessation of data collection (other than if required for safety reasons after consultation with the sponsor, safety monitor, and Data Safety and Monitoring Board).

Culture and characterization of clinical parasite isolates

P. falciparum blood-stage parasites were introduced into semi-automated culture from peripheral whole-blood samples of patients with clinical malaria and used to generate infective *Anopheles stephensi* mosquitoes, as described before (20). The origins of NF54 and NF135 strains of *P. falciparum* have been described previously (8, 9). The NF166 strain of *P. falciparum* originated in 2010 as a clinical isolate (parasitemia 0.5 to 1%) from a child who had recently visited Guinea (West Africa). The clone NF166.C8 was obtained by limiting dilution culturing and established as a stable producer of fertile gametocytes and subsequently oocysts in infected mosquitoes (table S2). To minimize a potential confounding effect, gametocytes used to infect mosquitoes for our CHMI studies are generated following a strict culture procedure. When a cryopreserved aliquot of any isolate from our MCB is thawed and brought into culture, new aliquots of the culture are frozen down again for future use after the first four rounds of subculture. The remaining culture is then maintained for the generation of gametocytes, generally between the 15th and 25th rounds of subculture. Because *in vitro* parasites lose their ability to generate gametocytes beyond ±25 rounds of subculture, such cultures are discontinued. The MCB of NF54 was generated from the estimated 10th to 15th subculture since its original isolation in the 1970s. For NF135, the MCB was generated from the sixth subculture since isolation, and for NF166 from the fourth subculture.

The identities of NF54, NF135.C10, and NF166.C8 were defined by assessing the polymorphic regions of three *P. falciparum* antigenic genes by PCR in a method adapted from Snounou *et al.* (21). Briefly, parasite DNA was isolated using QIAamp DNA Blood Mini Kit (Qiagen) and amplified with GoTaq G2 Flexi Polymerase (Promega) using specific primers for the polymorphic repetitive RII region of *P. falciparum* GLURP (22); the K1, MAD20, and R033 allelic variants of block 2 of MSP1 (23); and the IC1 and FC27 variants of block 3 of

MSP2 (24) (all primers from Invitrogen); 18S ribosomal DNA was used as a loading control. The sensitivity of NF166.C8, NF135.C10, and NF54 to dihydroartemisinin (Sigma-Tau), chloroquine diphosphate salt (Sigma-Aldrich), mefloquine, proguanil (British Pharmacopoeia), atovaquone (GlaxoSmithKline), and lumefantrine (Novartis) was tested by the Malaria SYBR Green I-Based Fluorescence Assay in triplicate experiments (25). See also table S2 and fig. S2.

Controlled human malaria infections

Mosquitoes in small cages were allowed to feed for 10min on the forearms of study subjects. All blood-engorged mosquitoes were dissected to confirm sporozoite carriage. Where necessary, study subjects were exposed to additional mosquitoes until precisely the predetermined number of infective bites was achieved. From day 5 after infection, subjects were seen twice daily as outpatients. Solicited and unsolicited adverse events and vital parameters were recorded at each visit and venous whole blood was drawn for thick blood smear, qPCR (see below), safety parameters, and exploratory assays. Adverse events were graded as mild (not interfering with),

moderate (interfering with), or severe (prohibiting daily activities). Respective grading of fever was 37.5° to 38.0°C, 38.0° to 39.0°C, and > 39.0C°. Adverse events were recorded by clinical investigators as being probably, possibly, or unlikely related to study procedures.

In CHMI-a, subjects were treated with a standard regimen of atovaquone-proguanil (Malarone, 1000/400 mg daily for 3 days) upon their first positive thick blood smear (defined as ≥2 parasites per 225 high-powered fields, equating to 0.5 ml of whole blood examined). In CHMI-b, subjects were treated with the same regimen as soon as two consecutive whole-blood samples were positive by qPCR (defined as > 500 parasites/ml, calibrated against a daily standard curve of known parasite concentrations). The change in methodology between studies was proposed (12) and implemented to minimize potential safety risks upon a request by Netherlands' Central Committee on Human Research (CCMO), following concerns over a cardiac SAE possibly related to CHMI in an earlier study (26). Sequential daily thick smears/qPCRs were continued after treatment in each subject until complete clearance of blood-stage parasites. CHMI-a whole-blood samples were also retrospectively analyzed by qPCR. These studies were approved by Netherlands' CCMO (NL41004.078.12 and NL48704.000.14).

Quantitative real-time fluorescence polymerase chain reaction

P. falciparum parasitemia was quantified by qPCR as described before (27) with some modifications. Briefly, isolated DNA was resuspended in 100 ml of H_2O, using 5 µl as template and the TaqMan MGB probe AAC AAT TGG AGG GCA AG-FAM. Samples for post hoc analysis were stored at −80°C. For each sample (including standard curve dilutions), two qPCR replicates were performed; if these values differed by >5%, then the sample was remeasured a third time. In addition, all samples were spiked with heterologous DNA as an extraction control; if the qPCR value of the extraction control differed by > 5% from the running average of all other samples, then the extraction process for that sample was repeated. Quantification of *P. falciparum* nuclei in wells of the human hepatocyte infectivity assay was performed using the same qPCR.

Primary human hepatocyte infectivity assay

In vitro sporozoite infectivity assays in human hepatocytes were performed, as described previously (6), at two different laboratories. Briefly, fresh primary hepatocytes derived from patients undergoing elective (partial) hepatectomy were plated in 96-well plates (50,000 cells per well). Two to five days after plating the human hepatocytes, sporozoites were dissected from the salivary glands of 80 to 110 infected mosquitoes per *P. falciparum* strain and added to the human hepatocyte wells in triplicate. In the first *in vitro* experiment (experiment A), sporozoites were harvested from the exact same batches of mosquitoes used on the same day to infect human subjects in CHMI-a; 40,000 sporozoites were added per human hepatocyte well. In subsequent *in vitro* experiments (B1 and B2), sporozoites were harvested from mosquitoes fed on independent gametocyte cultures used to subsequently infect subjects in CHMI-b, and 50,000 sporozoites were added per well. After 2 to 6 days, the number of human hepatocytes in each well harboring viable parasites was assessed by intracellular staining with anti–*P. falciparum* HSP-70 rabbit polyclonal antibody (SPC-186C/D, StressMarq Biosciences) followed by fluorescence microscopy; no instances of >1 invaded sporozoite per human hepatocyte were

observed. Discrimination between sporozoite invasion of/adherence to human hepatocytes was achieved by staining with fluorescent monoclonal antibodies against circumsporozoite protein (CSP) before cell permeabilization (28); the proportion of adherents (that is, HSP-70$^+$CSP$^+$-double positive, noninvaded parasites) was generally <5% at day 2 after infection and consistently <0.5% at day 6 after infection. The diameter of n = 19 to 49 liver-stage schizonts per strain was measured at day 2 and day 6 after infection by fluorescence microscopy and digital image analysis (LeicaDMI6000B). Total numbers of parasite nuclei per well were determined by qPCR in separate triplicate wells after discarding the culture supernatant. It was technically infeasible to perform both microscopy and qPCR on the same wells. The median number of merozoites per liver-stage schizont was calculated for each strain as follows: ([median #parasite nuclei in triplicate wells] − [median #adhered (noninvaded) parasites in triplicate wells])/([median #infected human hepatocytes in triplicate wells]). Investigators carrying out the *in vitro* hepatocyte infectivity assay were blinded as to the strain of the sporozoites.

Statistical analysis

Differences in continuous variables between groups of study subjects were compared by one-way ANOVA/Tukey's post hoc test (CHMI-a, three groups), unpaired *t* test (CHMI-b, two groups), or one-sample *t* test (CHMI-b, two groups when all values for one group were identical). Differences in categorical variables between groups were compared by χ^2/Fisher's exact test. Differences in *in vitro* invasive capacity between isolates were compared by repeated-measures ANOVA/Tukey's post hoc test (three groups). Correlations between *in vitro* and *in vivo* variables were assessed by Pearson's test on the mean value of each variable per strain; where appropriate, variables were first log-transformed. Two-sided $P < 0.05$ was considered statistically significant in all tests. Parasite densities below the lower quantification limit of the qPCR (35 parasites/ml in CHMI-a and 50 parasites/ml in CHMI-b) were plotted at 0.5× the log value of that limit. The magnitude of the initial wave of parasitemia emerging from the liver was defined as the highest parasite density measured by qPCR per subject between day 6.5 and 8.0 after infection.

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Supplementary Materials

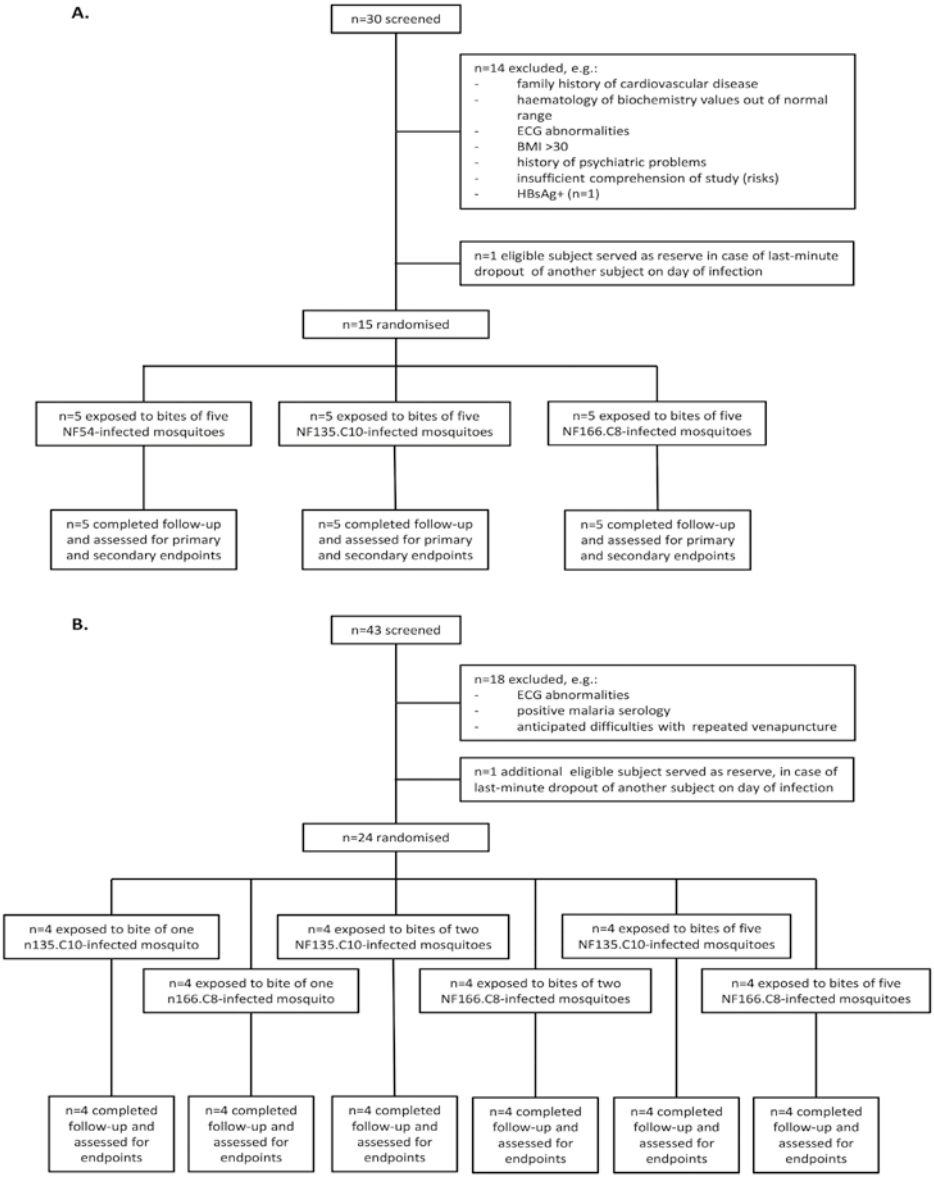

Figure S1. Study flow charts. A. CHMI-a (recruitment started August 2012, completed September 2012; day of infection 25-09-2012; final day of follow-up 30-10-2012); **B.** CHMI-b (recruitment started August 2014, completed October 2014; day of infection 07-10-2014; final day of follow-up 11-11-2014). **Note:** for comparison with CHMI-a, only the results of the two CHMI-b groups exposed to the bites of five infectious mosquitoes are presented in this paper.

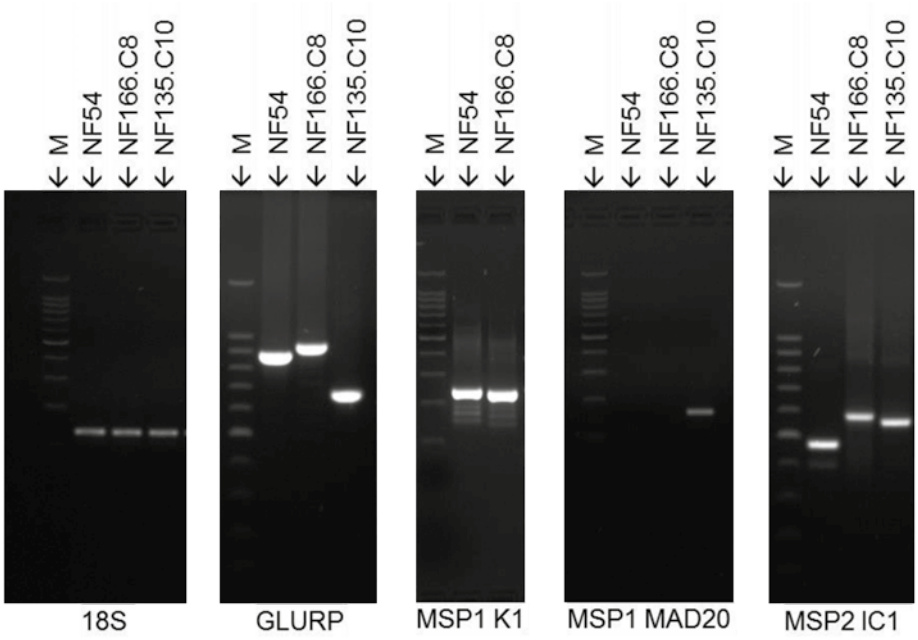

Figure S2. Genetic characterization of *P. falciparum* isolates. PCRs for 18S rDNA, GLURP, the K1, MAD20 & R033 allelic variants of MSP1 and the IC1 & FC27 variants of MSP2 were performed on the *P. falciparum* isolates NF54, NF166.C8 & NF135.C10. Note: NF54 and NF166.C8 failed to produce any MSP1 MAD20 band. M – 100bp marker.

Table S1. Study demographics.

	NF54	**NF135.C10**	**NF166.C8**
CHMI-a			
gender (male / total)	5 / 5	2 /5	2 / 5
age (median [range])	19 [18-21]	22 [18-27]	24 [18-35]
CHMI-b[1]			
gender (male / total)		0 / 4	3 / 4
age (median [range])		22.5 [19-27]	24 [19-25]

[1]Data only shown for the two CHMI-b groups exposed to bites of five infectious mosquitoes.

Table S2. Characteristics of *P. falciparum* isolates used in CHMI studies.

	NF54		**NF135.C10**		**NF166.C8**	
Country of origin	Netherlands (West Africa)		Cambodia		Guinea	
Year of isolation	1979		1993		2010	
Drug sensitivity (IC_{50}):						
Chloroquine (nM)	8.8		96		9.9	
Mefloquine (nM)	19		37		11	
Atovaquone (nM)	1.2		0.94		0.37	
Proguanil (μM)	0.57		169		0.29	
Dihydroartemisinin (nM)	0.36		0.19		0.05	
Lumefantrine (nM)	94		122		49	
Parasite batches used in individual CHMIs:	CHMI-a	CHMI-b	CHMI-a	CHMI-b	CHMI-a	CHMI-b
No. sub-cultures since thawing of Master Cell Bank aliquot	23	-	17	21	18	17
proportion of oocyst-infected mosquitoes	10/10	-	10/10	10/10	10/10	10/10
mean oocyst count/ mosquito (*n*=10)	20.1	-	15.6	32.5	15.7	15.2
proportion of sporozoite-infectious mosquitoes	9/10	-	9/10	10/10	10/10	10/10
mean sporozoite count/ mosquito (*n*=10)	$101x10^3$	-	$69x10^3$	$69x10^3$	$40x10^3$	$51x10^3$

Table S3. Overview of all comparable CHMI studies performed at our center.

Study# (*ref#*)	Year	Treatment threshold[1]	Number of subjects			Mosquito salivary gland sporozoite load		
			NF54	NF135.C10	NF166.C8	NF54	NF135.C10	NF166.C8
1[2] *(31)*	2000	TBS (NS)	5	-	-	N/A	N/A	N/A
2[2] *(31)*	2001	TBS (NS)	5	-	-	N/A	N/A	N/A
3[2] *(31)*	2002	TBS (NS)	5	-	-	N/A	N/A	N/A
4[2] *(31)*	2003	TBS (NS)	5	-	-	N/A	N/A	N/A
5 *(32)*	2007	TBS	5	-	-	31,500	-	-
6[3]	2008	TBS	18	-	-	72,800	-	-
7 *(33)*	2009	TBS	5	-	-	88,000	-	-
8 *(9)*	2010	TBS	4	3	-	69,000	12,500	
9 *(34)*	2011	TBS	5	-	-	79,500	-	-
10 *(19)*	2011	TBS	5	-	-	100,000	-	-
11 *(29)*	2012	TBS	4	-	-	98,250	-	-
12 (CHMI-a)[4]	**2012**	**TBS**	**5**	**5**	**5**	**101,250**	**69,000**	**40,000**
13 *(35)*	2012	TBS	-	5	-	-	38,250	-
14 *(36)*	2012	TBS	5	-	-	75,800	-	-
15 *(36)*	2013	qPCR	4	-	-	98,000	-	-
16 (CHMI-b)[4]	**2014**	**qPCR**	**-**	**4**	**4**	**-**	**69,000**	**51,000**
17	2015	qPCR	5	-	-	74,000	-	-
18[5]	2015	qPCR	5	5	5	26,500/ 44,300	18,000/ 23,500	59,500/ 17,000
Total			**90**	**22**	**14**			

[1]In initial studies at our centre, subjects were treated upon positive thick blood smear (TBS), whereas in more recent studies treatment was initiated upon positive quantitative PCR (qPCR). The lower detection threshold of the latter results in shorter pre-patent periods, lower parasitaemia at time of treatment and hence improved tolerability & safety for study subjects. In the very earliest studies, the methodology for performing thick blood smear analysis was not yet standardised (NS), which can result in subtly different detection thresholds between subjects and between studies. For meta-analysis of pre-patent periods and of highest parasite densities (usually at time of treatment), we have thus limited comparisons to those 10 studies (#5-14, including *n* = 56 NF54, *n* = 13 NF135.C10 and *n* = 5 NF166.C8 subjects) in which standardised thick blood smears were used to initiate treatment. For meta-analysis of time to first qPCR positivity and magnitude of the first wave of parasitaemia to emerge from the liver, all 18 studies are included.

[2]In the very earliest studies at our centre, subjects were exposed to the bites of between 4-7 *P. falciparum* NF54-infected mosquitoes; in all subsequent studies listed, all subjects were exposed to the bites of precisely 5 mosquitoes infected with the given isolate. No statistically significant difference was observed in the height of the first wave of parasitaemia in these first four studies compared to all later studies involving NF54.

[3]Subjects were immunised with a candidate malaria vaccine prior to CHMI, but where wholly unprotected against challenge.

[4]Studies for which directly-linked *in vitro* data on sporozoite invasion are available (boldface), as presented in this manuscript.

[5]Subjects were infected in two groups, for which two separate batches of *P. falciparum*-infected mosquitoes were used.

Table S4. Adverse events by severity grade during CHMI-a and CHMI-b studies.

table S4a. Adverse events by severity grade during CHMI-a

	NF54 (*n*=5)			NF135.C10 (*n*=5)			NF166.C8 (*n*=5)		
	Subjects[1]	**Duration[2]**	**Grade[3]**	**Subjects[1]**	**Duration[2]**	**Grade[3]**	**Subjects[1]**	**Duration[2]**	**Grade[3]**
All adverse events									
Fever[4]	4	2.0 [0.2-2.8]	2 [2-2]	3	1.4 [0.4-3.2]	2 [1-2]	3	2.8 [0.6-6.8]	3 [1-3]
Headache	5	3.8 [0.3-7.8]	1 [1-2]	5	1.8 [0.1-5.3]	2 [1-2]	5	4.3 [1.6-7.3]	2 [1-2]
Malaise/fatigue[5]	5	**5.3 [3.0-7.8]**	2 [1-3]	5	**2.7 [1.3-4.6]**	2 [1-3]	5	**2.7 [1.6-4.0]**	2 [1-3]
Myalgia	3	3.5 [1.6-5.9]	1 [1-2]	5	2.2 [1.0-4.0]	1 [1-2]	3	1.7 [0.8-3.3]	1 [1-2]
Arthralgia	-	-	-	1	4.1 .	1 .	-	-	-
Nausea[6]	2	1.3 [1.0-1.1]	1.5 [1-2]	3	1.1 [0.0-3.0]	1 [1-2]	2	1.1 [0.1-2.2]	1.5 [1-2]
Chills/rigors	3	1.4 [0.7-2.6]	2 [1-2]	3	0.6 [0.1-1.1]	1 [1-1]	2	0.9 [0.2-1.7]	1.5 [1-2]
Diarrhoea[6]	3	1.3 [0.0-3.9]	1 [1-1]	2	0.4 [0.0-0.8]	1 [1-1]	1	0.0 .	2 .
Abdominal pain[6]	3	2.4 [0.2-4.9]	2 [1-2]	1	0.3 .	1 .	3	0.9 [0.1-1.5]	1 [1-2]
Unsolicited[7]	4 (6)	3.0 [1.4-6.9]	1 [1-2]	2 (2)	2.0 [0.0-4.0]	1.5 [1-2]	5 (9)	4.8 [0.0-29]	1 [1-1]
Any[8]	5 (35)	3.0 [0.0-7.8]	1 [1-3]	5 (30)	1.8 [0.0-5.3]	1 [1-3]	5 (33)	3.0 [0.0-29]	1 [1-3]
Grade 3 AEs									
Fever[4]	-	-		-	-		2	3.9 [1.1-6.8]	
Malaise/fatigue	2	6.0 [5.0-7.0]		1	3.0 .		1	4.4 .	
Any[8]	2 (2)	6.0 [5.0-7.0]		1 (1)	3.0 .		3 (3)	4.0 [1.1-6.8]	

table S4b. Adverse events by severity grade during CHMI-b[9]

	NF135.C10 (*n*=4)			NF166.C8 (*n*=4)		
	Subjects[1]	**Duration[2]**	**Grade[3]**	**Subjects[1]**	**Duration[2]**	**Grade[3]**
All adverse events						
Fever	4	0.7 [0.2-1.9]	2 [1-3]	1	1.0 .	2 .
Headache	3	1.3 [0.0-3.6]	1 [1-2]	3	1.8 [0.3-2.9]	2 [1-3]
Malaise/fatigue	4	2.7 [1.4-3.6]	1 [1-2]	3	3.9 [0.7-8.3]	2 [1-2]
Myalgia	3	1.9 [1.0-3.3]	1 [1-2]	2	0.7 [0.6-0.7]	1 [1-1]
Arthralgia	1	1.0 .	1 .	-	-	-
Nausea[6]	4	0.7 [0.0-1.6]	1 [1-2]	1	0.2 .	1 .
Chills/rigors	3	1.3 [0.8-2.0]	2 [1-2]	3	0.7 [0.1-1.3]	1 [1-2]
Diarrhoea	-	-	-	-	-	-
Abdominal pain[6]	2	1.1 [0.1-2.1]	1 [1-1]	1	1.4 .	1 .
Unsolicited[7]	2 (2)	4.0 .	1 [1-1]	2 (4)	2.4 [0.0-8.3]	1 [1-2]
Any[8]	4 (27)	1.4 [0.0-4.0]	1 [1-3]	4 (18)	1.8 [0.0-8.3]	1 [1-3]
Grade 3 AEs						
Fever	1	1.9 .		1	2.9 .	
Any[8]	1 (1)	1.9 .		1 (1)	2.9 .	

[1]Number of subjects per group experiencing adverse event. Multiple similar events/episodes per subject were counted as one, with cumulative duration and maximum gradation recorded. For Unsolicited and Any adverse events, total numbers of events/episodes per group are shown in parentheses.

[2]In days; data represent mean [range] per group.

[3]AE gradation: 1=mild, 2=moderate, 3=severe; Fever gradation respectively: 37.5-38.0°C, 38.0-39.0°C, >39.0°C; data represent medians [range].

[4]Two NF135.C10 subjects and two NF166.C8 subjects (those with grade 3 symptoms) held persisting fever across the third and final day atovaquone-proguanil treatment, which subsequently resolved spontaneously.

[5]Mean difference in duration of malaise/fatigue: 2.6 [95% CI 0.0 to 5.1] days between NF54 and NF135.C10 ($P < 0.05$); 2.6 [95% CI 0.0 to 5.1] days between NF54 and NF166.C8 ($P < 0.05$); 0.0 [95% CI -2.6 to 2.5] days between NF135.C10 and NF166.C8 ($P > 0.05$); all comparisons by one-way ANOVA/Tukey's post-hoc test.

[6]Gastro-intestinal complaints, including nausea (but not vomiting), diarrhoea and abdominal pain were mainly experienced following intake of atovaquone-proguanil.

[7]Unsollicited adverse events in CHMI-a included 5 episodes of common cold, 2 of tender submandibular swelling, 1 of cold sore, 2 of anorexia, 1 of gastric reflux, 2 of syncope, 1 of hyperventilation syndrome, 1 of blurry vision, 1 of mild oedema orbitae and 1 of lumbago. In CHMI-b, there was 1 episode of atypical chest pain, 1 of dizziness, 1 of syncope, 1 of a minor transport accident, 1 of influenza-like illness (ongoing at the final day of follow-up) and 1 subject developed haematomas in both anticubital fossae.

[8]No other statistically significant difference in frequency, duration or gradation of any adverse events was observed between the three groups.

[9]Data only shown for the two CHMI-b groups exposed to bites of five infectious mosquitoes.

Table S5. Inclusion and exclusion criteria for CHMI studies.

CHMI-a	Inclusion criteria
1.	Males and females aged 18-35 years
2.	In general good health based on history, physical examination and basic haematology and biochemistry
3.	Negative pregnancy test for females
4.	Use of adequate contraception for females
5.	Signed informed consent, based on a thorough understanding of the concept and procedures of the study
6.	Volunteer agrees to allow informing his/her general practitioner about participation and agrees to sign a request for medical information from the GP concerning any contra-indications for participation in the study
7.	Willingness to undergo a Pf sporozoite challenge
8.	Agreement to stay in a hotel close to the trial center during part of the study (day 5 until three days post-treatment)
9.	Reachable (24/7) by mobile telephone during the whole study period
10.	Available to attend all study visits
11.	Agreement to refrain from blood donation to Sanquin or for other purposes, during the course of the study and thereafter following Sanquin guidelines.
12.	Willingness to undergo an HIV, HBV and HCV screening test
13.	Negative urine toxicology screening test at the screening visit and on the day before challenge
14.	Willingness to take a curative regimen of Malarone®
	Exclusion criteria
1.	History of malaria
2.	Plans to travel outside of the Netherlands during the study period
3.	Previous participation in any malaria vaccine study and/or positive serology for *P. falciparum*
4.	Symptoms, physical signs or laboratory values suggestive of systemic disorders, including but not limited to renal, hepatic, cardiovascular, pulmonary, skin, immunodeficiency, psychiatric and other conditions, which could compromise the health of the volunteer during the study or interfere with the interpretation of the study results
5.	History of diabetes mellitus or cancer (except basal cell carcinoma of the skin)
6.	Clinically significant ECG abnormalities at screening, or history of arrhythmia's or prolonged QT-interval
7.	Positive family history of cardiac disease in 1st or 2nd degree relatives < 50 years old
8.	An estimated ten year risk of fatal cardiovascular disease of ≥5%, as estimated by the Systematic Coronary Risk Evaluation (SCORE) system
9.	Body Mass Index (BMI) below 18 or above 30 kg/m2
10.	Any clinically significant deviation from the normal range in haematological or biochemical blood tests or urine analysis

Table S5. Continued.

11.	Positive HIV, HBV or HCV screening tests
12.	Participation in any other clinical study within 30 days prior to the onset of the study or during the study period
13.	Pregnant or lactating women
14.	Volunteers unable to give written informed consent
15.	Volunteers unable to be closely followed during the study period for social, geographic or psychological reasons
16.	Previous history of drug or alcohol abuse interfering with normal social function in the period of one year prior to study onset
17.	A history of psychiatric disease or convulsions
18.	Known hypersensitivity to anti-malarial drugs
19.	History of severe reactions or allergy to mosquito bites
20.	The use of chronic immunosuppressive drugs, antibiotics, or other immune modifying drugs within three months prior to study onset (inhaled and topical corticosteroids are allowed) or during the study period
21.	Contra-indications for Malarone® use, including treatment taken by the volunteers that interferes with Malarone®
22.	Any confirmed or suspected immunosuppressive or immunodeficient condition, including asplenia
23.	Co-workers of the departments of Medical Microbiology of the UMC St Radboud, the department of Internal Medicine of the Havenziekenhuis or the department of Medical Microbiology & Infectious Diseases of the Erasmus MC
24.	A history of sickle cell, thalassaemia trait or G6PD deficiency
CHMI-b	**Inclusion criteria**
1.	Subject is aged ≥ 18 and ≤ 35 years and in good health.
2.	Subject has adequate understanding of the procedures of the study and agrees to abide strictly thereby.
3.	Subject is able to communicate well with the investigator, is available to attend all study visits, lives in proximity to the trial centre (<10 km) or (if >10km) is willing to stay in a hotel close to the trial centre during part of the study (day 5 post-infection until three days post-treatment). Furthermore the subject will remain within the Netherlands during the study period and is reachable (24/7) by mobile telephone throughout the entire study period.
4.	Subject agrees to inform his/her general practitioner and (if applicable) medical specialist about participation in the study and to sign a request to release by the GP any relevant medical information concerning possible contra-indications for participation in the study.
5.	Subject agrees to refrain from blood donation to Sanquin or for other purposes throughout the study period and for a defined period thereafter according to current Sanquin guidelines.
6.	For female subjects: subject agrees to use adequate contraception and not to breastfeed for the duration of study.
7.	Subject has signed informed consent.

Table S5. Continued.

	Exclusion criteria
1.	Any history, or evidence at screening, of clinically significant symptoms, physical signs or abnormal laboratory values suggestive of systemic conditions, such as cardiovascular, pulmonary, renal, hepatic, neurological, dermatological, endocrine, malignant, haematological, infectious, immunodeficient, psychiatric and other disorders, which could compromise the health of the volunteer during the study or interfere with the interpretation of the study results. These include, but are not limited to, any of the following:
1.1	Body weight <50 kg or Body Mass Index (BMI) <18.0 or >30.0 kg/m2 at screening
1.2	A heightened risk of cardiovascular disease, defined as: an estimated ten year risk of fatal cardiovascular disease of ≥5% at screening, as determined by the Systematic Coronary Risk Evaluation (SCORE); history, or evidence at screening, of clinically significant arrhythmia's, prolonged QT-interval or other clinically relevant ECG abnormalities; or a positive family history of cardiac events in 1st or 2nd degree relatives <50 years old.
1.3	Functional asplenia, sickle cell trait/disease, thalassaemia trait/disease or G6PD deficiency.
1.4	History of epilepsy in the period of five years prior to study onset, even if no longer on medication.
1.5	Positive HIV, HBV or HCV screening tests.
1.6	Chronic use of i) immunosuppressive drugs, ii) antibiotics, iii) or other immune modifying drugs within three months prior to study onset (inhaled and topical corticosteroids and oral anti-histamines exempted) or expected use of such during the study period.
1.7	History of malignancy of any organ system (other than localized basal cell carcinoma of the skin), treated or untreated, within the past 5 years
1.8	Any history of treatment for severe psychiatric disease by a psychiatrist in the past year.
1.9	History of drug or alcohol abuse interfering with normal social function in the period of one year prior to study onset, or positive urine toxicology test for cocaine or amphetamines at screening or prior to infection.
2.	For female subjects: positive urine pregnancy test at screening or prior to infection.
3.	Any history of malaria, positive serology for *P. falciparum*, or previous participation in any malaria (vaccine) study.
4.	Known hypersensitivity to or contra-indications (including co-medication) for use of atovaquone-proguanil (Malarone®) or artemether-lumefantrine (Riamet®), or history of severe (allergic) reactions to mosquito bites.
5.	Receipt of any vaccinations in the 3 months prior to the start of the study or plans to receive any other vaccinations during the study period or up to 8 weeks thereafter.
6.	Participation in any other clinical study in the 30 days prior to the start of the study or during the study period.
7.	Being an employee or student of the department of Medical Microbiology of the Radboudumc, the department of Internal Medicine or Laboratory of the Havenziekenhuis or the department of Medical Microbiology & Infectious Diseases of the Erasmus MC.
8.	Any other condition or situation that would, in the opinion of the investigator, place the subject at an unacceptable risk of injury or render the subject unable to meet the requirements of the protocol.

CHAPTER 3

Controlled human malaria infection with graded numbers of *Plasmodium falciparum* NF135.C10- or NF166.C8-infected mosquitoes

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Abstract

Controlled human malaria infections (CHMIs) with *Plasmodium falciparum* (*Pf*) parasites are well-established. Exposure to five *Pf* (NF54)-infected *Anopheles* mosquitoes results in 100% infection rates in malaria-naïve volunteers. Recently, *Pf* clones NF135.C10 and NF166.C8 were generated for application in CHMIs. Here, we tested the clinical infection rates of these clones, using graded numbers of *Pf*-infected mosquitoes. In a double-blind randomized trial, we exposed 24 malaria-naïve volunteers to bites from one, two, or five mosquitoes infected with NF135.C10 or NF166.C8. The primary endpoint was parasitemia by quantitative polymerase chain reaction. For both strains, bites by five infected mosquitoes resulted in parasitemia in 4/4 volunteers; 3/4 volunteers developed parasitemia after exposure to one or two infected mosquitoes infected with either clone. The prepatent period was 7.25 ± 4.0 days (median ± range). There were no serious adverse events and comparable clinical symptoms between all groups. These data confirm the eligibility of NF135.C10 and NF166.C8 for use in CHMI studies.

Introduction

Controlled human malaria infections (CHMIs) are a well-accepted tool used since the 1980s for the exploration of immunology and pathophysiology of malaria infections and for evaluation of candidate vaccines and drugs.[1] The majority of studies have been conducted with 3D7 and its parental strain NF54.[1] Most volunteers have been infected using five laboratory-reared *Plasmodium falciparum* (*Pf*)–infected *Anopheles* mosquitoes, which reproducibly result in optimal infection rates in malaria-naïve volunteers.[2,3] Reducing the number of NF54 or 3D7 *Pf*-infected mosquitoes to one or two reduces the infection rate.[4,5]

Given the diversity of *Pf* isolates in the field, the value of CHMI trials can be increased by expanding the portfolio with *Pf* clonal isolates from different geographical origins.[6] The *Pf* isolate NF54 most likely originates from Africa.[7] The more recently generated clones are NF135.C10 and NF166.C8, originating from Cambodia and Guinea, respectively. Initial studies with NF135.C10 and NF166.C8 show that these clones are safe and give high infection rates with five infectious mosquitoes (80% [8/10] and 100% [5/5], respectively).[8,9] However, these clones show more effective hepatocyte invasion and liver merozoite development as compared with NF54, which results in higher first peak of parasitemia and shorter prepatent period.[10] In order to establish an NF135.C10 or NF166.C8 CHMI model with comparable parasite dynamics as obtained in NF54-infected volunteers, the objective of the current study was to compare infection rates and dynamics of parasitemia as well as clinical manifestations using one, two or five NF135.C10- or NF166.C8-infected mosquitoes.

Materials and Methods

The study is a single-center, double-blind, randomized trial. The flowchart, screening procedures, and inclusion and exclusion criteria were described previously.[10] Briefly, 24 healthy male and female volunteers, aged 18–35 years, were randomly assigned to six groups and exposed to bites from five mosquitoes of which five, two, or one were infected with either NF135.C10 or NF166.C8. Uninfected mosquitoes were added to preserve blinding of all trial personnel except the staff members preparing and aliquotting infected mosquitoes.

Anopheles stephensi mosquitoes were reared and infected according to standardized protocols.[11,12] The average number of sporozoites per mosquito used in this study was 69,000 for NF135.C10 and 51,000 for NF166.C8. Mosquito feeding was allowed for 10 minutes and infectivity of mosquitoes was assessed after feeding by dissecting salivary glands for the presence of sporozoites. Feeding was repeated until the predefined number of infected bites was reached. Eight volunteers needed two sessions, one needed three sessions (all in the five- or two-mosquito bite groups).

After CHMI, volunteers were followed-up twice daily from day 5 post-infection until two consecutive blood samples were positive by quantitative polymerase chain reaction (qPCR) (≥500parasites/mL), or, when they remained negative, until day 13. At this point, volunteers were treated with atovaquone/proguanil and followed on days 1, 2, 3, and 7 after treatment

and on day 35 post-infection. Follow-up was prolonged if volunteers remained qPCR-positive or symptomatic. Throughout the study, high-sensitive troponin T, lactate dehydrogenase (LDH), and platelet counts were measured as safety parameters. The first peak of parasitemia was calculated as the geometric mean of *Pf* parasites per milliliter between day 6.4 and 8.4.[13]

The trial was registered at www.clinicaltrials.gov, identifier NCT02149550. Ethical approval from the Central Committee on Research Involving Human Subjects (NL48704.000.14) was obtained.

All statistical analyses were performed with IBM SPSS statistics for Windows (Version 23.0; IBM Corp., Armonk, NY). Mean prepatent period, first peak of parasitemia, and the frequency of adverse events (AEs) between the groups were assessed by using unpaired *t*-test, one-way analysis of variance (ANOVA), or their nonparametric variants. Adverse events were reported as mild (grade 1), moderate (grade 2), or severe (grade 3).

Results

In total, 20 of 24 volunteers developed parasitemia; all volunteers exposed to five infected mosquitoes and 3/4 volunteers exposed to one or two infected mosquitoes with either strain (Table 1).

The prepatent period in the NF135.C10 groups ranged from 7 to 9 days and did not differ significantly between the dosage groups (median [range] five mosquitoes: 7.5 [7.0–9.0] versus two mosquitoes: 9.0 [7.5–9.0] versus one mosquito: 7.0 [7.0–7.0]; P = 0.06, Table 1).

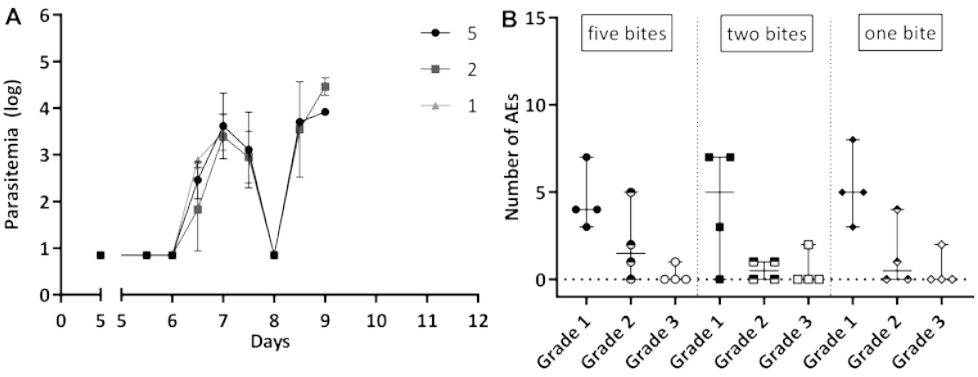

Figure 1. Comparison of one, two, and five NF135.C10-infected mosquito bites (**A**). Kinetics of parasitemia in *Pf*/mL (log) at days post-controlled human malaria infection until qPCR positive (two consecutive samples > 500 *Pf*/mL). Data represent the geometric means and error bars. (**B**). Number of graded adverse events (AE) per volunteer. Symbols represent individual volunteers, and horizontal lines and whiskers represent median and range, respectively. *Pf* = *Plasmodium falciparum*.

Table 1. The number of volunteers developing parasitemia and the prepatent period in days after infection with graded numbers of NF135.C10- or NF166.C8- infected mosquitoes

			No. of infectious bites				
			Five, two, or one	**Five**	**Two**	**One**	***P* value**
Strain	NF135.C10	Subjects PCR positive (*n*/total)	10/12	4/4	3/4	3/4	-
		Prepatent period (days (median [range]))	7.5 [7.0-9.0]	7.5 [7.0–9.0]	9.0 [7.5–9.0]	7.0 [7.0–7.0]	0.06
	NF166.C8	Subjects PCR positive (*n*/total)	10/12	4/4	3/4	3/4	-
		Prepatent period (days (median [range]))	7.0 [7.0-11.0]	7.0 [7.0–7.0]	7.0 [7.0–11.0]	9.0 [7.5–9.0]	0.09

There was no difference in first peak of parasitemia in parasites/mL (geometric mean (GM) [95% confidence interval (CI)] five mosquitoes: 2.9 [1.8–4.0], versus two mosquitoes: 2.4 [1.0–3.8], versus one mosquito: 3.0 [2.3–3.7]; P = 0.32) (Figure 1A). All parasitemic volunteers experienced one or more AEs. When comparing subjects challenged with either five, two, or one NF135. C10-infected mosquitoes, there were no differences in the total number of grade 1, 2, or 3 AEs (*P* = 0.81, *P* = 0.60, and *P* = 0.87, respectively) (Figure 1B), or the number of solicited AEs (headache, nausea, malaise, chills and myalgia, data not shown). A few unsolicited AEs were reported, two or three per group, all grade 1.

For the NF166.C8 clone, no significant difference was found in the prepatent period between the three dosage groups either (median [range] five mosquitoes: 7.0 [7.0–7.0] versus two mosquitoes: 7 [7.0–11.0] versus one mosquito: 9.0 [7.5–9.0]; P = 0.09, Table 1). In contrast to NF135.C10, the first peak of parasitemia was significantly higher in volunteers bitten by higher numbers of NF166.C8-infected mosquitoes (GM [95% CI] five mosquitoes: 3.8 [3.3–4.2], versus two mosquitoes: 2.4 [−0.9 to 5.6], versus one mosquito: 2.2 [1.7–2.7]; P = 0.04, Figure 2A). There were no differences in the number of grade 1, 2, or 3 AEs (*P* = 0.11, *P* = 0.33, and *P* = 0.90, respectively) (Figure 2B), nor in the number of solicited AEs between the volunteers challenged with either five, two, or one NF166.C8-infected mosquitoes (data not shown). A few unsolicited AEs were reported, four or six per group, all grade 1.

When comparing all subjects who developed parasitemia with either strain, the prepatent period was similar for NF135.C10 and NF166.C8 (median [range] 7.5 [7.0–9.0] versus 7.0 [7.0–11.0]; *P* = 0.65 see Table 1) as was the height of the first peak of parasitemia (GM [95% CI] NF135. C10: 2.8 [2.4–3.2] parasites/mL versus NF166.C8: 2.9 [2.1–3.6]; *P* = 0.48). In addition, there were no significant differences in the number of grade 1, grade 2, or grade 3 AEs between the two strains (*P* = 0.91, *P* = 0.07, *P* = 0.96).

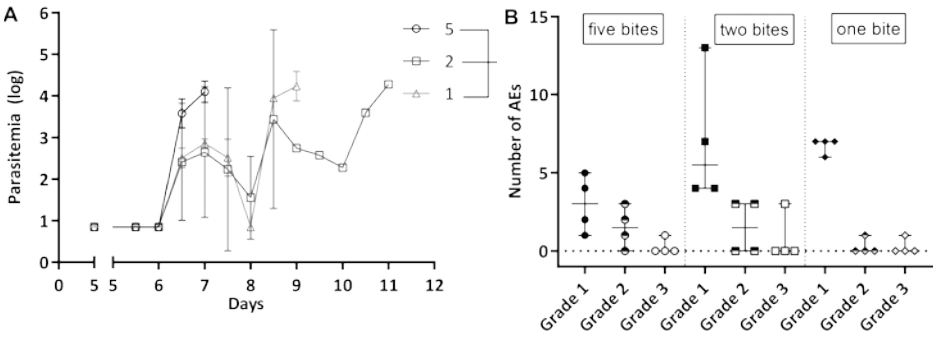

Figure 2. Comparison of one, two, and five NF166.C8-infected mosquito bites (A). Kinetics of parasitemia in Pf/mL (log) at days post-controlled human malaria infection until qPCR positive (two consecutive samples > 500 Pf/mL). Data represent the geometric means and error bars. Significant difference in first peak of parasitemia (geometric mean of day 6.4–8.4) between five, two, or one infected mosquito bites, P = 0.04 (**B**). Number of graded adverse events (AEs) per volunteer. Symbols represent individual volunteers, and horizontal lines and whiskers represent median and range, respectively. Pf = *Plasmodium falciparum*.

Upon antimalarial drug treatment, LDH was elevated (> 248 U/L, max 499 U/L) in 10 (50%) malaria-positive volunteers. Platelets were decreased (< 150 × 109/L, min 85 × 109/L) in 11/20. There was no relation with the *Pf*-clone used or the number of bites, and these changes in safety parameters were deemed clinically insignificant. The changes normalized by the end of the study. Throughout the trial hs-troponin T remained lower than 14.0 ng/L in all volunteers.

Discussion

This study shows that CHMI with five bites of NF135.C10- or NF166.C8-infected mosquitoes results in 100% patent parasitemia, which is comparable with the outcomes of previous CHMIs with NF54, 3D7, or 7G8.[3,14,15] Lowering the number of infectious bites of both test clones to one or two bites reduced the patency to 75% (3/4 individuals infected), which is similar to previous trials with 3D7-or NF54-infected mosquitoes showing parasitemia in 40-60% of the cases.[4,5] The kinetics of emerging NF135.C10 parasitemia remained relatively similar irrespective of the number of infected mosquitoes used. Theoretically, a relation with the number of infectious mosquito bites could be expected, as seen in NF166.C8. However, the variance in first peak of parasitemia in NF135.C10 was large, and these small groups did not allow to find differences. Parasite release from the liver may be subject to variation in sporozoite numbers delivered by the blood-feeding mosquitoes. We previously estimated from CHMI studies that on average 21 sporozoites per mosquito infect hepatocytes, whereas others estimated a release in the range of 1–369 per mosquito.[16,17] In addition, the probability of infection decreases with a lower

sporozoite load (0–1,000) per mosquito.[18] However, in our studies mosquitoes are infected with >25,000 sporozoites/mosquito where no such relation exists.[19]
Differences in patency between one, two, or five infected bites suggest there is a strain-independent threshold to be overcome before successful infection occurs which can stochastically result in zero successful events. Such bottlenecks may include the frequency of mosquito probing, the number of sporozoites entering the blood stream, and the number that invade hepatocytes. After hepatocyte invasion, the efficiency of parasite multiplication

may differ between strains with a potential higher first peak of parasitemia and correspondingly shorter prepatent periods for NF135.C10 and NF166.C8. This difference in multiplication inside hepatocytes is unlikely related to differences in fitness of these clones, as the IC50 of CSP-antibodies blocking *in vitro* hepatocyte development is comparable.[9]
Data from this and other studies with NF135.C10- and NF166.C8-infected volunteers showed that there is no increase in the number or severity of AEs compared with NF54-infected volunteers, despite the higher first peak of parasitemia.[10] The prevalence and severity of AEs will likely further reduce if antimalarial treatment is initiated upon more stringent qPCR cut-off values.[20]

In conclusion, this study with limited numbers of volunteers per group shows that bites of five mosquitoes of NF135.C10 and NF166.C8 of either clone consistently gives 80–100% patent parasitemia in the three studies performed with these heterologous clones. In future CHMI studies using clones NF135.C10 and NF166.C8 and lower numbers of mosquito bites may suffice to achieve blood-stage dynamics which are more similar to NF54, albeit reaching lower infection rates.

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CHAPTER 4

A double-blind, placebo-controlled phase 1/2a trial of the genetically attenuated malaria vaccine PfSPZ-GA1

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Abstract

Immunization with attenuated *Plasmodium* sporozoites can induce protection against malaria infection, as shown by *Plasmodium falciparum* (Pf) sporozoites attenuated by radiation in multiple clinical trials. As alternative attenuation strategy with a more homogeneous population of Pf sporozoites (PfSPZ), genetically engineered *Plasmodium berghei* sporozoites (SPZ) lacking the genes *b9* and *slarp* induced sterile protection against malaria in mice. Consequently, PfSPZ-GA1 Vaccine, a Pf identical double knockout (PfΔ*b9*Δ*slarp*), was generated as a genetically attenuated malaria parasite vaccine and tested for safety, immunogenicity, and preliminary efficacy in malaria-naïve Dutch volunteers. Dose-escalation immunizations up to 9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine were well tolerated without breakthrough blood-stage infection. Subsequently, groups of volunteers were immunized three times by direct venous inoculation with cryopreserved PfSPZ-GA1 Vaccine (9.0×10^5 or 4.5×10^5 PfSPZ, $N = 13$ each), PfSPZ Vaccine (radiation-attenuated PfSPZ, 4.5×10^5 PfSPZ, $N = 13$), or normal saline placebo at 8-week intervals, followed by exposure to mosquito bite controlled human malaria infection (CHMI). After CHMI, 3 of 25 volunteers from both PfSPZ-GA1 groups were sterilely protected, and the remaining 17 of 22 showed a patency ≥9 days (median patency in controls, 7 days; range, 7 to 9). All volunteers in the PfSPZ Vaccine control group developed parasitemia (median patency, 9 days; range, 7 to 12). Immunized groups exhibited a significant, dose-related increase in anti-Pf circumsporozoite protein (CSP) antibodies and Pf-specific interferon-γ (IFN-γ)–producing T cells. Although no definite conclusion can be drawn on the potential strength of protective efficacy of PfSPZ-GA1 Vaccine, the favorable safety profile and induced immune responses by PfSPZ-GA1 Vaccine warrant further clinical evaluation.

Introduction

A recent resurgence in *Plasmodium falciparum (*Pf) malaria cases after years of control underscores the need for a highly efficacious vaccine for elimination (1). The Pf circumsporozoite protein (CSP) subunit vaccine RTS,S/AS01E (Mosquirix, GlaxoSmithKline) is the only malaria vaccine to move beyond phase 3 clinical trials, although it provides only short-term and partial clinical vaccine efficacy (2).

In the past decade, there has been a growing interest in attenuated whole Pf sporozoite (PfSPZ) vaccines based on the idea that this whole-organism immunization will be able to induce the protection needed against the breadth of antigens present in the parasite. The first approach to immunizing humans with radiation-attenuated PfSPZ was developed almost 50 years ago (3) and has now been translated to a vaccine, consisting of radiation-attenuated, metabolically active, aseptic PfSPZ that meet regulatory standards for direct venous inoculation (DVI). This product, Sanaria PfSPZ Vaccine, has shown an excellent safety profile in 1595 subjects aged 5 months to 65 years in 20 clinical trials in the United States, Europe, and Africa (4–6). Furthermore, it has provided protection against controlled human malaria infection (CHMI) and malaria infections in the field (4, 7, 8).

Radiation induces random DNA damage in the parasite genome, generating a heterogeneous nonreplicating population of PfSPZ. These PfSPZ invade hepatocytes, partially develop, and then arrest at an early stage in the liver (9). As an alternative to radiation-based attenuation, genetic modification generates a homogeneous formulation of PfSPZ, which stop development in the liver at a well-defined point (10). In rodent models, immunization with genetically attenuated malaria SPZ can induce similar, or even greater, protective immunity compared to radiation-attenuated malaria SPZ (11). The intrinsic and irreversible nature of the genetic attenuation greatly reduces safety risks during manufacturing of PfSPZ. Consequently, several liver-arresting genetically attenuated Pf parasites have been generated (12–14), two of which have been tested for safety in volunteers by mosquito bite (13, 15).

We engineered attenuated PfSPZ by deletion of two genes encoding *slarp* and *b9*, each governing independent and critical processes for successful liver-stage development (12). Pf double-knockout (Pf*Δb9Δslarp*) SPZ were capable of invading primary human hepatocytes *in vitro*, but arrested growth early after invasion and were not detected at days 2 to 7 after infection, similar to PfSPZ Vaccine. Pf*Δb9Δslarp* parasite development was fully abrogated in the liver of humanized mice (12). SPZ of the equivalent rodent *Plasmodium berghei*–attenuated parasite (Pb*Δb9Δslarp*) also showed aborted liver-stage development while retaining the capacity to induce fully protective immunity in both the BALB/c and C57BL/6 mouse models (12). These preclinical data justified formulation and clinical assessment of Pf*Δb9Δslarp*.

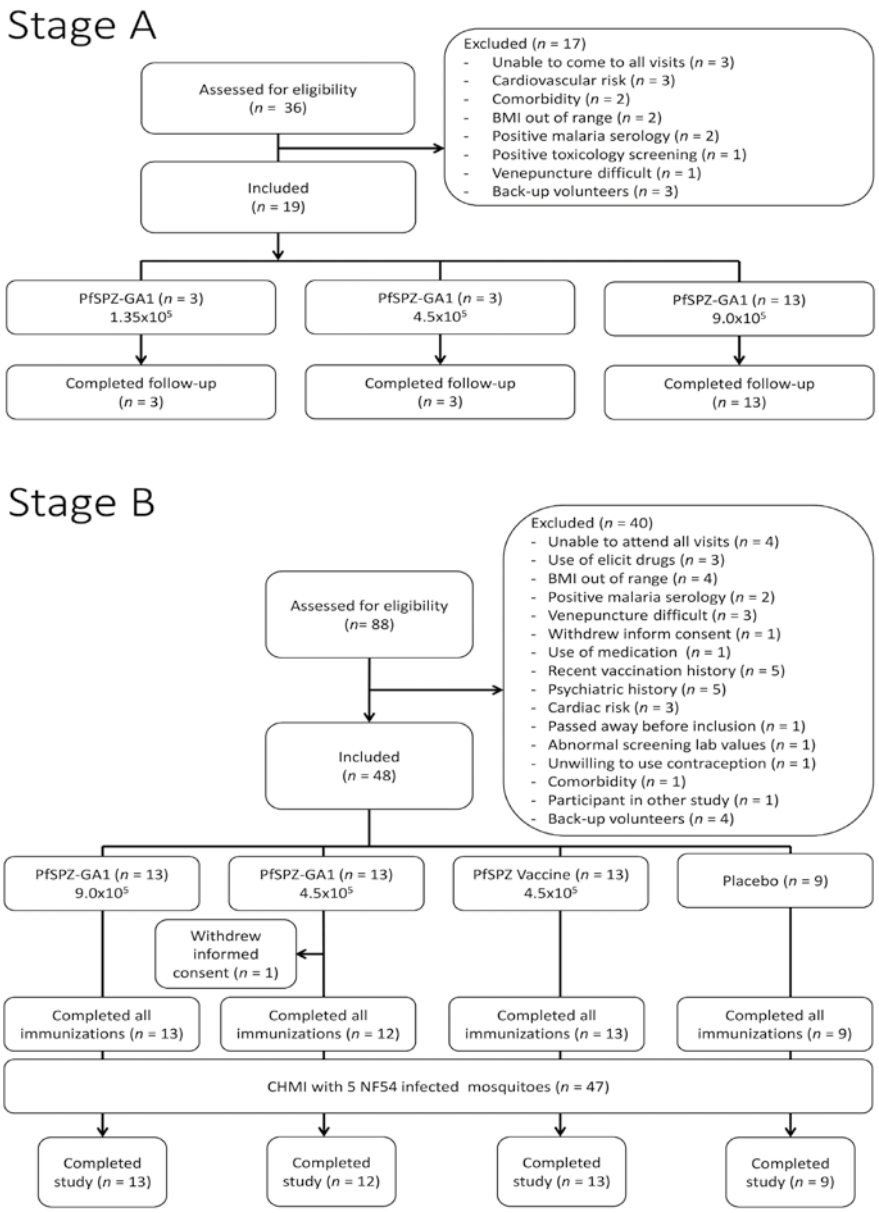

Figure 1. Study flow chart. Manufacture of aseptic, purified, and cryopreserved PfΔ*b9*Δ*slarp* PfSPZ (Sanaria PfSPZ-GA1 Vaccine) was performed in compliance with Good Manufacturing Practice (16). We report the first-in-human evaluation of PfSPZ-GA1 Vaccine (NCT0316121). We tested safety and immunogenicity of PfSPZ-GA1 Vaccine and subsequently examined the protective vaccine efficacy against a homologous CHMI with wild-type (WT) Pf (NF54) and compared this to a previously tested regimen of PfSPZ Vaccine.

Results

Study population

In total, 124 malaria-naïve adults were screened for participation in the study from 1 May to 28 November 2017. Nineteen volunteers were selected as volunteers in the safety dose-escalation stage A of the study, and 48 were selected for the immunogenicity and preliminary efficacy stage B. In addition, six backup volunteers were enrolled in stage B to replace any dropouts before immunization. One volunteer withdrew informed consent after the second immunization in stage B of the trial for reasons unrelated to the trial; all others completed follow-up (Fig. 1). In total, 34 of 67 (51%) were males. Mean age of the volunteers was 23 years old (SD, 4; range, 18 to 34), and mean body mass index (BMI) was 23.5 kg/m2 (SD, 3.0; range, 18 to 30) (Table 1). Volunteers in stage A were immunized once with escalating doses up to 9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine, whereas in stage B volunteers were randomized double blind to receive three doses of 4.5×10^5 or 9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine, 9.0×10^5 PfSPZ of PfSPZ Vaccine, or saline placebo at 8-week intervals.

Table 1. Volunteer demographics.

		STAGE A			STAGE B	Total
		Group A1	Group A2	Group A3		
Number of volunteers		3	3	13	48	67
Age (years)	Mean	23	25	23	23	23
	SD	3	3	5	3	4
	Median	23	26	23	23	23
	Min, Max	20, 26	20, 29	18, 34	18, 33	18, 34
Sex	Male	1	2	5	26	34
	Female	2	1	8	22	33
BMI	Mean	25.3	23.9	23.6	23.3	23.5
	SD	0.7	2.7	2.9	3.1	3.0
	Median	25.1	24.9	23.6	22.5	23.4
	Min, Max	25, 26	18, 26	19, 29	18, 30	18, 30

Safety results

No serious adverse events occurred during this trial. None of the blood samples taken for blood-stage infection at any time point after DVI of 1.35×10^5, 4.5×10^5, or 9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine in stage A and after any of three immunizations with 4.5×10^5 or 9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine in stage B were positive for parasite DNA. Blood samples were tested for erythrocytic-stage parasites by quantitative polymerase chain reaction (qPCR) every day from day 6 to day 21 after immunization and on day 28 in stage A and on day 14 after each immunization in stage B (Fig. 2).

All immunizations with PfSPZ-GA1 Vaccine and PfSPZ Vaccine were well tolerated, and there were no significant differences in incidence or severity of adverse events between vaccine and placebo groups in stage B. A total of 66 related adverse events were reported after immunization (table S1). DVI was successful after a single needle stick in 93% of injections (151 of 162 injections), and after three attempts, all but one DVI was successful. Volunteers reported no or mild pain during injection; only one volunteer reported severe pain once for a few seconds during needle insertion. Bruising after DVI was the most commonly reported local adverse event, occurring in 7 of 67 (10%) of volunteers. Headache and fatigue/malaise were the most frequently reported systemic adverse events (reported by 31 and 14 volunteers, respectively) in both intervention and placebo groups, of which three events were severe. One severe unsolicited adverse event probably related to immunization occurred when a volunteer experienced a vasovagal reaction during immunization. There were no clinically significant laboratory abnormalities.

The most common adverse events after CHMI with Pf WT NF54 were headache (52% of volunteers) and fatigue (51%). One volunteer (placebo group) reported severe chills 2 days after atovaquone/proguanil treatment for blood test–positive Pf malaria. Two volunteers (placebo group and PfSPZ Vaccine group) reported severe dizziness on the first and third day of atovaquone/proguanil treatment. All adverse events resolved without sequelae.

There were two cases of mild (grade 1) highly sensitive troponin T elevation to a maximum of 19 ng/ml (reference, <14 ng/ml) 10 to 12 days after CHMI at the time when blood samples were positive for Pf by qPCR: one (9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine group) deemed probably related and one (4.5×10^5 PfSPZ of PfSPZ-GA1 Vaccine) deemed possibly related to CHMI. Both volunteers were asymptomatic, and electrocardiogram did not show abnormalities. Both volunteers were treated with atovaquone/proguanil on the first day of troponin elevation, at which time blood samples were positive for Pf. The highly sensitive troponin T concentration decreased to normal range within a day, and volunteers experienced no sequelae.

Protective efficacy against CHMI

To obtain a preliminary measure of PfSPZ-GA1 vaccine efficacy (VE), the immunized volunteers in the stage B study underwent CHMI with Pf NF54 WT parasites by mosquito bite 3 weeks after the final immunization. The volunteers were monitored on a daily basis, and blood samples were tested for the presence of parasites by qPCR (Fig. 2). Although the primary endpoint of proportion protected was not significantly different between any vaccine groups and the placebo control group, all vaccine groups showed a significant delayed time to positive qPCR as compared to the placebo (Fig. 3; log-rank test, $P = 0.0003$). All volunteers in the placebo group developed parasitemia, with a median of 7 days after CHMI (seven volunteers at day 7, one at day 8, and one at day 9). All 13 volunteers immunized with the control 4.5×10^5 PfSPZ of PfSPZ Vaccine developed parasitemia, with a median delay of 2 days (median, 9; range, 7 to 12 days, compared with placebo Mann-Whitney, $P = 0.0078$). After CHMI, 3 of 25 volunteers from both PfSPZ-GA1 groups were sterilely protected. Immunization with 4.5×10^5 PfSPZ of PfSPZ-GA1 Vaccine resulted in 11 of 12 volunteers developing blood-stage parasitemia, with a median delay of 2 days (median, day 9; range, 7 to 12 days, compared with placebo Mann-Whitney, $P = 0.0005$). In the highest-dose PfSPZ-GA1 group, 11 of 13 volunteers became qPCR positive, with a median 4-day delay (median prepatent period, 11 days; range, 7 to 12 days, compared with placebo Mann-Whitney, $P = 0.0018$). This study was not powered to detect significant differences in time to positive qPCR between vaccine groups.

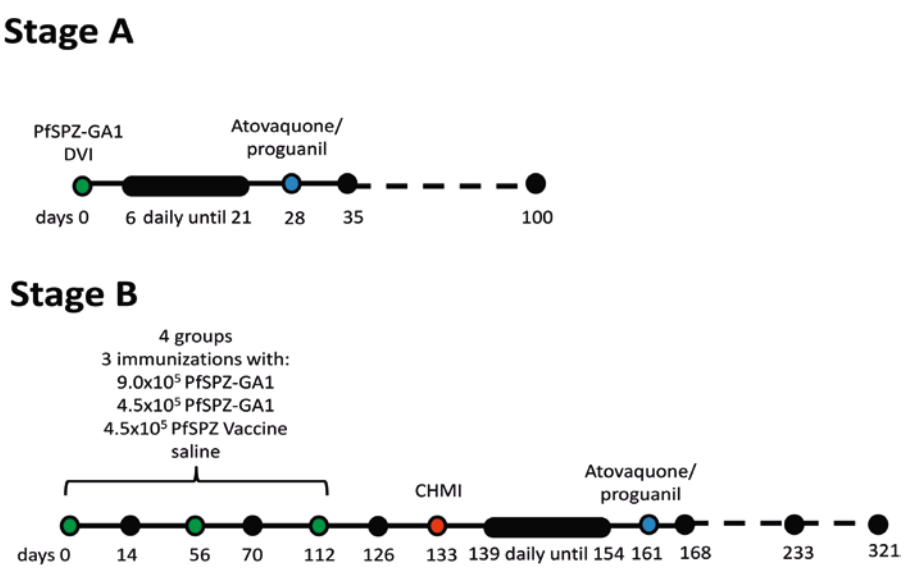

Figure 2. Study design. In stage A study, volunteers were immunized by direct venous inoculation (DVI) with either 1.35 × 10^5, 4.5 × 10^5, or 9.0 × 10 PfSPZ of PfSPZ-GA1 Vaccine (*n* = 3, 3, or 13, respectively; green circle), after which blood samples were taken on a daily basis from day 6 until day 21 (black circles). At day 28 (blue circle), all volunteers were treated with a curative regimen of atovaquone/proguanil. Final visits were at days 35 and 100. In stage B study, four groups of volunteers were immunized three times (green circles) with either 4.5 × 10^5 (*n* = 13) or 9.0 × 10^5 PfSPZ (*n* = 13) of PfSPZ-GA1 Vaccine or 4.5 × 10^5 PfSPZ of PfSPZ Vaccine (*n* = 13) or saline placebo *(n* = 9) by DVI, with blood samples taken for blood-stage parasitemia at day 14 after every immunization. CHMI by mosquito bite with WT Pf NF54 (red circle) was performed 3 weeks after the final immunization, after which daily follow-up was performed from day 139 to day 154. All volunteers received curative treatment with atovaquone/proguanil (blue circle) and came for three final follow-up visits.

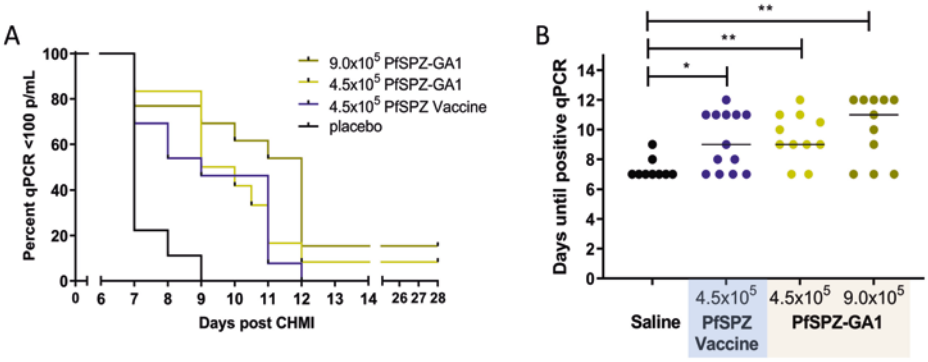

Figure 3. Parasitemia after CHMI. (**A**) Kaplan-Meier showing number of volunteers without blood-stage parasitemia as measured by qPCR between 0 to 28 days after CHMI (log-rank, *P* = 0.0003) and (**B**) days until patency [qPCR > 100 p/ml, for the 9.0 × 10^5 PfSPZ-GA1 Vaccine (dark green), 4.5 × 10^5 PfSPZ-GA1 Vaccine (green), 4.5 × 10^5 PfSPZ Vaccine (blue), and placebo group (black)]. Lines indicate median. (B) shows significance by Mann-Whitney test: **P* < 0.05 and ***P* < 0.01.

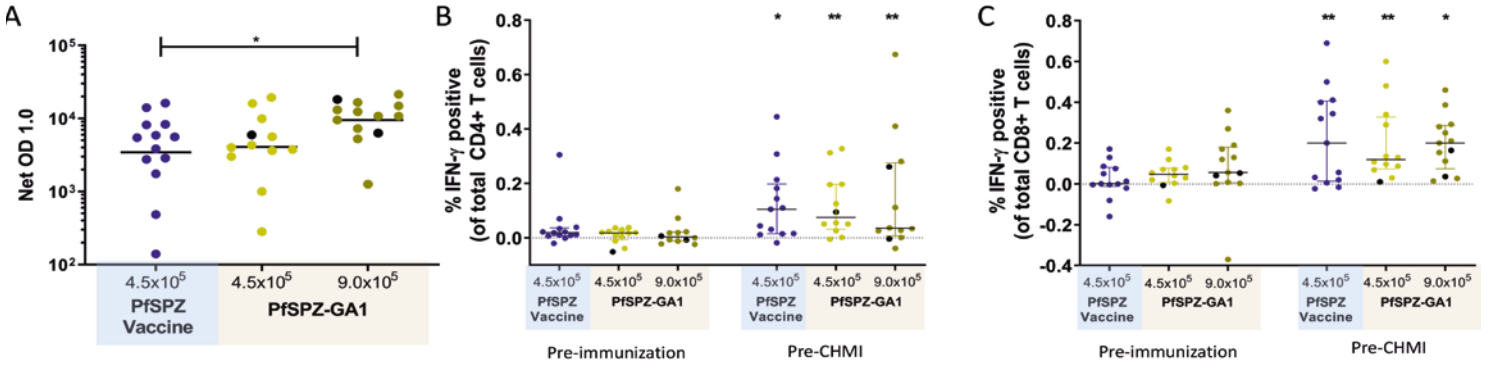

Figure 4. Immune responses after three vaccine doses. (**A**) Anti-PfCSP antibody titers as determined by ELISA for the 9.0 × 10^5 PfSPZ-GA1 Vaccine (dark green), 4.5 × 10^5 PfSPZ-GA1 Vaccine (green), and 4.5 × 10^5 PfSPZ Vaccine (blue) groups 14 days after the final immunization. Fully protected volunteers shown in black lines indicate geomeans. One-way ANOVA post hoc Tukey: **P* < 0.05. Number of (**B**) IFN-γ–producing $CD4^+$ and (**C**) $CD8^+$ T cells determined by flow cytometry after stimulation with infected RBC before immunization and the day before CHMI for the 9.0 × 10^5 PfSPZ-GA1 Vaccine (dark green), 4.5 × 10^5 PfSPZ-GA1 Vaccine (green), 4.5 × 10^5 PfSPZ Vaccine (blue), and placebo group (black). Fully protected volunteers are displayed in black. Lines indicate medians with interquartile range, and dotted line indicates zero response. Paired *t* test of pre-CHMI data with pre-immunization data: **P* < 0.05 and ***P* < 0.01.

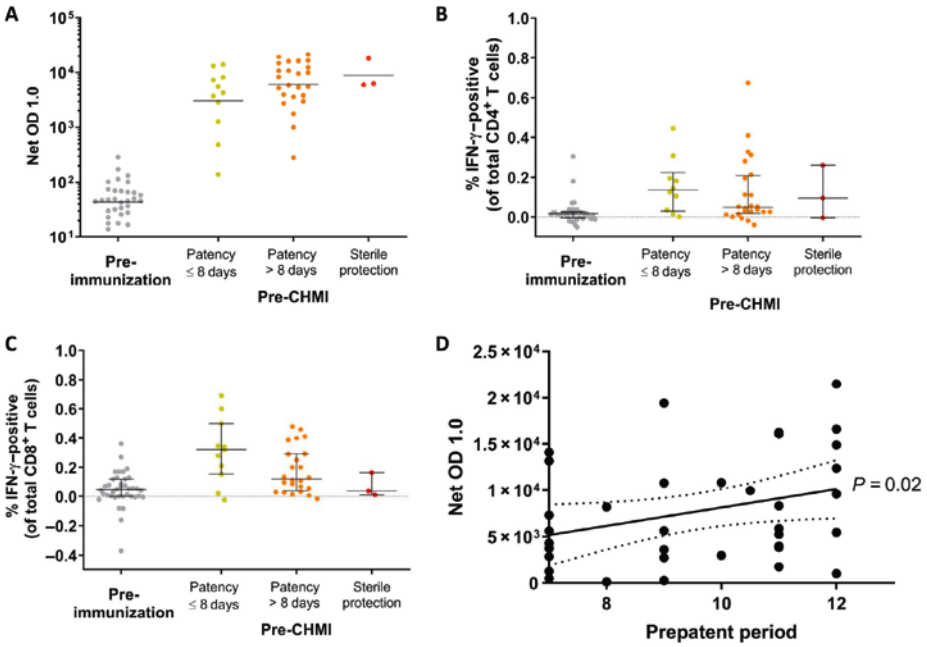

Figure 5. Relationship between immune parameters and protection. (**A**) Relationship between anti-PfCSP antibody titers (lines indicate geomean), % of (**B**) IFN-γ–producing $CD4^+$, or (**C**) $CD8^+$ T cells (lines indicate median and interquartile ranges) and the protection status of volunteers. Protection grouped by day of positive qPCR (patency) ≤8 or >8 or "sterile" if qPCR negative until day 28. One-way ANOVA, P = 0.05. (D) Correlation of anti-PfCSP antibody titer 14 days after the final immunization with prepatent period. Pearson correlation P = 0.02 and r = 0.32.

Immunogenicity

All immunized groups showed a significant increase in antibody titers against PfCSP between pre-immunization and pre-CHMI time points ($P < 0.0001$, paired t test overall; Fig. 4A). Immunization with 4.5×10^5 PfSPZ of PfSPZ Vaccine and PfSPZ-GA1 Vaccine induced similar anti-PfCSP antibody titers, whereas immunization with 9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine produced significantly higher anti-PfCSP titers [Fig. 4A; one-way analysis of variance (ANOVA) Tukey post hoc mean difference, 5573; 95% confidence interval (CI), 332 to 30,823; $P = 0.04$].

Peripheral blood cells from all immunized groups exhibited a significant increase in $CD4^+$ and $CD8^+$ T cells producing interferon-γ (IFN-γ) upon stimulation with Pf-infected red blood cells after three immunizations (PfRBC; Fig. 4, D and E; $P < 0.03$) as compared to baseline. In total, 35 and 32% of all immunized volunteers were IFN-γ responders for CD4+ or CD8+ T cells, respectively, with 46% of volunteers showing an increase in at least one subset.

To examine whether there was an association between an increase in antibody or cellular responses and protection, data from immunized individuals were segregated on the basis of protection status (Fig. 5A). The anti-PfCSP antibody titers correlated significantly with time until

positive qPCR-based blood-stage patency (Pearson correlation $r = 0.32$; 95% CI, −0.01 to 0.59; $P = 0.02$, $R^2 = 0.1$; Fig. 5D). However, cellular responses did not correlate with protection (one-way ANOVA for patency ≤8 days, >8 days, and full protection, $P = 0.05$).

We thus demonstrate that immunization with three doses of 4.5×10^5 PfSPZ of PfSPZ Vaccine and PfSPZ-GA1 induced similar anti-PfCSP antibody responses and that there was a dose-dependent increase in anti-PfCSP responses after immunization with 9.0×10^5 PfSPZ of PfSPZ-GA1. Moreover, anti-PfCSP antibody responses correlated with protection.

Discussion

Here, we report the first-in-human administration and efficacy data of the live, injectable, nonreplicating, genetically attenuated PfSPZ, PfSPZ-GA1 Vaccine. PfSPZ-GA1 Vaccine was safe and well tolerated, and no blood-stage infections were observed in 45 volunteers after 97 injections, totaling more than 6×10^7 PfSPZ administered by DVI. PfSPZ-GA1 Vaccine was immunogenic and induced both antibody and $CD4^+$ and $CD8^+$ T cell responses, with a potency analogous to the comparator PfSPZ Vaccine. Homologous CHMI through the bites of WT (Pf NF54)–infected mosquitoes 3 weeks after the last immunization resulted in three fully protected individuals and delays in time to patency in 17 of 25 volunteers at both dosages of PfSPZ-GA1 Vaccine. The delay in time to patency correlated with increased anti-PfCSP antibody responses. This study shows that an injectable, double gene deletion attenuated parasite vaccine is safe and immunogenic in humans. Previously, two genetically attenuated parasites (GAPs) have undergone safety evaluation in healthy volunteers in an experimental setting through the bites of infected *Anopheles* mosquitoes (13, 15). One GAP, lacking the two genes *p52* and *p36*, showed a blood-stage infection in a single volunteer after being exposed to 200 infectious bites (15). The breakthrough blood parasites were confirmed as having the *p52* and *p36* gene deletion genotype, indicating that deletion of these two genes was not sufficient to result in a complete growth arrest in the liver stage. This same incomplete attenuation phenotype (at high infection doses) had also been observed in rodent malaria parasites lacking the same genes (17). In a subsequent study, another GAP was analyzed, which additionally included a deletion of the *slarp* gene, also referred to as *sap1*. This triple-knockout GAP (Pf*Δp52Δp36Δsap1*) was administered to healthy volunteers through the bites of 150 to 200 infectious *Anopheles* mosquitoes, and no breakthrough blood infections were observed (13). No protective efficacy studies of the triple-knockout GAP have been reported yet. In contrast to Pf*Δp52Δp36Δsap1*, PfSPZ-GA1 Vaccine was administered as an injectable vaccine.

We found a delay in patency up to day 12 after CHMI as compared to a 7- to 9-day patency in controls, reflecting a 2-log reduction in parasites released from the liver in volunteers vaccinated with both doses of PfSPZ-GA1. However, the interpretation of the vaccine efficacy data is complicated by the unexpected low efficacy of the PfSPZ Vaccine reference group. The dose of the control PfSPZ Vaccine was chosen on the basis of a previous study in which three doses of 4.5×10^5 PfSPZ of PfSPZ Vaccine by DVI protected 13 of 15 volunteers from mosquito bite CHMI 3 weeks after the last immunization (18). The same dose was selected for PfSPZ-GA1 in group 1 to enable a comparison of the two vaccines' immunogenicity. As anticipated when designing the trial, the PfSPZ Vaccine group allows us to put the vaccine efficacy results in perspective of

previous trials with the PfSPZ Vaccine as reference. This difference in vaccine efficacy of PfSPZ Vaccine between the study in the United States and our study may be explained by either (i) differences in the stringency of the CHMI or (ii) differences in the immunogenicity of PfSPZ Vaccine in the two studies. With regard to the first possibility, both studies used mosquito bite–based CHMI at 3 weeks after the last immunization. Mosquito bite CHMI is more similar to natural infections as it includes the possibly relevant SPZ skin stage, where antibodies against SPZ may have an effector function (19, 20). However, in the Epstein *et al*. study (18), the 3D7 clone of the NF54 strain of Pf was used in the CHMI, whereas in this study we used the NF54 strain of Pf, which is not clonal. Small genotypic differences between these two strains have been identified (21), but it remains unclear whether these are relevant and result in differences in prepatent period as observed in independent clinical trials (22). Unfortunately, direct comparisons have not been performed. Because PfSPZ-GA1 was created in an NF54 background, we would expect the NF54 CHMI to be more homologous as compared to the 3D7 CHMI. In addition, the primary parasitological outcome variable differed between the trials (qPCR in The Netherlands and thick smear in the United States), so prepatent periods cannot be directly compared, making it difficult to assess challenge stringency. However, for both strains, five mosquito bites are needed to achieve a virtually 100% infection rate and ultimately cannot account for the lower than expected vaccine efficacy. Thus, we do not think that differences in stringency of CHMI can explain the difference in VE between the two studies. Moreover, we have broad experience with this mosquito bite CHMI model, including studies in which we show 100% vaccine efficacy by the chemoprophylaxis with SPZ approach against homologous Pf NF54 CHMI (23–25).

Both PfSPZ Vaccine and PfSPZ-GA1 Vaccine were immunogenic and induced anti-PfCSP antibodies and PfRBC-specific T cells. There was a positive association between anti-PfCSP antibodies and the protection status of the volunteers as measured by prepatent period. However, less than half of volunteers had significant induction of T cell responses, and $CD8^+$ responses were inverse related to prepatency. Whether this reflects a compartmental shift of $CD8^+$ T cells to nonlymphoid tissues, as observed in animal models (26), will require further study. This is in contrast with other PfSPZ Vaccine studies (7, 8), in which typically most volunteers show induction of $CD4^+$ T cell responses, although $CD8^+$ T cell responses have been variable. Possibly a difference in the *in vitro* cell stimulus (PfRBC versus PfSPZ) could also explain this difference, and therefore, in future studies with GAP vaccines, it would be of importance to also compare these two stimuli. However, anti-PfCSP antibody titers in our study were also significantly lower than those found in the Epstein *et al*. study (18) after immunization with PfSPZ Vaccine using the same schedule and dose of 4.5×10^5 PfSPZ (median level [net optical density (OD), 1.0] at 2 weeks after the third dose of 19,044 versus 5465).

On the basis of these data, we consider a lower vaccine immunogenicity of PfSPZ Vaccine compared to the Epstein *et al*. study (18) to be a likely explanation for the decreased vaccine efficacy of PfSPZ Vaccine observed in our study. However, the true cause of the decreased immunogenicity remains unclear. Retrospective evaluation did not reveal any procedural complications or deviations from established protocols in vaccine transport, storage, or administration. In addition, the trial in our study was performed in two centers, with different teams performing vaccine preparation and administration and yet both showed similar immunogenicity results. Vaccine lot–specific problems also do not seem a likely explanation, given experiences in other sites with parallel trials. Although we observed that PfSPZ-GA1 Vaccine appeared to be as immunogenic as PfSPZ Vaccine, at an equivalent dose, the unexpected low vaccine efficacy of the PfSPZ Vaccine comparator limits our ability to draw firm conclusions on

the VE of PfSPZ-GA1 Vaccine. However, given the suboptimal vaccine efficacy of PfSPZ-GA1, the next-generation genetically attenuated PfSPZ vaccines should aim at enhanced potency either by increasing dose or potentially through an arrest later in the liver stage.

An alternative to Pf gene deletion mutants as whole SPZ vaccine is the use of *Plasmodium* species that are nonpathogenic for humans expressing selected Pf target proteins. Transgenic murine *P. berghei* SPZ expressing the Pf CSP were recently tested in a clinical trial for safety, immunogenicity, and protective efficacy against a CHMI (27).

This study demonstrates that a genetically attenuated, live parasite vaccine, PfSPZ-GA1, can be safely administered to malaria-naïve volunteers by DVI. Genetically attenuated PfSPZ have advantages over other whole PfSPZ vaccination strategies, because they can improve the safety and consistency of manufacturing. Although the potential protective efficacy of PfSPZ-GA1 Vaccine cannot be fully appreciated in this trial, the data show clear immunogenicity combined with a favorable safety and tolerability profile. The current trial underscores the clinical potential of genetically attenuated vaccines, boosting further development of such malaria vaccine strategies.

Materials and Methods

Study design

The study was designed as a multicenter phase 1, open-label, dose-escalating trial to assess safety, tolerability, and immunogenicity of PfSPZ-GA1. In the initial, open-label safety stage of the trial (stage A), single escalating doses of PfSPZ-GA1 were administered by DVI to three groups of healthy adults at the Leiden University Medical Center (LUMC). Group A1 (n = 3) was inoculated with 1.35×10^5 PfSPZ of PfSPZ-GA1 Vaccine, group A2 (n = 3) was inoculated with 4.5×10^5 PfSPZ of PfSPZ-GA1 Vaccine, and group A3 (n = 13) was inoculated with 9.0×10^5 PfSPZ of PfSPZ-GA1 Vaccine. For this initial proof-of-concept study, a dose of 1.35×10^5 PfSPZ of PfSPZ-GA1 Vaccine was chosen because this is the lowest dose at which PfSPZ Vaccine has shown to induce protective immunity (18), whereas after three doses of 9.0×10^5 PfSPZ Vaccine >90% VE was to be expected. This is based on a study where three doses of 4.5×10^5 PfSPZ induced >80% protection (18). In the follow-on efficacy stage of the trial (stage B), a total of 48 volunteers were included at LUMC (n = 24) and Radboud University Medical Center (RUMC) (n = 24), with double-blind randomization over four study groups according to a randomization list prepared by the study head pharmacist. Randomization was stratified per study site. The investigator, site personnel, and the sponsor were masked to treatment assignment. The site pharmacist or qualified employees were not masked and prepared the assigned vaccines. Groups 1 and 2 received three immunizations with PfSPZ-GA1 Vaccine at doses of 9.0×10^5 (n = 13) and 4.5×10^5 (n = 13) PfSPZ. Group 3 received three immunizations with the control PfSPZ Vaccine at a dose of 4.5×10^5 PfSPZ (n = 13), and group 4 was injected three times with normal saline as placebo (n = 9). All immunizations in stage B were administered at 8-week intervals. Three weeks after the final immunization, all stage B volunteers were exposed to five bites of Pf NF54–infected *Anopheles stephensi* mosquitoes according to previously described procedures to assess vaccine efficacy (28). The primary objective of the study was to investigate the safety and tolerability of PfSPZ-GA1 Vaccine, by analysis of (i) the presence of blood-stage parasites after inoculation and (ii) the frequency and magnitude of adverse events. A

secondary objective was the VE of PfSPZ-GA1 Vaccine against mosquito bite CHMI with Pf NF54 SPZ, as assessed by the presence or absence of parasitemia after CHMI. The presence of blood-stage parasites after inoculation with PfSPZ-GA1 Vaccine and the frequency and magnitude of adverse events after immunization were primary endpoints. The presence of blood-stage parasites after immunization was a stopping criterium.

We calculated a sample size of 13 immunized subjects per group and 9 infectivity controls for the first CHMI to show with a power of 80% that a 50% blood-stage parasite positive rate in the immunized group and 100% in the control group are significantly different ($\alpha < 0.05$, two-tailed), assuming that one subject drops out from the immunized group (final $N = 12$).

The clinical trial was conducted under a U.S. Food and Drug Administration (FDA) Investigational New Drug (IND) application and was approved by the central committee for research involving human subjects in The Hague [Centrale Commissie Mensgebonden Onderzoek (Central Committee on Research Involving Human Subjects) (CCMO; NL56657.000.16)]. It was performed in The Netherlands under a license from the Dutch Ministry of Infrastructure and Environment (Ministerie van Infrastructuur en Milieu) for deliberate release of genetically modified organisms (IM-MV 15-004 and IM-MV 15-009). The study was registered at ClinicalTrials.gov (NCT03163121). Primary data are reported in data file S1.

Production of PfSPZ-GA1

The genetically attenuated Pf NF54 parasite, PfΔ*b9*Δ*slarp* (12), lacks two genes, *b9* and *slarp*, which are vital for liver-stage development (12). Master and working cell banks were generated from the clone Pf NF54 parasite, PfΔ*b9*Δ*slarp*, filed under an FDA Master File and IND application, resulting in the product referred to as PfSPZ-GA1 Vaccine. PfSPZ-GA1 parasites were tested sensitive to the anti-malarial drugs chloroquine, mefloquine, artemether/lumefantrine, atovaquone/proguanil, and pyrimethamine.

Manufacture of PfSPZ-GA1 Vaccine bulk product followed the identical manufacturing schema of PfSPZ (NF54) Vaccine (16) except for several tests of vialed final products that were specific to PfSPZ-GA1 Vaccine. These tests included a PCR test for identity that confirmed the genetic signature of PfΔ*b9*Δ*slarp* (12), the potency assay that documented 3-day parasites that were developed in HCO4 cells *in vitro*, and the 6-day safety assay that confirms the absence of late-stage developing parasites *in vitro*. The manufacturing process generated aseptic *A. stephensi* mosquitoes that were infected with PfΔ*b9*Δ*slarp* (12). PfSPZ were harvested, purified, vialed, cryopreserved, and shipped in liquid nitrogen vapor phase at −150° to −196°C. On the day of administration, vials of PfSPZ-GA1 Vaccine were thawed and diluted using phosphate-buffered saline and 25% human serum albumin (CSL Behring) to the correct dose in a sterile environment.

Participants

A total of 67 healthy malaria-naïve male and female volunteers aged 18 to 35 years were recruited for the study. All included volunteers were in good health as assessed by medical history, physical examination, general chemistry and hematology evaluation, and an electrocardiogram. All included volunteers provided informed consent, and females were counseled to use adequate

contraception. A detailed list of inclusion and exclusion criteria is provided in the Supplementary Materials.

Procedures

Volunteers were immunized by a trained nurse administering 0.5 ml of the vaccine by DVI through a 25-gauge needle. Volunteers were observed for 30 min after every immunization. Local adverse events and pain scores were assessed immediately. In stage A, volunteers visited the trial facility daily from day 6 to day 21 after every immunization to report adverse events and to collect blood samples for assessment of parasites by qPCR. During the immunization period, all volunteers were treated with a curative regiment of atovaquone/proguanil when qPCR was positive for malaria or at day 28 after immunization. Complete blood counts and general chemistry laboratories were performed on days 6, 14, 21, 30, and 35 after immunization. Platelet counts, lactate dehydrogenase, and highly sensitive troponin T tests were performed daily to detect possible myocarditis in an early stage, in line with previously established protocols (29, 30). Blood samples for immunological assays were taken at baseline and at days 6, 14, 21, 28, 35, 100, and 188. In stage B of the clinical trial, visits were on day 14 after every immunization and the day before immunization for safety assessments. Three weeks after the third and final immunization, volunteers were exposed to the bites of five mosquitoes infected with the homologous Pf NF54 strain (CHMI). All mosquitoes were checked for a blood meal and infectivity by dissection (28). Further details on the CHMI with Pf are reported in table S2. After CHMI, volunteers visited the trial center on a daily basis from day 6 to day 21. All volunteers were treated with a curative regimen of atovaquone/proguanil if they were qPCR positive or, alternatively, at day 28 after CHMI. Final visits took place at days 35, 100, and 188 after CHMI. Blood samples for immunological analysis were taken before and 14 days after each immunization, before CHMI, and at days 6, 14, 21, 35, 100, and 188 after CHMI.

Adverse events

Solicited and unsolicited adverse events after DVI were recorded at every visit until 35 days after immunization. Solicited local adverse events were tenderness, induration, bruising/extravasated blood, erythema, swelling, pain, and pruritis. Solicited systemic adverse events were fever, rash, urticaria, pruritis, edema, headache, fatigue, malaise, chills, myalgia, and arthralgia. All volunteers were instructed to fill out a diary card, listing daily temperature and any adverse events up to day 35 after immunization. Causality of all adverse events was assessed by the investigators as definitely related, probably related, possibly related, unlikely related, or not related to the study procedures. In dichotomous analysis, the latter two were regarded as “unrelated” and the first three categories as “related.” All adverse events were graded as mild (grade 1), moderate (grade 2), severe (grade 3), or serious (grade 4). Review of all safety data by an independent safety monitoring committee was performed at 28 days after each immunization in stage A, before continuing dose escalation to the next group and on day 28 after CHMI in stage B.

Blood-stage parasitemia

To examine whether PfSPZ-GA1 were fully attenuated and incapable of establishing a blood-stage infection, blood samples were monitored for parasites by qPCR (31). Blood samples were considered negative if no signal was detected in 50 cycles or the Pf load was <100 Pf/ml. Any sample with a load of >100 Pf/ml was considered positive. Parasite densities were determined with the use of a trendline of standardized control samples between 20 and 10^6 Pf/ml.

Immunology

Exploratory endpoints included immune responses after immunization with PfSPZ-GA1 Vaccine. Antibodies were detected by enzyme-linked immunosorbent assay (ELISA) against PfCSP (32). Cellular immune responses were analyzed using peripheral blood mononuclear cell (PBMC) samples obtained 1 day before the first immunization and 21 days after the third immunization. Cells were isolated using heparin cell preparation tubes according to previously published protocols (32). After thawing, cells were stimulated, as described previously (33). In short, PBMCs were cultured at 2.5×10^6 cells/ml in a final volume of 200µl per well in RPMI 1640 (Dutch Modification; Gibco) with gentamicin (5 mg/ml; Centraform), 100 mM pyruvate (Gibco), 200 mM GlutaMAX (Gibco), and 10% heat-inactivated pooled human A+ serum (Sanquin, Nijmegen, The Netherlands). Cells were stimulated with purified NF54 schizonts (PfRBC) or uninfected red blood cells (uRBC) at a concentration of 2.5×10^6 RBC/ml for 24 hours. Brefeldin A (10 µg/ml; Sigma-Aldrich) and monansin (2 µM; eBioscience) were added during the last 4 hours of stimulation. Cells were stained with fixable viability dye labelled with eFlour780 (eBioscience), CD3–phycoerythrin (PE)–Dazzle549 (BioLegend; clone OKT3), CD4–fluorescein isothiocyanate (FITC) (BioLegend; clone OKT4), CD8-Alexa Fluor 700 (BioLegend; clone HIT8A), pan-γδTCR-PE (Beckman Coulter; clone IMMU510), and CD56 peridinin chlorophyll protein (PerCP)–Cy5.5 (BioLegend; clone HCD56) for 30 min at 4°C. Cells were subsequently permeabilized using Foxp3 fixation/permeabilization buffer (eBioscience) and stained for intracellular cytokines with IFN-γ–PE-Cy7 (BioLegend; clone 4S.B3), interleukin-2 (IL-2)–BrilliantViolet510 (BioLegend; clone MQ1-17H12), and tumor necrosis factor–α (TNF-α)–Alexa Fluor 647 (BioLegend; clone MAb11). Analysis was performed using a Gallios flow cytometer (Beckman Coulter) and FlowJo software (version 10.0.8 for Apple OS). Background cytokine production after stimulation with uRBC was subtracted from PfRBC responses. On an individual level, we defined IFN-γ responders as those volunteers with a percentage increase in IFN-γ–producing cells greater than twice the SD of all pre-immunization samples.

Statistical analysis

Adverse events were evaluated by tabulating according to intention to treat analysis. The proportion of volunteers in each group who reported mild, moderate, or severe adverse events was calculated, and analysis was primarily descriptive. The secondary endpoint of the study was the presence of parasitemia (by qPCR) after CHMI with the (WT) Pf NF54 strain in stage B of the study. Differences between groups were evaluated by log-rank test.

Differences in immunological parameters between groups were assessed by comparing mean values between the groups using one-way ANOVA when comparing several groups or a two-tailed Student's *t* test or nonparametric equivalents. Paired tests were used if pre-exposure values were compared with post-exposure values, and unpaired tests were used if comparisons were made between groups. For discrete variables, the $\chi 2$ test or Fisher's exact test was used (two-tailed). All statistical analyses were performed with SPSS version 23.

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Supplementary

Inclusion and exclusion criteria

Inclusion criteria

1. Subject is aged ≥ 18 and ≤ 35 years and in good health.

2. Subject has adequate understanding of the procedures of the study and agrees to abide strictly thereby.

3. Subject is able to communicate well with the investigator, is available to attend all study visits.

4. Furthermore, the subject will remain within the Netherlands or within reasonable travelling distance from the Radboudumc from day -1 till day +28 after each parasite exposure. After CHMI, subjects have to be reachable by phone (24/7) from day -1 until day 35.

5. Subject agrees to inform his/her general practitioner (GP) about participation in the study and to sign a request to release by the GP, and medical specialist when necessary, any relevant medical information concerning possible contra-indications for participation in the study.

6. Subject agrees to refrain from blood donation to Sanquin or for other purposes throughout the study period and for a defined period thereafter according to Sanquin guidelines (3 years minimum, depending on serology).

7. Non-pregnant, non-lactating females of reproductive potential (i.e., have a uterus and are neither surgically sterilized nor post-menopausal) should agree to use adequate contraception and not to breastfeed for the duration of study.

8. Subject agrees to refrain from intensive physical exercise (disproportionate to the subjects' usual daily activity or exercise routine) for twenty-one days following each immunization and during the malaria challenge period.

9. Subject has signed informed consent.

Exclusion criteria

1. Any history, or evidence at screening, of clinically significant symptoms, physical signs or abnormal laboratory values suggestive of systemic conditions, such as cardiovascular, pulmonary, renal, hepatic, neurological, dermatological, endocrine, malignant, hematological, infectious, immune-deficient, psychiatric or other disorders, which could compromise the health of the volunteer during the study or interfere with the interpretation of the study results. These include, but are not limited to, any of the following:

a. Body weight <50 kg or Body Mass Index (BMI) <18.0 or >30.0 kg/m2 at screening

b. A heightened risk of cardiovascular disease, defined as:

i. An estimated ten-year risk of fatal cardiovascular disease of ≥5% at screening, as determined by the Systematic Coronary Risk Evaluation (SCORE);

ii. History, or evidence at screening, of clinically significant arrhythmia's, prolonged QT-interval or other clinically relevant ECG abnormalities; or

iii. a positive family history of cardiac events in first or second degree relatives (according to the system used in medical genetics) <50 years old.

c. Functional asplenia, sickle cell trait/disease, thalassemia trait/disease or G6PD deficiency.

d. History of non-febrile seizure at any time prior to study onset, even if no longer on medication.

e. Positive HIV, HBV or HCV screening tests.

f. Chronic use of i) immunosuppressive drugs, ii) antibiotics, iii) or other immune modifying drugs within three months prior to study onset (excluding inhaled and topical corticosteroids and incidental use of oral anti-histamines) or expected use of such during the study period.

g. History of malignancy of any organ system (other than localized basal cell carcinoma of the skin), treated or untreated, within the past five years.

h. Any history of treatment for severe psychiatric disease by a psychiatrist in the past year.

i. History of drug or alcohol abuse interfering with normal social function in the period of one year prior to study onset, positive urine toxicology test for cocaine or amphetamines at screening or prior to infection or positive urine toxicology test for cannabis prior to infection.

2. For female subjects: breastfeeding, or positive urine pregnancy test at screening or prior to immunization or prior to CHMI.

3. Any history of malaria, positive serology for *P. falciparum*, or previous participation in any malaria (vaccine) study or CHMI.

4. Known hypersensitivity to or contra-indications (including co-medication) for use of atovaquone/proguanil or artemether/lumefantrine, or history of severe (allergic) reactions to mosquito bites.

5. Receipt of any vaccinations in the 3 months prior to the start of the study or plans to receive any other vaccinations during the study period or up to 8 weeks thereafter.

6. Participation in any other clinical study in the 30 days prior to the start of the study or during the study period.

7. Being an employee or student of the department of Medical Microbiology or Infectious Diseases of the Radboudumc or the LUMC.

8. Any other condition or situation that would, in the opinion of the investigator, place the subject at an unacceptable risk of injury or render the subject unable to meet the requirements of the protocol or would compromise the integrity of the data.

Table S1. Number and severity of adverse events after immunization. Number of volunteers reporting solicited local and systemic adverse events possibly, probably or definitely related to immunization. Percentages are given between parenthesis. Data collected until 35 days after each immunization. No rash, induration, edema, chills or arthralgia were reported.

			PfSPZ-GA1 Vaccine			**PfSPZ Vaccine**	**Placebo**
			1.35x10^5	**4.5x10^5**	**9.0x10^5**	**4.5x10^5**	
		Grade	***n*=3**	***n*=16**	***n*=26**	***n*=13**	***n*=9**
Local	Tenderness	1	0 (0)	0 (0)	2 (8)	1 (8)	0 (0)
	Bruising	1	1 (33)	0 (0)	3 (12)	3 (23)	0 (0)
	Erythema	1	0 (0)	0 (0)	0 (0)	0 (0)	1 (11)
	Swelling	1	0 (0)	0 (0)	1 (4)	2 (15)	0 (0)
	Pain	1	0 (0)	0 (0)	2 (8)	1 (8)	1 (11)
	Pruritis	1	0 (0)	0 (0)	2 (8)	0 (0)	1 (11)
Systemic	Fever	1	0 (0)	1 (6)	1 (4)	1 (8)	0 (0)
		2	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		3	0 (0)	1 (6)	0 (0)	0 (0)	0 (0)
	Headache	1	0 (0)	5 (31)	11 (42)	4 (31)	3 (33)
		2	1 (33)	1 (6)	2 (8)	2 (15)	1 (11)
		3	0 (0)	0 (0)	0 (0)	1(8)	0 (0)
	Fatigue/malaise	1	1 (33)	2 (13)	3 (12)	2 (15)	1 (11)
		2	0 (0)	1 (6)	1 (4)	0 (0)	1 (11)
		3	0 (0)	0 (0)	1 (4)	0 (0)	1 (11)
	Myalgia	1	0 (0)	0 (0)	1 (4)	0 (0)	0 (0)
		2	0 (0)	0 (0)	2 (8)	0 (0)	1 (11)
		3	0 (0)	1 (6)	0 (0)	0 (0)	0 (0)

Table S2. Bite numbers used for CHMI for the different study arms. Mosquitoes were 100% infected with a mean of 106,000 sporozoites per mosquito.

	Infection		
	Number of sessions median (range)	Number of Infected bites median (range)	Number of Uninfected bites median (range)
PfSPZ-GA1 9 x10^5	1 (1-3)	5	0 (0-2)
PfSPZ-GA1 4.5 x10^5	1 (1-2)	5	0 (0-2)
PfSPZ Vaccine 4.5 x10^5	1 (1-2)	5	0 (0-1)
Placebo	1 (1-2)	5	0 (0-3)

II

Development of a controlled human schistosomiasis infection model

CHAPTER 5

Establishing the production of male *Schistosoma mansoni* cercariae for a controlled human infection model.

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Abstract

To accelerate the development of novel vaccines for schistosomiasis, we set out to develop a human model for *Schistosoma mansoni* infection in healthy volunteers. During natural infections, female schistosomes produce eggs that give rise to morbidity. Therefore, we produced single-sex, male *Schistosoma mansoni* cercariae for human infection without egg production and associated pathology. Cercariae were produced in their intermediate snail hosts in accordance with the principles of good manufacturing practice (GMP). The application of GMP principles to an unconventional production process is a showcase for the controlled production of complex live challenge material in the European Union or under Food and Drug Administration guidance.

Schistosomiasis ranks among the leading neglected tropical diseases in terms of disability-adjusted life years, with roughly 252 million people infected worldwide [1]. *Schistosoma mansoni* and *Schistosoma haematobium* are responsible for the majority of cases [2]. Morbidity in schistosomiasis is caused by the inflammatory response to tissue-deposited eggs, which induce granulomas and fibrosis that can lead to portal hypertension or bladder cancer [2].

Current schistosomiasis control programs rely on the mass administration of praziquantel, but transmission of schistosomes persists, primarily because of high reinfection rates [3]. Alternative tools to break transmission and eliminate schistosomiasis are urgently needed. The development of a highly efficacious vaccine would be a major asset to schistosomiasis control programs.

Vaccination studies in mice and nonhuman primates prove that immunity to schistosomes can be induced by repeated exposure to radiation-attenuated cercariae [4, 5]. Based on such landmark studies, stage-specific antigens have been identified that reduce the worm burden by >40% [6]. Currently there are 4 vaccine candidates in the clinical stage of development (Sh28GST, Sm-TSP-2, Sm14, and Smp80) [6, 7]. Typically, these vaccine candidates undergo phase 1 testing for safety, after which efficacy needs to be demonstrated in large-scale field trials in schistosome-endemic areas. To obtain an estimate of the vaccine induced protective efficacy, trials of long duration and/or large population size are necessary.

To decrease the risk of downstream efficacy failure, healthy volunteers have been deliberately exposed to infectious agents to test malaria, dengue, and influenza vaccines [8]. Such controlled human infection (CHI) trials provided early efficacy estimates that were used to guide further clinical development. The availability of a CHI model for schistosome infection could revolutionize the development of schistosomiasis vaccines.

To ensure the safety of participants in a human schistosome infection trial, a challenge inoculum should be available that complies with regulatory requirements for human use and cannot induce egg-associated morbidity. We therefore took an important conceptual step to produce single-sex, male, schistosome cercariae in accordance with good manufacturing practice (GMP) principles. This process for producing live parasites, which complies with the highly demanding regulatory environment in place currently, is a showcase for the production of complex live challenge material in the European Union (EU) or under Food and Drug Administration guidance.

Methods

Production Principles

Manufacture of single-sex cercariae for the purpose of controlled human schistosome infections was performed within the quality control and quality assurance system of the Interdivisional GMP facility of Leiden University Medical Center (LUMC). The product was characterized as an auxiliary medicinal product (EU regulation 536/2014) or previously as a noninvestigational medicinal product according to the definition of EU clinical trial directive 2001/20/EC. The principles of GMP as outlined in European Commission directive 2003/94/ EC were used in the production process wherever possible.

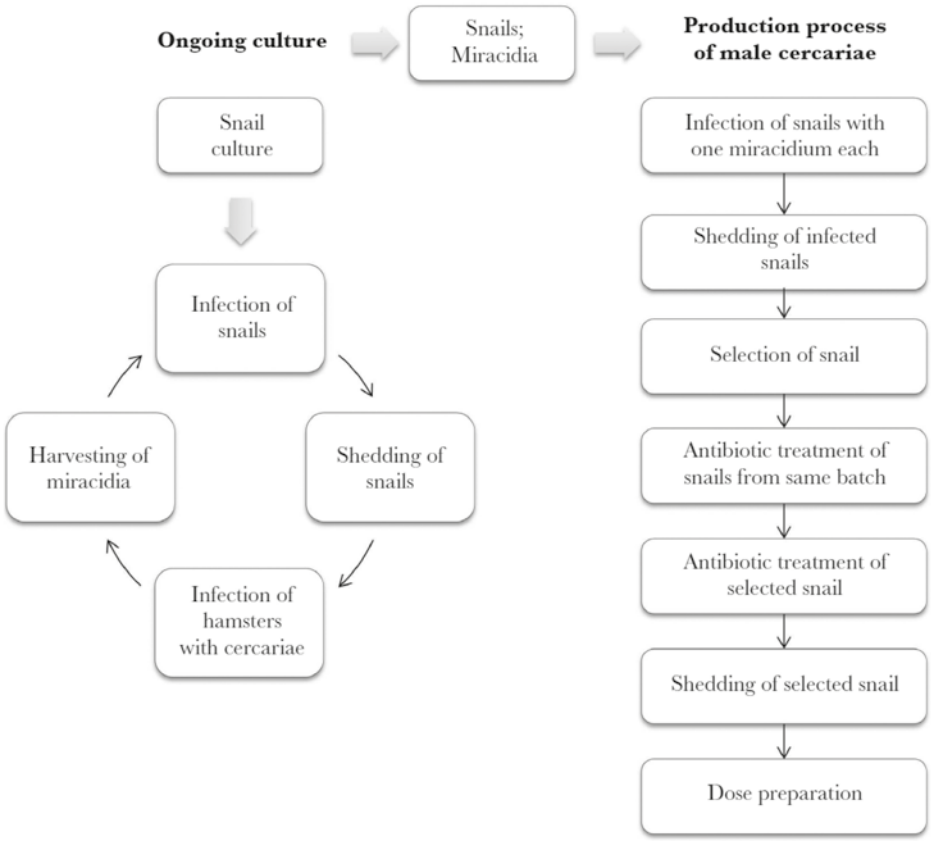

Figure 1. Schematic representation of the production process.

Cercariae were produced in a biosafety level 3 laboratory environment with controlled humidity, temperature, and pressure. The production process (Figure 1) was standardized in 34 standard operating procedures, which were kept in the institutional document management system. A product dossier was created according to section 2.7 of EC CT-1 guideline 2010/C 82/01 for pharmacological products. All disposables, reagents, solvents, culture media, and starting materials were released for use by the quality control (QC) officer and the qualified person (QP) responsible for advanced cell therapy medicinal products. All manufacturing steps and batch numbers of all used materials were entered and logged in real time in a GMP-compliant digital database, using a portable tablet. The use of a tablet facilitated communication between manufacturers and QC officers without breaching hygienic procedures established for containment of the biosafety level 3 environment. A separate read-only account was held by the QC officer and the QP. Monitored data were locked by the QP to prevent editing. All QC testing was independently analyzed by the QC officer, and thereafter the product was released by the QP for use.

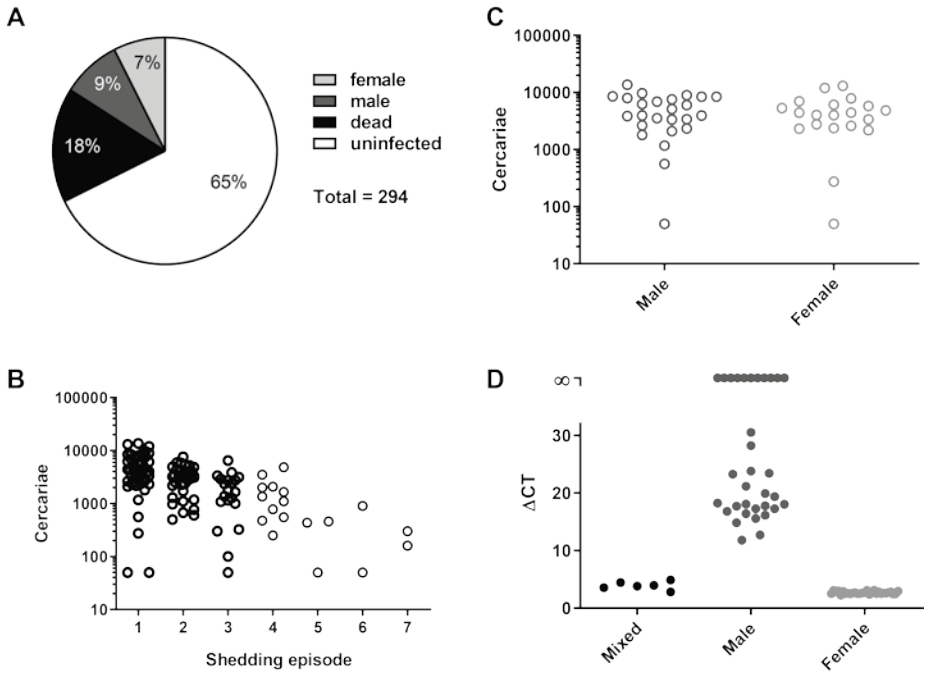

Figure 2. A. Percentages of uninfected, dead, and infected Biomphalaria glabrata snails with either male or female cercariae after single-miracidium infection. **B**. Number of cercariae produced by each snail for each weekly shedding episode, starting from week 5 after infection. **C.** Number of male or female cercariae from shedding episode 1 or 2. **D**. Results from sex-specific polymerase chain reaction analysis, shown as the difference in cycle threshold (ΔCt) between the ITS2 gene and the W1 repeat, in 47 cercarial samples from preclinical production.

Origin of Snails and Parasites

The *S. mansoni* Puerto Rico strain used for production was obtained together with its *Biomphalaria glabrata* snail host by Prof C. F. A. Bruijning in 1955 (Prof A. M. Deelder, personal communication). This isolate has been maintained in laboratory culture in the LUMC by routine passaging of the schistosomes through laboratory hamsters and/or mice as the definitive host and laboratory-cultured *B. glabrata* as the intermediate host. Adult worms were genetically characterized to confirm their origin (Supplementary Materials).

Production of Cercariae

S. mansoni miracidia were obtained by light-induced hatching of *S. mansoni* eggs isolated from an infected hamster [9]. After infection by a single miracidium [10], *B. glabrata* snails of the same batch (ie, infection day) were housed and labeled individually, and they shed cercariae after 5 weeks. Cercariae were counted, and their viability was determined by calculating the percentage of the counted larvae that were moving.

Identity and Sex Confirmation

The identity and sex of cercariae were determined by a purpose-made multiplex real-time polymerase chain reaction (PCR) targeting *Schistosoma*-specific ITS2 sequences [11], as well as the *S. mansoni* W1 repeat [12] (Supplementary Materials). The W1 copy number is much lower in male as compared to female schistosomes [13]. Using DNA of duplicate cercaria samples unequivocally resulted in an ITS2 cycle threshold (Ct) between 15 and 30. Sex was determined on the basis of the difference in Ct between W1 and ITS2, with males having a ΔCt of >10, and females having a ΔCt of <4. Sensitivity of the quantitative PCR was confirmed in samples containing only 1 cercaria (mean ITS2 Ct, 23). To validate the PCR finding, the sex of single-sex cercaria samples was confirmed by assessment of the morphology of adult worms after hamster infection.

Bioburden Testing

The bioburden in shed waters was determined at the ISO-15189–certified clinical medical microbiology laboratory of the LUMC (Supplementary Materials). *Aeromonas* species, *Staphylococcus aureus*, β-hemolytic streptococci, and *Pseudomonas aeruginosa* were defined as pathogenic bacteria potentially causing skin infections upon transfer with cercariae. Cultures were optimized for detection of these bacteria. To reduce the bioburden, snails were kept in an environment containing 2 mg/L ciprofloxacin and 4 mg/L gentamicin for 24 hours before clinical use.

One week before use, the bioburden in shed water was assessed before and after antibiotic treatment. After shedding for clinical use, cercariae were counted, their viability was recorded, and doses were prepared by 2 persons separately.

Results

Preclinical Production Data

In preparation for the production of clinical trial material, 24 preclinical batches of miracidium-infected snails were produced, averaging 12 snails per batch. Of those 294 snails, 246 (84%) were still alive at week 5. In total, 47 snails (19%) shed cercariae, of which 25 (53%) were male (Figure 2A).

Viability of cercariae was always very high (>90% in all batches), but the number of cercariae per shed varied considerably per snail and over time. At week 5, snails shed approximately 3500 cercariae (geometric mean [GM], 3416 cercariae; 95% confidence interval [CI], 2386–4889), decreasing to 1261 cercariae (95% CI, 704–2259) at week 7 ($P < .0001$, by the Mann-Whitney U test; Figure 2B). The number of cercariae per shed was not affected by the cercarial sex ($P = .6$, by the Mann- Whitney U test; Figure 2C). On the basis of these data, cercariae from shedding episode 2 were preferably selected for clinical use.

Sex Identification by Quantitative PCR

Sex and identity were unequivocally confirmed in all cercarial samples (Figure 2D). Samples obtained from snails shedding mixed male and female cercariae always showed ΔCt values similar to those containing females only (ΔCt W1-ITS2 range, 2.83–4.88 for mixed-sex cercariae and 2.24–3.11 for female cercariae). Samples containing only male cercariae always showed different ΔCt values (range, 11.83 to undetectable). Thus, the ΔCt difference of >6 between male and female cercarial samples ensured that male sex could always be confirmed with certainty.

Bioburden

All samples that underwent bioburden testing were found to contain *Aeromonas veronii* biovar sobria susceptible to ciprofloxacin, gentamicin, and trimethoprim-sulfamethoxazole. No other pathogenic bacteria were cultured. Because *Aeromonas* species are incidentally reported as causative agents of skin infections, production snails were pretreated with antibiotics before clinical use of cercariae. After this treatment, all samples were free of *Aeromonas* species. After antibiotic treatment, waterborne bacteria such as *Chryseobacterium* species and *Stenotrophomonas maltophilia*, which are not known to cause infections in healthy volunteers, persisted.

In-Process Testing and Release

S. mansoni miracidia and hamsters were defined as starting materials and were released by the QC officer. Based on the critical steps in the production process, 3 in-process tests were defined: (1) confirmation of single-miracidium infection of snails by a second person, (2) daily examination

of snail viability, and (3) determination of the number and viability of cercariae after the first shedding episode (ie, >100 and >90%).

A 2-step release procedure was established, with a conditional release procedure immediately after dose preparation, followed by a final release procedure when bioburden tests were completed 3 days later. Criteria for conditional release were based on (1) absence of potentially pathogenic bacteria after antibiotic treatment of 2 snails from the same batch, (2) identity confirmation of cercarial species and male sex by quantitative PCR, (3) absence of potentially pathogenic bacteria resistant to ciprofloxacin and gentamicin, and (4) presence of >100 cercariae with >90% viability.

The shelf life of the product at 28°C was determined at 2 hours, when 96% (95% confidence interval [CI], 91%–100%) of cercariae were confirmed viable by microscopy. At 4 hours viability was more variable (mean, 96%; 95% CI, 81%–100%), and at 24 hours viability had dropped to 14% (95% CI, 4%–24%).

Discussion

In summary, we were able to establish a rigorous production process for male *S. mansoni* cercaria that complied with current regulatory standards for human use. Despite the complex, unconventional production process that includes a snail host, production was in accordance with the principles of GMP, with clearly defined release criteria, which were supervised by a QC officer and a QP responsible for advanced cell therapy medicinal products.

CHI trials are increasingly used for the down selection of novel drugs or vaccines [8]. However, specific guidelines regarding the production of challenge material are often lacking. Whereas the Food and Drug Administration states that stage-appropriate GMP is required, European Medicines Agency guidelines for auxiliary medicinal products state that full GMP may not be required, but deviations must be justified [14]. In this specific case, the production process in snails is exceptional and requires thorough risk analysis and intense collaboration of pharmacists, clinicians, and technicians. Paramount to the safety of volunteers in a controlled human schistosome trial is the ability to determine cercaria sex so that egg-induced pathology cannot occur. To this end, we have developed technologies and procedures to ensure unique identification and tracking of individual snails.

To confirm the identity and sex of cercariae, we established a sensitive and specific multiplex real-time PCR. We confirmed high variability in the copy numbers of the female-specific W1 repeat in male cercariae, corroborating previous data [13]. Nevertheless male cercariae could be confidently distinguished from female or mixed-sex cercarial samples, based on a large difference in Ct between male and female/mixed-sex samples. Should the production of female cercariae be necessary in the future, multiple individual cercariae from 1 snail should be tested to confirm cercarial sex.

We fully characterized the bioburden associated with the snail microbiome. We focused on excluding microbiological contaminants that might confer a risk of adverse events when applied to cercaria-penetrated skin. We believe that the current remaining nonpathogenic bioburden does not pose any risk for volunteers. In addition, the rearing of sterile snails has not been

reported previously and might be biologically challenging, owing to increasing evidence that the microbiome is particularly essential for the well-being of nonvertebrates [15].

Both the *B. glabrata* snails and the *S. mansoni* parasites have been kept in the laboratory for >60 years. Although the origin of the isolates lacked a full historical paper trail, the main advantage of such an old and highly established laboratory culture is the naivety to praziquantel, excluding possible resistance this drug. Nevertheless, for clinical use, extensive follow-up is needed to ensure full cure after praziquantel administration. We performed genotyping to confirm the parasite strain as *S. mansoni* from Puerto Rico. Future efforts will focus on establishing a master and working bank of parasites, although cryopreservation of schistosome cercariae, miracidia, or eggs has not been performed and may be challenging.

In conclusion, through an interdisciplinary approach, we were able to achieve highly controlled production of viable male *S. mansoni* cercariae. Following these efforts, the next steps will be the use of cercariae for infection of healthy volunteers in a proof-of-concept clinical trial to find a safe and infectious dose. Establishing a CHI model for schistosomiasis will be a game-changing step to accelerate the development of novel vaccines and drugs for this devastating disease.

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Supplementary

Origin of parasites

Genomic DNA was extracted from pooled worms using the DNeasy tissue kit (QIAgen) according to the manufacturer's protocol. The mitochondrial cox1 region was PCR amplified and sequenced as described in Webster et al., 2013 [1]. The sequences were compared to the *S. mansoni* cox1 database to find the closest identity, which was a 99% match to Genbank Accession: HE601612 (Mitochondrial genome of *S. mansoni* Puerto Rico).

Sex-identity *Schistosoma* multiplex qPCR

DNA from at least 50 cercariae was isolated with the QIAamp DNA mini kit spin columns (QIAgen, Hilden, Germany) and diluted 1:100. The multiplex PCR amplified a 77-bp fragment from the *Schistosoma*-specific ITS2 sequences (with primers Ssp48F, Ssp124R and probe Ssp78T) [2] and a 121-basepair fragment from the female specific *Schistosoma mansoni* W1 repeat (with primers SmW1-238F, SmW1-358R and probe SmW1-291T (Biolegio, Netherlands)) [3]. Amplification was performed in 20 µl PCR mix and 5 µl of cercarial DNA. Table 1 shows the concentrations of the PCR mix used with the sequences of all primers and double-labelled probes. The CFX real-time detection system (Bio-Rad laboratories) was used with amplification program of 15 min at 95°C followed by 50 cycles of 15s at 95°C, 30s at 60°C, and 30s at 72°C. Negative and positive (mixed, male and female) control samples were included.

Table 1. PCR mix and primer/probe sequences of the sex-identity *Schistosoma* multiplex PCR.

Components	Concentration	Per sample	Sequence
H_20		2,73	
$MgCl_2$	25 mM	3,50	
BSA	5 mg/ml	0,50	
Primer Ssp48F	25 µM	0,06	5'-GGTCTAGATGACTTGATTGA GATGCT-3'
Primer Ssp124R	25 µM	0,06	5'-TCCCGAGCGTGTATAATGTC ATTA-3'
Probe Ssp78T	10 µM	0,13	FAM-5'-TGGGTTGTGCTCGAGT CGTGGC-3'-BHQ1
Primer SmW1-238F	25 µM	0,20	5'-TGTTTGTGGATGCGATGGTG-3'
Primer SmW1-358R	25 µM	0,20	5'-TGTGCACAAGCAACGATTCC-3'
Probe SmW1-291T	10 µM	0,13	YAK-5'-GCGATGATGCATTAGG GTGTGTGGT-3'-BHQ1
HotStarTaq Master Mix		12,50	

Bioburden testing

Shed water was plated on Trypcase Soy Agar with 5% sheep blood, Cystine lactose electrolyte deficient agar, Columbia agar with colistin and nalidixic acid, Xylose Lysine Desoxycholaat Agar (Biomerieux, Marcy-l'Étoile, France) and Cefsulodin Irgasan Novobiocine agar (Media Products, Groningen, The Netherlands) and incubated aerobically for 48 hours at 35°C. Plates were checked daily for the growth of bacteria. Identification was performed by MALDI TOF mass spectrometry (MALDI Biotyper, Bruker Daltonik GmbH, Bremen, Germany) and susceptibility testing in a Vitek 2 instrument (Biomerieux) or, in case of streptococci by disk diffusion (Oxoid, Thermofisher, Landsmeer, The Netherlands).

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CHAPTER 6

Katayama syndrome without *Schistosoma mansoni* eggs.

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Annals of Internal Medicine 2019 May 21;170(10):732-733

Background

Katayama syndrome is a self-limited illness that occurs several weeks after infection with schistosome larvae (cercariae). Its symptoms are typical of an acute inflammatory response.

Objective

To provide insight into the cause of Katayama syndrome.

Case Report

We exposed 2 human participants to male cercariae of *Schistosoma mansoni* in an experimental infection that met all applicable laws and regulations, was approved by the Ethical Research Committee of Leiden University Medical Center (study P16.111), and was registered at ClinicalTrials.gov (NCT02755324).

The first patient was a healthy 24-year-old medical student with a history of hay fever and mild atopic dermatitis. We exposed her to 30 male cercariae of *S. mansoni* on her lower arm for 30 minutes. She felt itching and developed a typical localized cercarial rash that lasted 2 weeks. Four weeks after exposure, she started having fever, night sweats, myalgia, and headache, but no cough. Her temperature increased above 38.5 °C for 10 days (Figure, top). At that time, her complete blood count showed lymphocytopenia without eosinophilia, but her eosinophil count increased 1 week later (Figure, bottom). A chest radiograph was normal, and bacterial blood and urine cultures were negative. Serum aminotransferase levels increased to 4 times the upper limit of detection. We found no acute viral infections in serologic studies or in repeated molecular diagnostic tests on throat swabs. At week 6, the patient's fever decreased to between 37.5 and 38.0 °C in the evenings; she had night sweats for 3 weeks and reported fatigue, malaise, and mild to severe headaches for a total of 8 weeks. These symptoms were relieved by intermittent acetaminophen and nonsteroidal anti-inflammatory drugs and did not require corticosteroids.

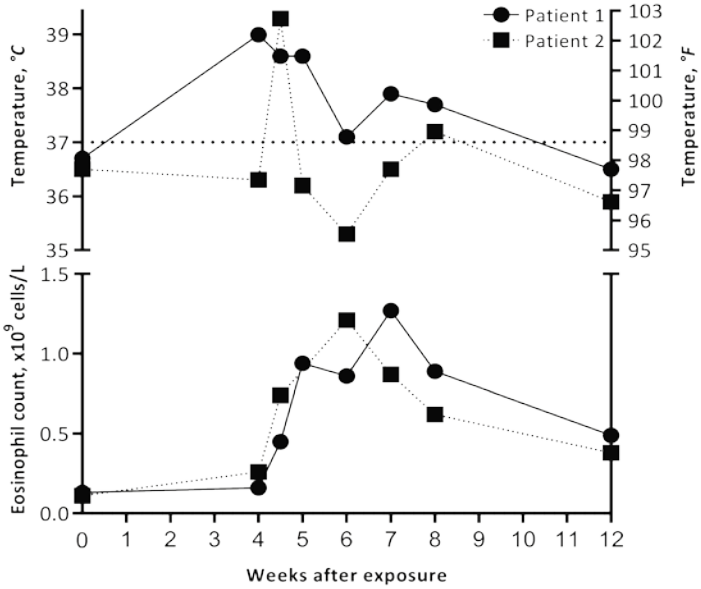

Figure. Highest temperature recorded by the physician or trial participant (top) and eosinophil count (bottom). The horizontal dashed line represents normal body temperature.

The second patient was a healthy 26-year-old psychology student who also had a history of hay fever and mild atopic dermatitis. We exposed her to 30 male cercariae of *S. mansoni* on her lower arm for 30 minutes. She developed nonitching cercarial dermatitis that lasted 6 weeks. Four weeks after exposure, she reported fever and severe headaches for 2 days (Figure, top). Molecular diagnostic tests could not identify viral infections on a throat swab. After the patient's fever abated, she had mild headaches for 5 days. One week after onset of symptoms, her complete blood count showed eosinophilia (Figure, bottom) and she reported periorbital edema of the left eye. We treated her with cetirizine for 2 days, and the edema resolved.

All symptoms eventually subsided, and both patients recovered completely. At the end of the study (week 12), we treated them with praziquantel, 40 mg/kg of body weight, as part of the study protocol.

Table. Quantitative Data of the Anti–Adult Worm IgM (IFA) and Anti-Egg IgG (EIA) in Serum and of the Parasite Antigen Detection Assays for CCA in Urine and CAA in Serum.

Test	week						
	0	4	5	6	7	8	12
Patient 1							
Schistosoma serology							
IFA*	<1:16	1:32	1:256	1:1024	>1:1024	>1:1024	>1:1024
EIA†	<1:32	<1:32	-	-	-	-	<1:32
Parasite antigen							
Urine POC-CCA	Negative	Trace	-	-	-	Trace	Negative
Serum UCP-LF CAA	Negative	Trace	Positive	Positive	Positive	Positive	Positive
Patient 2							
Schistosoma serology							
IFA*	<1:16	<1:16	1:64	1:1024	>1:1024	>1:1024	>1:1024
EIA†	<1:32	<1:32	-	-	-	-	<1:32
Parasite antigen							
Urine POC-CCA	Negative	Trace	-	-	-	Trace	Negative
Serum UCP-LF CAA	Negative	Negative	Negative	Positive	Positive	Positive	Positive

EIA = enzyme immunoassay; IFA = immunofluorescent assay; POC-CCA = point-of-care–circulating cathodic antigen; UCP-LF CAA = upconverting phosphor-lateral flow circulating anodic antigen. *Cutoff, 1:16. †Cutoff, 1:32.

Discussion

Both participants developed Katayama syndrome, as evidenced by typical illness at the expected time after exposure without detectable viral or bacterial pathogens (1). In addition, both were diagnosed with schistosomiasis by parasite antigen testing in serum (upconverting phosphorlateral flow circulating anodic antigen) and had antiworm IgM antibodies detectable by immunofluorescent assay 1 week after the onset of fever (Table) (2, 3). Selection of male cercariae by molecular tests for the experimental infection ensured that *Schistosoma* egg production would not occur (4). In addition, the monosexual infection was supported by the absence of IgG antibody responses to egg antigens on enzyme immunoassay (Table) and the lack of detectable *Schistosoma* DNA in feces (3).

Katayama syndrome has been described as an immune complex–mediated hypersensitivity response to schistosomes or their eggs in previously unexposed persons (1). The antigens that trigger the syndrome are unknown. Our cases prove that it can occur in the absence of eggs and suggest that newly expressed antigens on developing worms may be the cause. The classic type 1 immunologic responses in Katayama syndrome (for example, urticaria; angioedema; and particularly periorbital edema, which occurred in one participant) suggest that worm-specific IgE has a role in this process. These IgE-mediated conditions often respond well to antihistamines and corticosteroids (5).

Katayama syndrome can be remarkably heterogeneous in terms of clinical signs and symptoms (1). Because symptoms are generally mild, transient, and nonspecific, the diagnosis is easily overlooked. Our cases show that both eosinophilia and seroconversion may become apparent after severe symptoms have subsided, that Katayama syndrome can be diagnosed as early as 5 weeks after exposure, and that it is not necessarily related to egg deposition.

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CHAPTER 7

A controlled human *Schistosoma mansoni* infection model to advance novel drugs, vaccines and diagnostics

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Abstract

Schistosomiasis treatment relies on the use of a single drug, praziquantel, which is insufficient to control transmission in highly endemic areas[1]. Novel medicines and vaccines are urgently needed[2,3]. An experimental human model for schistosomiasis could accelerate the development of these products. We performed a dose-escalating clinical safety trial in 17 volunteers with male *Schistosoma mansoni* cercariae, which do not produce eggs (clinicaltrials.gov NCT02755324), at the Leiden University Medical Center, the Netherlands. The primary endpoints were adverse events and infectivity. We found a dose-related increase in adverse events related to acute schistosomiasis syndrome, which occurred in 9 of 17 volunteers. Overall, 5 volunteers (all 3 of the high dose group and 2 of 11 of the medium dose group) reported severe adverse events. Worm-derived circulating anodic antigen, the biomarker of the primary infection endpoint, peaked in 82% of volunteers at 3–10 weeks following exposure. All volunteers showed IgM and IgG1 seroconversion and worm-specific cytokine production by $CD4^+$ T cells. All volunteers were cured with praziquantel provided at 12 weeks after exposure. Infection with 20 *Schistosoma mansoni* cercariae led to severe adverse events in 18% of volunteers and high infection rates. This infection model paves the way for fast-track product development for treatment and prevention of schistosomiasis.

Introduction

Worldwide, 290 million people are infected with schistosomes, mainly *Schistosoma haematobium* and *Schistosoma mansoni*[4]. The endemicity is determined by the presence of the fresh water snail intermediate host. Snail-derived cercariae penetrate the human skin and migrate into the vascular system, where mature male and female worms mate and produce ~300 eggs per day. *S. mansoni* eggs provoke inflammatory responses, which can lead to liver cirrhosis and portal hypertension[5].

Current treatment and control of schistosomiasis relies on the use of a single drug, praziquantel. Mass drug administration with praziquantel does not protect from reinfection[1] and thus provides insufficient control in highly endemic areas, creating the need for a vaccine[2].

Several schistosome antigens have been put forward as possible *S. mansoni* vaccine candidates, of which three are in clinical development: Sm-TSP-2, rSm14/GLA-SE and Sm-p80[6–8]. These candidates aim for >40% reduction in worm load for World Health Organization endorsement[9], but higher levels are preferred[10,11]. To obtain efficacy data, large phase II and III field trials in *Schistosoma*-endemic areas are needed[12]. In addition, increasing concerns of praziquantel resistance create a need for anti-schistosomal drug development[3]. Controlled human infection (CHI) trials can select drug and vaccine candidates early in clinical development and help prevent late clinical failure[12]. We thus aimed to develop a schistosome CHI model to aid vaccine and drug development and better characterize human anti-schistosome immune responses.

Between September 2016 and January 2018, 35 healthy adult volunteers were screened, of which 17 were included in the trial and completed follow up (Fig. 1). Baseline demographics between dose groups were comparable (Supplementary Tables 1 and 2).

Volunteers were exposed to 10, 20 or 30 cercariae in water on the forearm skin for 30 min, after which they were followed bi-weekly for adverse events and infectivity. After exposure, water was pipetted off the skin and inspected for remaining cercariae. We found tails of roughly half the number of the cercariae that they were exposed to, with clear differences between exposure groups (r = 0.70, P = 0.002; Supplementary Table 1).

Results

Safety

The majority of volunteers (15 of 17, 88%) experienced pruritus during or after exposure, but no topical treatment was required (Supplementary Table 3). All but one volunteer (16 of 17, 94%) developed a mild local cercarial dermatitis within 2 d after exposure (Fig. 2a–c), which lasted longer in higher dose groups (10 cercariae: median 1 week (range 1–2); 20 cercariae: 3 weeks (1–9); 30 cercariae: 6 weeks (2–7), r = 0.45, P = 0.07).

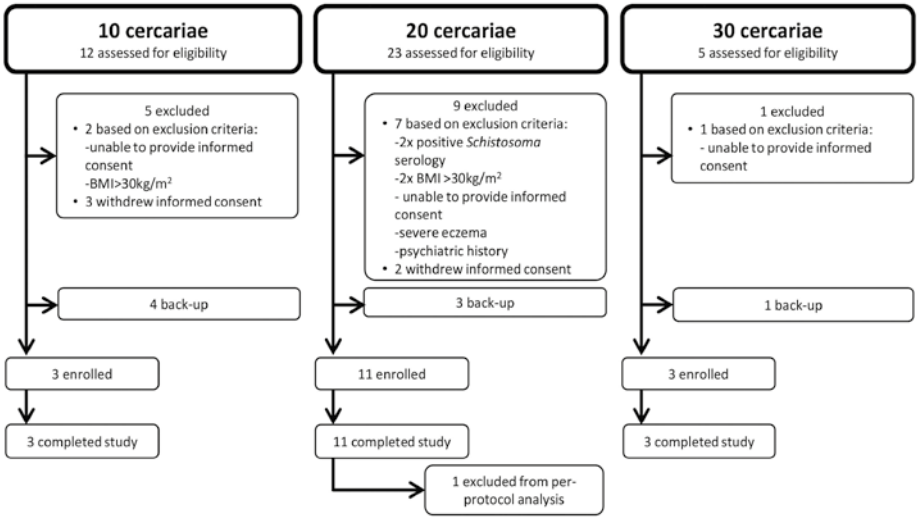

Figure 1. Study flow diagram. The inclusion and exclusion of volunteers. The first group (*n* = 3 volunteers) was exposed to 10 cercariae and the second group (n = 3 volunteers) to 30 cercariae. Thereafter the dose was de-escalated to 20 cercariae for the third group (n = 3 volunteers) and validated in a fourth group (*n* = 8 volunteers). In total eight volunteers served as backup for included volunteers in case an included volunteer might drop out before exposure to cercariae.

There were no serious adverse events (AEs) (an event that is life-threatening or requires hospitalization), but nine severe related AEs (resulting in the inability to perform daily activity) were reported in volunteers from the 20 (*n* = 2) or 30 cercariae (*n* = 3) group. Seven of these severe AEs were symptoms of an acute schistosomiasis syndrome (*n* = 5). In the 30 cercariae group all volunteers (*n* = 3) experienced severe AEs, starting 2.5 to 5.0 weeks after exposure (Fig. 2d–f) as follows: headache (*n* = 2), fever (*n* = 2), syncope (*n* = 1), nausea (*n* = 1) and elevated liver enzymes (*n* = 1) (Supplementary Table 3). In one volunteer this episode was followed by mild to moderate headaches, malaise, fatigue and nocturnal sweats for up to 6 weeks as previously described[13], but steroid treatment was declined. Given the burden of these prolonged symptoms of an acute schistosomiasis syndrome (Katayama symptoms), defined as moderate to severe symptoms of malaise, fatigue, fever, night sweats, flu-like symptoms or headache, between 2 to 7 weeks after exposure, the dose was de-escalated to 20 cercariae. Subsequently, 2 of 11 (18%) volunteers reported one severe symptom of an acute schistosomiasis syndrome (headache, nocturnal fever and sweats).

In addition to these five volunteers with severe AEs there were four volunteers, all exposed to 20 cercariae, with moderate symptoms of an acute schistosomiasis syndrome (flu-like symptoms, *n* = 3; and malaise, *n* = 2). Eight volunteers did not experience any symptoms of an acute schistosomiasis syndrome.

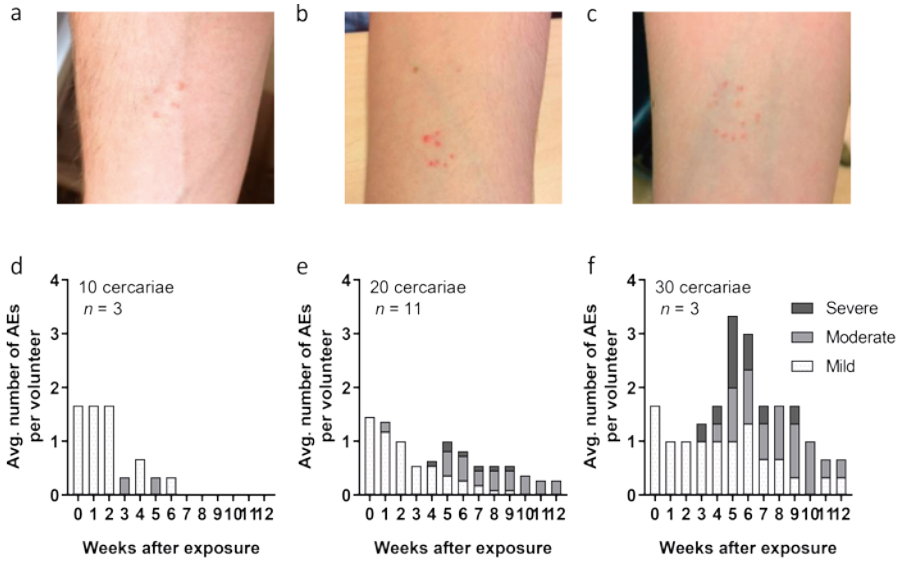

Figure 2. Adverse events. a–c, Pictures of volunteers with the most pronounced cercarial dermatitis taken 5 d after infection from the group infected with 10 cercariae (**a**), 20 cercariae (**b**) or 30 cercariae (**c**). **d–f**, The average number of related AEs per volunteer at each week for volunteers exposed to 10 cercariae (**d**), 20 cercariae (**e**) or 30 cercariae (**f**). For each AE the highest grading score is plotted. White bar, mild AE; light gray bar, moderate AE; dark gray bar, severe AE.

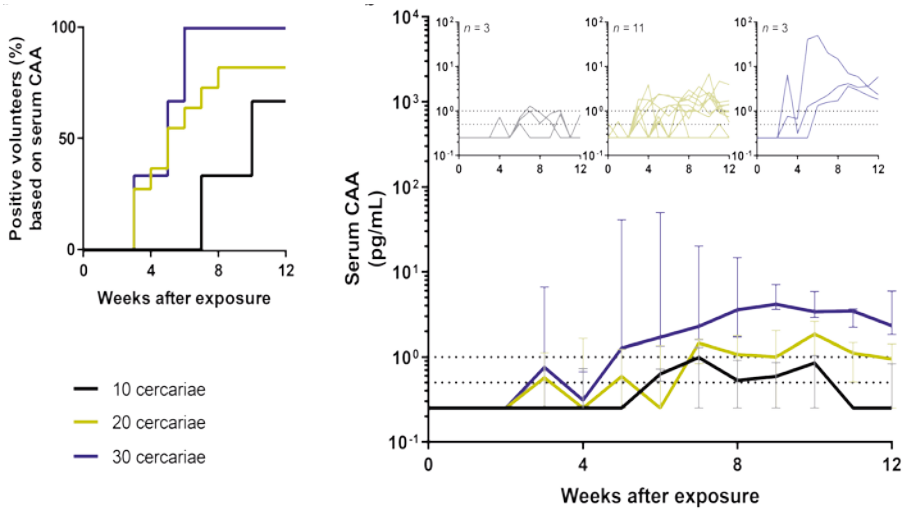

Figure 3. Pre-patent period and serum CAA levels. a, The time to patency (serum CAA level > 1 pg ml^{-1}) in weeks per dose group (log-rank test, $P = 0.21$). **b**, The serum CAA levels for individual volunteers in pg ml^{-1} (top) and the median serum CAA levels and interquartile range for groups infected with 10, 20 or 30 cercariae (bottom). The gray, yellow and blue lines represent data of individual volunteers or groups exposed to 10 ($n = 3$), 20 ($n = 11$) or 30 ($n = 3$) cercariae respectively.

Eosinophils

Eosinophils increased in 11 of 17 (65%) volunteers peaking between week 2 and 8 after infection (0.1–8.4 × 10^9 l^{-1}; Extended Data Fig. 1). Eosinophils were not related to the dose or symptoms (Extended Data Fig. 2a).

Infection rates by antigen detection assays

Both worm-excreted circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) were measured to determine the presence and degree of infection. In two of three volunteers exposed to 10 cercariae the serum CAA was higher than 1 pg ml^{-1}. In the higher dose groups, 9 out of 11 (82%) volunteers exposed to 20 cercariae and all volunteers exposed to 30 cercariae crossed this threshold. The time to patency was comparable between these groups (range 3–8 weeks, Fig. 3a).

Cercarial dose and serum CAA levels were related (10 cercariae median at week 7–12: 0.4 pg ml^{-1} (range 0.3–0.8); 20 cercariae: 1.2 pg ml^{-1} (0.3–1.9); and 30 cercariae: 3.6 pg ml^{-1} (2.0–6.5), $r = 0.70$, $P = 0.002$) (Fig. 3b). The volunteer experiencing the most severe AEs had at least sevenfold higher serum CAA levels (maximum 49.9 pg ml^{-1}) than other volunteers. There was no significant correlation between serum CAA levels and symptoms of an acute schistosomiasis syndrome (Extended Data Fig. 2b) or eosinophils (data not shown).

Urine CAA levels were variable (Extended Data Fig. 3a,b), but correlated with serum CAA ($r = 0.58$, $P < 0.0001$). The point-of-care rapid test for CCA (POC-CCA) was positive in 12% (2 of 17) of volunteers within 12 weeks after exposure and did not correlate with serum or urine CAA levels.

Praziquantel treatment

After a single 40 mg kg^{-1} dose of praziquantel treatment 12 weeks after exposure, serum CAA levels dropped below the detection limit in 8 out of 14 (57%) positive volunteers. The remaining 43% of volunteers were treated again with praziquantel, after which all remained undetectable until 1 year after exposure (Extended Data Fig. 4a–c).

Serology

All volunteers showed seroconversion of IgM against adult worms by immunofluorescence assay (IFA) (Fig. 4a,b). Seroconversion became apparent at week 4 in two volunteers and by week 6 in all. In addition, seven volunteers showed anti-soluble egg antigen (SEA) IgG seroconversion respectively at week 4 (1 of 16), week 12 (1 of 16) or week 20 (5 of 16). The absence of eggs was confirmed by a negative feces *Schistosoma* PCR at week 12 in all volunteers.

All volunteers showed an increase in adult worm antigen (AWA)-specific total IgG over time, with seroconversion above 2× s.d. of all baseline values in 12 of 16 volunteers at week 20. These responses were primarily IgG1, with seroconversion in all volunteers by week 16 (Fig. 4c,d).

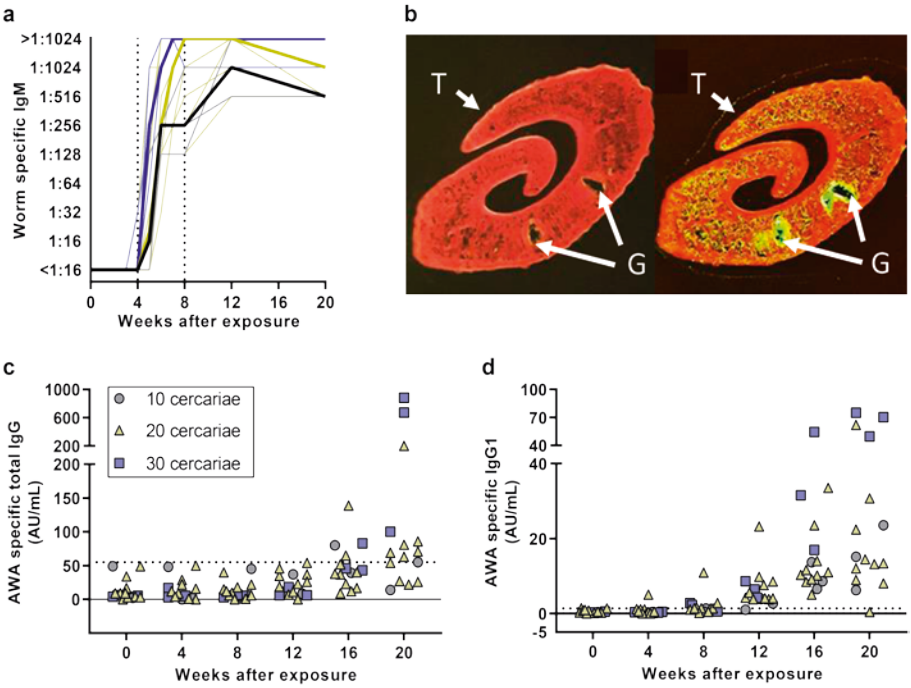

Figure 4. Humoral immune response. a, IgM against adult worm antigens over time. The gray, yellow and blue lines represent data of individual volunteers exposed to 10 (*n* = 3), 20 (*n* = 10) or 30 (*n* = 3) cercariae respectively and the darker lines represent the median of each group. **b**, IFA showing reactivity of volunteer IgM antibodies to Rossman-fixed *S. mansoni* adult worm sections (right) and pre-exposure negative serum (left). T, tegument; G, gastrodermis. **c**, AWA-specific total IgG for individual volunteers over time. **d**, AWA-specific IgG1 for individual volunteers over time. The gray circles, yellow triangles and blue squares represent data of individual volunteers exposed to 10 (*n* = 3), 20 (*n* = 10) or 30 (*n* = 3) cercariae respectively.

There was a clear dose response in AWA-specific total IgG and IgG1 levels (IgG week 20: $r = 0.70$, $P = 0.003$; IgG1 week 16: $r = 0.56$, $P = 0.02$), and a trend toward higher IgG1 levels and symptoms of an acute schistosomiasis syndrome ($P = 0.08$) (Extended Data Fig. 2c). No changes over time were found in total IgE or AWA-specific IgE and IgG4 compared to baseline (data not shown).

Cytokines

Cytokine and chemokine measurements in serum revealed increases in innate chemokines interferon (IFN)-γ- inducible protein (IP)-10 (Fig. 5a,b) and macrophage inflammatory protein (MIP)-1β (Fig. 5c), the latter of which was significantly higher in volunteers with symptoms of an acute schistosomiasis syndrome ($P = 0.01$ at week 8, Fig. 5d). There were no detectable changes in the other circulating chemokines or cytokines measured in serum (data not shown).

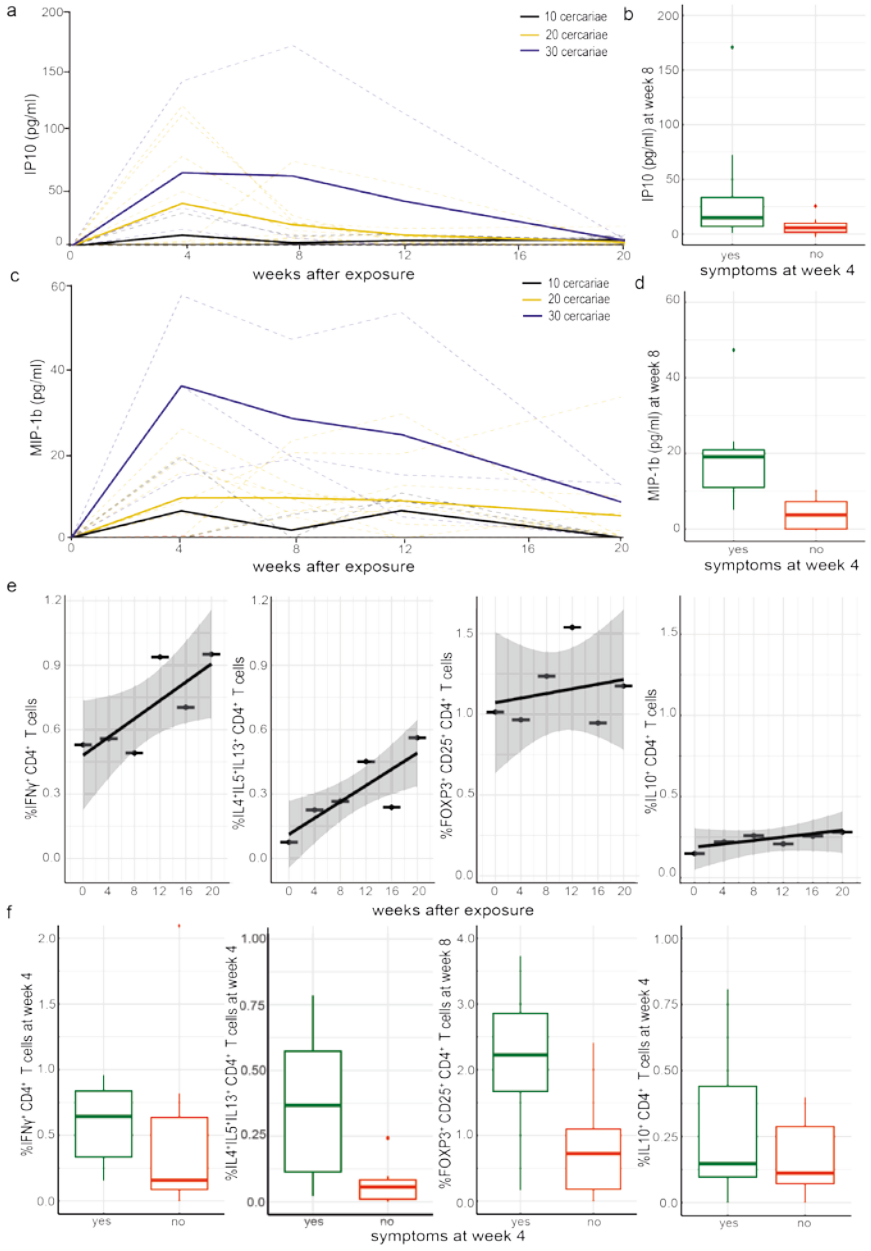

Figure 5. Cytokine responses. a, Ex vivo IP-10 over time for individual volunteers (dotted line) and the mean of each group (line). **b**, IP-10 levels at week 8 in volunteers with (yes, *n* = 8) or without (no, *n* = 8) symptoms of an acute schistosomiasis syndrome (two-sided Mann–Whitney *U*-test, *P* = 0.16). **c**, Ex vivo MIP-1β over time for individual volunteers (dotted line) and the mean of each group (line). **d**, MIP-1β levels at week 8 in volunteers with (yes, *n* = 8) or without (no, *n* = 8) symptoms of an acute schistosomiasis syndrome at week 4 (two-sided Mann–Whitney *U*-test, *P* = 0.01). **e**, The percentage of IFN-γ-producing CD4⁺ T cells (*P* = 0.01), Th2 cytokine (IL-4, IL-5 and IL-13)-producing CD4⁺ T cells

(P = 0.004), FOXP3$^+$CD25$^+$CD4$^+$ T cells (P = 0.49) and IL-10-producing CD4$^+$ T cells (P = 0.91) over time for all volunteers (n = 16), showing average of all volunteers at each week and fitted linear regression line with 95% confidence interval (linear mixed model with two-sided Student's t-test, using the Satterthwaite's degrees of freedom method). **f**, The percentage of IFN-γ-producing CD4$^+$ T cells (P = 0.28), Th2 cytokine (IL-4, IL-5 and IL-13)-producing CD4+ T cells (P = 0.01), FOXP3$^+$CD25$^+$CD4$^+$T cells (P = 0.02) and IL-10-producing CD4$^+$ T cells (0.56) in volunteers with (yes, n = 8) or without (no, n = 8) symptoms of an acute schistosomiasis syndrome. In all boxplots, the lower and higher hinge are respectively the first and third quartiles and the middle line is the median (second quartile). The whiskers extend from the hinge to the largest value no further than 1.5× the interquartile range or distance between the first and third quartile. The largest and lowest visible points beyond the whiskers extend this range and can then be considered, respectively, the maxima and minima.

Cellular immunology

Overall, the frequency of antigen-specific IFN-γ (P = 0.01) and Th2 cytokine (interleukin (IL)-4, IL-5 and IL-13, P = 0.004)-producing CD4$^+$ T cells increased over time (Fig. 5e and Extended Data Fig. 5a,b), but FOXP3$^+$CD25$^+$CD4$^+$ regulatory T cells (P = 0.49) or the production of the regulatory cytokine IL-10 (P = 0.91, Fig. 5e) did not. However, in volunteers with symptoms of an acute schistosomiasis syndrome, both IFN-γ and Th2 cytokine-producing CD4$^+$ T cells and CD25$^+$FOXP3$^+$ regulatory T cells were higher at week 4 and week 8 respectively, although not significantly for all (P = 0.28, P = 0.01 and P = 0.02, respectively; Fig. 5f). There were no differences in IL-10-cytokine-producing CD4$^+$ T cells at week 4 (Fig. 5f). At all timepoints 17% of IFN- γ$^+$CD4$^+$ T cells also produced IL-2.

Data integration

To understand which immunological and microbiological datasets were associated with the occurrence of symptoms of an acute schistosomiasis syndrome, we performed data integration using parallel generalized canonical correlation and partial least squares discriminant analysis. Eosinophil data decreased model accuracy and were thus removed (Extended Data Fig. 6a,b). At baseline, 9 of 16 (56.3%) participants were accurately predicted by the model with leave-one-out cross-validation, which increased to 13 of 16 (81.3%) correct classifications at week 12 and further to 15 of 16 (93.8%) when data from all timepoints were included (Fig. 6a,b). Thus, the model was able to accurately classify participants by the presence of an acute schistosomiasis syndrome. Permutation analysis confirmed that symptoms were strongly associated with the measured immunological and microbiological parameters over the infection course (n = 1,000; 99.6th percentile, Extended Data Fig. 6c). We identified Th2 cytokines at week 4 and FOXP3 regulatory T cells at weeks 12 and 16, MIP-1β at weeks 8 and 12 and levels of serum CAA at week 9 as important features elevated in symptomatic participants by leave-one-out cross-validation (Fig. 6c). Levels of Th2 cytokines upon stimulation at week 4 correlated with serum CAA at week 9, whereas concentrations of MIP-1β in plasma correlated with FOXP3 regulatory T cell numbers (Extended Data Fig. 6d).

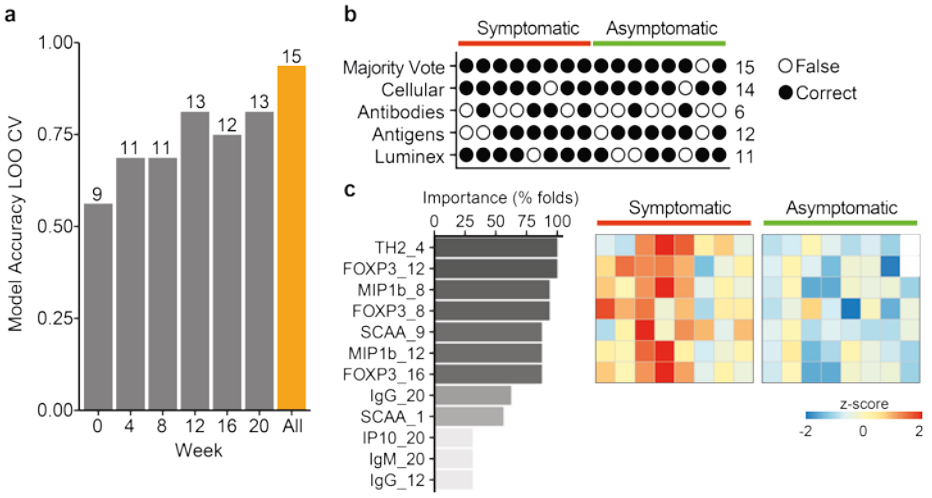

Figure 6. Immunological and microbiological data integration. Cellular, antibody, antigen and Luminex data were combined with generalized canonical correlation analysis and simultaneously associated with presence of symptoms through discriminant analysis. **a**, Proportion of correct predictions of each model assessed using leave-one-out cross-validation. Gray bars indicate data from one specific timepoint and orange bars indicate the model, including all data across timepoints. The number of correct predictions is indicated above each bar. **b**, Individual predictions across folds for the full model per dataset, including majority vote, with weighing to break ties. Each symbol represents one prediction, with volunteers in columns and datasets in rows. Filled circles indicate a correct prediction and open circles a false prediction. Volunteers with (red, *n* = 8) or without (green, *n* = 8) symptoms of acute schistosomiasis syndrome are indicated, as are the number of correct predictions per dataset. **c**, Consensus features selected in at least 25% of the folds are indicated and ranked by frequency of presence among folds. The number after each feature indicates the week of measurement. The *z* score-normalized levels of the seven features present in at least 75% of the folds are indicated in the heat map, where each column corresponds to one volunteer. Missing data points from one asymptomatic participant are depicted in white.

Discussion

This pilot study shows that experimental exposure to 20 male cercariae results in a detectable and well-tolerated *S. mansoni* infection in 82% of volunteers. This infection rate resembles that of other human infection models[14]. All volunteers were cured after 1–2 doses of praziquantel. The dose-escalating design of the study revealed a concomitant increase in AEs. The occurrence of a severe acute schistosomiasis (Katayama) syndrome in one volunteer prompted us to lower the dose. At a dose of 20 cercariae, 2 of 11 volunteers reported severe AEs, which is comparable with other human infection models. For example, in experimental malaria infections, volunteers generally experience three to four AEs, of which one would be severe for several days[15]. In typhoid infections ~50% of volunteers report severe symptoms and 55% experiences fever[16] and in cholera infection studies 40% of volunteers experience 1.6–8.0 liters of diarrhea[17]. We thus conclude that 20 cercariae may be the optimal dose that was both well tolerated and detectable. A relationship between dose and symptoms, as suggested by epidemiological data[18], cannot be

statistically confirmed with the current number of volunteers. Regardless of dose, all symptoms had resolved without sequelae at 12 weeks.

The follow up of volunteers in our study unequivocally showed that anti-adult worm IgM (100% seroconversion by week 6) or serum CAA (10 of 17 positive by week 6) are the earliest diagnostic markers currently available. This is in accordance with previous studies in travelers[19–21]. We found levels of serum CAA comparable to those in low endemic settings[22]. In nonhuman primate models these levels reflect 5–24 cercariae reaching adulthood[8,23]. Although serum CAA levels were more stable compared to urine CAA, the higher volume input of urine allows for more sensitive detection[24]. As suggested in previous studies, the urine POC-CCA rapid test was not suitable to detect very low intensity infection[25]. The main advantage of measuring serum CAA levels, as opposed to antibody detection, is the potential to follow up after treatment and confirm cure. In doing so, we found low cure rates with a single dose of 40 mg kg^{-1} praziquantel. Although a 60 mg kg^{-1} dose seemed more effective, the subsequent changes in pharmacokinetic and pharmacodynamic parameters need to be evaluated to conclude this with certainty. Because of the synchronous development of adult worms and the absence of reinfection in our model, the CHI design allows for screening of existing and new (stage-specific) anthelmintics.

The controlled schistosome infection model presented here clearly differs from infections in endemic settings, where doses are not controlled and infection occurs repeatedly. In addition, the single-sex infection lacks production of eggs that drive chronic regulatory and modified Th2 responses[26]. The presence of IgG to soluble egg antigen, indicates cross-reactive epitopes between eggs and worms. The induction of chemokines IP-10 and MIP-1β aligned with the increase in both antigen-specific $CD4^+$ T cell IFN-γ and Th2 cytokine production, respectively. The IFN-γ production contrasts with predominantly Th2 profiles in epidemiological studies, which may be driven by egg-related responses[26]. Although we detected increases in $FOXP3^+CD25^+CD4^+$ T cells, these mainly occurred in symptomatic volunteers and were found at a later timepoint than IFN-γ- or Th2 cytokine-producing T cells. This would be in line with the induction of regulatory T cells to prevent deleterious immune responses[27], different from the chronic regulation found in endemic infections. These important differences in antigen exposure and subsequent immunological responses between single-sex infections and endemic chronic egg production may limit the use of the model to study anti-fecundity effects. However, the immunological observations from our model are comparable to acute infection models in travelers and baboons, where a mixed Th1 and Th2 response also dominates[28,29]. Integrated data analysis revealed that acute schistosomiasis symptoms were particularly associated with antigen-specific Th2 cytokine production and circulating MIP-1β and IgM production, but could not be predicted at baseline. In contrast to volunteers with symptoms, those without symptoms predominantly showed IgG1 antibody responses. Similarly to what has been performed for other infectious diseases[30], a trial with repeated controlled infections could address whether these IgM or IgG1 antibodies have a protective effect. Currently, the clinical and immunological data from baseline alone cannot predict who will become symptomatic. However, the analysis of longitudinal responses reveals a clear profile predicting presence or absence of symptoms in 15 out of 16 participants. Unraveling the interplay between symptoms, immune responses and resistance to schistosome reinfection provides an opportunity for identification of new antigens for vaccine development[31].

Immunological models for protection against *S. mansoni* were developed in rodents and nonhuman primates by repeated exposure to radiation attenuated cercariae[23,32]. In humans, three candidate *S. mansoni* vaccines are currently in clinical development[6]. A phase I study with rSm14/GLA-SE showed a good safety profile and immunogenicity[7]. Phase I safety results for Sm-

TSP-2 are expected soon (ClinicalTrials.gov identifier: NCT02337855), while Sm-p80 is currently entering phase I testing on the basis of promising efficacy data in baboons[8]. The limited funding opportunities and large sample size required for phase III testing preclude testing of all three candidates in phase III trials. Despite the differences between chronic *S. mansoni* infection in the field and the controlled human *S. mansoni* infection, the model provides an opportunity to obtain preliminary efficacy data on these vaccines and reduce the costs by allowing selection of the most promising candidates, which may be co-formulated to maximize benefit[12].

Future vaccine studies aim at a 75% infection reduction in worm burden and egg output[10]. As there is a clear relationship between worm burden and serum CAA levels[24,33], we propose that the reduction in worm burden can be determined by measuring the median serum CAA level between week 7–12. Assuming an 80% power and $\alpha = 0.05$, this would require a group size of 11 individuals per arm. Currently the main limitation of our model is the use of male schistosomes only. Consequently vaccine targets that are more commonly expressed on females, such as the Sm-p80 vaccine candidate[8], cannot be fully evaluated. A female worm infection model would be of value to dissect mechanisms of action and sex-specificity of vaccines.

We conclude that this controlled human *S. mansoni* infection model results in an 82% infection rate with few severe side effects at a dose of 20 cercariae. In addition, this model provides insight into the onset of symptoms of a schistosome infection, the ensuing immune response and the performance of diagnostic tests over time. Notably, this model paves the way for cost-effective and rapid proof-of-concept testing of new vaccines and drugs.

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Methods

Study design

This phase I trial (ClinicalTrials.gov identifier: NCT02755324) was an open-label dose-escalation study at the Leiden University Medical Center (LUMC).

Male cercariae were produced as previously reported[34]. The initial dose was 10 cercariae (n = 3), which was escalated to 30 (n = 3) and subsequently de-escalated to 20 cercariae (n = 3) on the basis of AEs. The 20 cercariae infection was then validated in another group (n = 8). The viability of cercariae was confirmed by imaging of cercariae penetrating skin explants[35]. The cercariae were applied to the volunteer's forearm in 0.5–1 ml of water for 30 min, the number of remaining cercariae were counted by microscopy. Volunteers were observed for 30 min after exposure.

Volunteers were followed bi-weekly between week 0–24 and on week 52. During each visit, AEs were recorded. Symptoms of an acute schistosomiasis syndrome were defined as moderate to severe symptoms of malaise, fatigue, fever, night sweats, flu-like symptoms or headache, between 2–7 weeks after exposure. Safety reports were reviewed regularly by an external safety monitor, who advised on dose escalation. Blood and urine samples were collected at all visits.

The pre-patent period was defined as the time until serum CAA levels were above 1.0 pg ml^{-1} [24]. At week 12 all volunteers were treated with 40 mg kg^{-1} praziquantel in two doses. A second regimen of 60 mg kg^{-1} praziquantel in two doses was provided if serum CAA levels persisted 3–6 weeks after treatment. Cure was defined as serum CAA levels ≤0.5 pg ml^{-1}.

The study was approved by the LUMC Institutional Medical Ethical Research Committee (Institutional Review Board P16.111). It was performed according to the European Clinical Trial Directive 2001/20/EC, in accordance with ICH-GCP guidelines and the Declaration of Helsinki[36,37].

Informed consent procedure

Healthy 18–45-year-old *Schistosoma*-naïve volunteers were screened by medical history, general physical examination and safety laboratory tests. Informed consent was obtained from all volunteers.

Through advertisements, volunteers provided their email address and received written information. When they so wished, volunteers could schedule a screening visit at least 3 d after having received the information. They were then requested to complete an application form, which included a questionnaire regarding their health.

During the 1.5–2-h screening visit, the study purpose and procedures were explained and questions answered. The possible AEs and right of withdrawal were explained to the volunteers. The informed consent form was signed and a full physical exam was performed. All volunteers were required to consent to an HIV, hepatitis B (HBV) and hepatitis C (HCV) serological screening, urine toxicology and (for females), a pregnancy test at screening.

At the infection day (14 d to 23 weeks after screening), informed consent was reconfirmed, and a final check of inclusion and exclusion criteria was performed, including a focused physical exam. Volunteers were exposed to *male Schistosoma mansoni* cercariae after baseline assessment and safety laboratory tests.

The first volunteer was included on 27 October 2015 and the last volunteer was included on 1 February 2018.

All three volunteers gave permission to use the photographs (Fig. 2a–c) taken of their skin after cercarial exposure for publication.

Inclusion and exclusion criteria

Inclusion criteria

- Volunteer is aged ≥ 18 and ≤ 45 years and is in good health.
- Volunteer has adequate understanding of the procedures of the study and agrees to abide strictly thereby.
- Volunteer is able to communicate well with the investigator and is available to attend all study visits.
- Volunteer will remain within Europe (excluding Corsica) during the study period and is reachable by mobile telephone from week 3 to week 12 of the study period.
- Volunteer agrees to refrain from blood donation to Sanquin or for other purposes throughout the study period.
- For females: volunteer agrees to use adequate contraception and not to breastfeed for the duration of study.
- Volunteer has signed informed consent form.

Exclusion criteria

- Any history, or evidence at screening, of clinically significant symptoms, physical signs or abnormal laboratory values suggestive of systemic conditions, such as cardiovascular, pulmonary, renal, hepatic, neurological, dermatological, endocrine, malignant, hematological, infectious, immune-deficient, psychiatric and other disorders, which could compromise the health of the volunteer during the study or interfere with the interpretation of the study results. These include, but are not limited to, any of the following:
- body weight <50 kg or body mass index <18 or >30 kg m^{-2} at screening;
- positive HIV, HBV or HCV screening tests;
- the use of immune-modifying drugs within 3 months before study onset (inhaled and topical corticosteroids and oral anti-histamines exempted) or expected use of such during the study period;
- history of malignancy of any organ system (other than localized basal cell carcinoma of the skin), treated or untreated, within the past 5 years;
- any history of treatment for severe psychiatric disease by a psychiatrist in the past year;
- history of drug or alcohol abuse interfering with normal social function in the period of 1 year before study onset;
- any clinically significant abnormalities (including extended QT interval) on electrocardiogram.
- The chronic use of any drug known to interact with praziquantel, artesunate or lumefantrine (artesunate combined with lumefantrine served as alternative treatment of schistosomiasis in an earlier phase of infection) metabolism (for example, phenytoin, carbamazepine, phenobarbital, primidone, dexamethasone, rifampicin, cimetidine, flecainide, metoprolol,

imipramine, amitriptyline, clomipramine, class IA and III anti-arrythmics, antipsychotics, antidepressants, macrolides, fluorquinolones, imidazole- and triazole antimycotics and antihistamines). Because lumefantrine may cause extension of QT-time, chronic use of drugs with effect on QT interval are excluded from the study.
- For female volunteers: positive urine pregnancy test at screening
- Any history of schistosomiasis or treatment for schistosomiasis
- Positive serology for schistosomiasis or elevated serum or urine CAA at baseline
- Known hypersensitivity to or contraindications (including co-medication) for use of praziquantel, artesunate or lumefantrine
- Being an employee or student of the Department of Parasitology or Infectious Diseases of the LUMC.

Adverse events

Grading

- Mild (grade 1): awareness of symptoms that are easily tolerated and do not interfere with usual daily activity
- Moderate (grade 2): discomfort that interferes with or limits usual daily activity
- Severe (grade 3): disabling, with subsequent inability to perform usual daily activity, resulting in absence or required bed rest
- Serious AE: any untoward medical occurrence in a patient or trial participant, which does not have a causal relationship with the treatment, and:
    - is fatal, and/or
    - is life-threatening for the volunteer, and/or
    - makes hospital admission or an extension of the admission necessary, and/or
    - causes persistent or significant invalidity or work disability, and/or
    - manifests itself in a congenital abnormality or malformation, and/or
    - could, according to the person that carries out the research, have developed to a serious undesired medical event, but was, however, prevented due to premature interference.

Causality

Unrelated
- Not related: a relationship to the administration of *the S. mansoni* male cercariae cannot be reasonably established; another etiology is known to have caused the AE or is highly likely to have caused it.
- Unlikely related: a relationship to the administration *of S. mansoni* male cercariae is unlikely; however, it cannot be ruled out.

Related
- Possibly related: there is a potential association between the event and administration of the *S. mansoni* male cercariae; however, there is an alternative etiology that is more likely.

- Probably related: administration of the *S. mansoni* male cercariae is the most likely cause; however, there are alternative reasonable explanations, even though less likely.
- Definitely related: administration of the *S. mansoni* male cercariae is the cause; another etiology causing the adverse event is not known.

Parasitological assays

CAA was measured in serum and urine by the previously described upconverting phosphor lateral flow (UCP-LF CAA) assay[24,38]. The presence of urine CCA was determined by the POC-CCA (Rapid Medical Diagnostics). The *Schistosoma* PCR on feces was performed according to previous descriptions (ISO 15189:2012-certified)[39].

Antigen detection assays

Upconverting phosphor lateral flow assay for circulating anodic antigen
Both serum and urine were analyzed for schistosome CAA using a UCP-LF CAA assay. The assay was performed as described previously[24,38]. In brief, 500 µl of serum (or 4 ml of urine) samples or standards were diluted 1:1 in 4% trichloro-acetic acid (TCA) (or diluted 5:1 in 12% TCA for urine samples) and incubated for 5 min at room temperature. Serum samples were centrifuged for 10 min at 13,000 r.p.m. (and urine samples were centrifuged for 45 min at 4,000 r.p.m.). Then 500 µl of supernatants of serum (or 4 ml of urine) was applied to 0.5-ml (or 4.0-ml) Amicon filtration devices (Amicon Ultra-0.5 (or Amicon Ultra4), Millipore) and concentrated to approximately 20 µl by centrifugation for 25 min at 13,000 r.p.m. for serum (or 60 min at 4,000 r.p.m. for urine). The concentrates were diluted 1:5 in LF assay buffer and incubated in microtiter plate wells at 37 °C for 1 h while shaking. LF strips were inserted into the wells and incubated for 3 h or overnight, before being read on a modified Packard FluoroCount microtiter plate reader[24]. A TCA-soluble fraction of *S. mansoni* adult worm antigen with known CAA concentration was used as a reference standard for the quantification of the antigen. Predefined cutoff values were used, where a serum CAA concentration above 1 pg ml^{-1} was defined as positive, below 0.5 pg ml^{-1} as negative and between 0.5 and 1.0 as undecisive[24].

Point-of-care circulating cathodic antigen
Urine samples were tested for the presence of schistosome CCA using a commercially available rapid diagnostic test (POC-CCA, batch no. 170622073, Rapid Medical Diagnostics) according to the manufacturer's procedure. Readings above a trace line were considered as positive.

Immune responses

Clinical diagnostics

Schistosome-specific antibodies, IgM against adult worms and anti-SEA IgG were determined according to ISO 15189:2012-certified routine diagnostic in-house IFA and ELISA respectively, which have been in use for the clinical diagnosis of schistosomiasis at the LUMC for decades[40,41].

IgM adult worm antibodies by IFA

IgM antibodies against adult worms were detected by an in-house IFA assay as previously described[42]. This IFA, as well as the ELISA described below, are currently in use at LUMC as the routine antibody detecting assays for the diagnosis of imported schistosomiasis and feature in all laboratory quality assessment requirements (ISO 15189:2012-certified), including successful participation in an external quality assessment scheme (UK-NEQAS). In brief, sections of Rossman's fixed male adult worms were incubated with a twofold dilution series of serum samples starting at 1:8 dilution. Following incubation with goat anti-human IgM (u-chain specific)-FITC antibody (Sigma-Aldrich; F5384), slides were examined using a fluorescence microscope. A negative control and a positive reference serum were run in parallel at each slide. The titer was determined as the dilution of the sample at which the fluorescence of adult worm gut epithelium was still visible. Samples were considered positive if titers were above 1:8. Previous studies showed that the IgM detected in this IFA is mainly directed against CCA[42].

IgG against soluble egg antigen

IgG antibodies directed against *S. mansoni* SEA were detected by a previously described in-house ELISA with some minor modifications[40]. Crude SEA was prepared from *S. mansoni* eggs collected from the livers of infected hamsters[43,44]. A concentration of 5 µg protein ml^{-1} was diluted in 100 µl of 0.1 M sodium carbonate buffer (pH 9.6) and coated overnight at 4 °C in 96-well plates (Polysorb NUNC). Plates were stored at −80 °C until use. After thawing, plates were washed and blocked in 1% BSA in PBS for 1 h at 37 °C. A dilution series of serum samples from 1:16 to 1:2,048 in a solution with 5% FCS and 0.05% Tween in PBS were incubated for 1 h at 37 °C. Plates were washed and incubated for 1 h at 37 °C with mouse anti-human IgG alkaline phosphatase 1:10,000 (Sigma-Aldrich; A2064) in 4% BSA and 0.05% Tween/PBS. Para-nitrophenyl phosphate (pNPP) substrate (Sigma-Aldrich; P5994) in 0.1 M diethanolamine buffer (pH 9.6) (Merck) was added for 1 h at room temperature after washing. Plates were read with the Multiskan EX reader at 405 nm. A negative control and a positive reference serum were run in parallel at each plate. The titer was determined as the dilution of the sample at which the extinction is higher or equal to the reference standard. Samples were considered positive if titers were above 1:16.

Experimental immunological assays

Serum was analyzed for total IgE, *S. mansoni* AWA-specific IgE and IgG by ELISA[45–47]. Data were expressed as arbitrary units (AU ml^{-1}). Peripheral blood mononuclear cells (PBMCs) were evaluated for their phenotype and function after 24-h stimulation with crude *S. mansoni* AWA by flow cytometry.

Adult worm antigen-specific IgE, IgG, IgG1 and IgG4

S. mansoni AWA-specific IgE and IgG was measured by ELISA modified from previous protocols[45,46]. In brief, adult *S. mansoni* worms were collected from hamsters and crude AWA was prepared as described previously[48]. MaxiSorp plates (Nunc) were coated overnight with 5 µg ml^{-1} AWA diluted in carbonate buffer pH 9.6. After blocking with 5% BSA/PBS, the sera were diluted with a solution with 5% FCS and 0.05% Tween in PBS and the presence of IgG1 or IgG4 was shown by using horseradish peroxidase (HRP)-labeled anti-human IgG1 or HRP-labeled anti-human IgG4 (1:3,000 dilution; Sanquin). For measuring total anti-AWA IgG antibody, alkaline phosphatase-conjugated anti-human IgG (1:5,000 dilution; Sigma) was added, whereas for the anti-AWA IgE

assay, the plate was incubated with biotinylated goat anti-human IgE (1:1,000 dilution; Vector Laboratories) followed by streptavidin HRP conjugate (1:10,000 dilution; Sanquin). IgG1 and IgG4 assays were developed using tetramethylbenzine, stopped with 10% H_2SO_4 and the absorbencies were measured at 450 nm. For total IgG and IgE, the color was developed by addition of pNPP (Sigma) diluted in diethanolamine buffer and optical density was measured at 405 nm. For all four assays, the levels of antibody present in a given sample were expressed in AU ml^{-1} or International Units (IU ml^{-1}) according to the standard curve of pooled sera from inhabitants of an *S. mansoni* endemic area in Ghana. The levels of antibody present in a given samples were expressed in AU ml^{-1} for total IgG, IgG1 and IgG4 or in IU ml^{-1} for IgE. Seroconversion was defined as antibody levels above 2× s.d. of the baseline.

Total IgE

Total IgE levels were measured as previously described[47]. Briefly, MaxiSorp plates were coated overnight with rabbit anti-human IgE (Dako). Plates were blocked with PBS 5% BSA followed by incubation of diluted samples in PBS 0.05% Tween-20. As a reference, the World Health Organization standard of human serum IgE (NIBSC) was used, starting at a concentration of 90 IU ml^{-1}. After a washing step, the plates were incubated with IgE biotinylated goat anti-human IgE antibody (Vector Laboratories) followed by an incubation with streptavidin alkaline phosphatase conjugate (Boehringer Mannheim). The color was developed by addition of pNPP (Boehringer Mannheim) diluted in diethanolamine buffer and optical density was measured at 405 nm. The results were expressed in IU ml^{-1}. Seroconversion was defined as antibody levels above 2× s.d. of the baseline.

Ex vivo cytokines

Serum samples were tested for the presence of different cytokines using a commercially ProcartaPlex Multiplex Immunoassay (17-plex, lot 178863000, Invitrogen) according to the manufacturer instructions. The following cytokines were measured: IFN-α, IFN-β, IL-1β, IL-10, IL-12p70, IL-13, IL-15, IL-2, IL-22, IL-23, IL-4, IL-6, IP-10, MCP-1, MIP-1α, MIP-1β and TNF-α using the Bioplex 200 Luminex (Bio-Rad).

Cellular immunology

Between week 0 and 24 after exposure every 4 weeks, human PBMCs were isolated from whole blood collected in heparin. Cells of two heparin tubes were diluted at least 1:2 with HBSS (ThermoFisher) at room temperature. Ten milliliters of ficoll at room temperature was added, followed by 25 min centrifugation at 400g with low brake. Cells were collected and washed with HBSS, counted and frozen in 10% DMSO, in RPMI Hepes (Invitrogen), with 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 1 mM pyruvate/2 mM glutamate and 10% FCS (Bodinco). Subsequently, PBMCs were thawed in RPMI Hepes, with 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 1 mM pyruvate/2 mM glutamate and 10% FCS and rested overnight at 37 °C with 5% CO_2. Cells were counted and transferred to a 96-well round bottom plate (Corning) with 500,000 cells per well. Cells were stimulated with AWA (50 µg ml^{-1}) for 24 h. Staphylococcal enterotoxin B (SEB) (Sigma-Aldrich) 200 ng ml^{-1} was used as a positive control and RPMI Hepes (Invitrogen), with 100 U ml^{-1} penicillin, 100 µg $m^{l-}1$ streptomycin, 1 mM pyruvate/2 mM glutamate and 10% FCS as a negative control. After 4 h of incubation 5 mg ml^{-1} brefeldin A (Sigma) was added to SEB-stimulated wells and after 20 h to AWA- and medium-stimulated wells. After a total stimulation of 24 h, the cells were stained with Aqua (Invitrogen) and fixed with 3.9% formaldehyde (Sigma). After fixating,

the cells were stained with the following antibodies: CD3, CD4, IFN-γ, IL-2, Th2-cytokines (IL-4, IL-5, IL-13), TNF and IL-10 (Supplementary Table 4). Human FC block was used to avoid nonspecific interactions. The cells were measured with the FACSCanto II (BD Biosciences; Supplementary Fig. 1). The data were analyzed with FlowJo 10.5 software for MAC OS. The gating was placed with the help of fluorescence minus one controls, the medium as a negative control and SEB as a positive control. The leftover, aqua-stained and fixed cells were frozen in 10% DMSO, in RPMI Hepes (Invitrogen), with 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 1 mM pyruvate/2 mM glutamate and 10% FCS (Bodinco) and stored at −80 °C. The cells were thawed at 37 °C and stained with the following antibodies: CD3, CD4, CD25, CD127 and FOXP3 (Supplementary Table 4). As before, human FC block was used to avoid nonspecific interactions. The cells were measured with the FACSCanto II (BD Biosciences; Supplementary Fig. 1). The data were analyzed with FlowJo 10.5 software for MAC OS. The gating was placed with help of fluorescence minus one controls, the medium as a negative control and SEB as a positive control.

Statistical analysis

All 17 volunteers were included in the intention-to-treat analysis (safety analysis and parasitological assays). One volunteer was excluded from the per-protocol analysis (all immunological readouts) on the basis of high baseline AWA-specific IgG and IgG1 levels. Samples from individual volunteers were measured once and plotted as single values.

Demographics and the presence of symptoms between groups were analyzed with a Mann–Whitney *U*-test, time to patency with a log-rank test and correlations with a Spearman's rank test. Changes in the frequency of cytokine-producing cells over time were analyzed using a linear mixed model. Time was considered as the fixed effect and the volunteer ID as a random effect for the intercept. *P* values based on Student's *t*-tests were obtained using the Satterthwaite's degrees of freedom method. In the cytokine boxplots, the negative values (after subtracting the medium condition) were set to zero to prevent a negative cytokine response. However, the statistical analysis was performed on the unaltered data. All statistical tests were two-tailed with α set at the 0.05 level.

Analyses were performed using IBM SPSS statistics for Windows, v.23.0 (IBM Corp.) and R (v.3.5). The R packages used were ggplot2, lme4 and lmerTest[49–51].

P values were considered significant when $P < 0.05$.

MixOmics and data integration

Data integration was performed using the mixOmics package in R (v.6.8.0)[52,53]. This method allowed us to correlate across datasets, while associating features with outcome. Feature selection was performed using Lasso-like penalization for each of the datasets. The number of components was set at two and tuning was performed to find the minimum number of features needed per dataset (in a range from 1–3 per component) and the correlation between datasets was entered into the design matrix. The number of features included in the final model was 3, 3, 2 and 5 for cytokines, antigens, antibodies and cellular responses, respectively. A correlation of 0.75 between datasets was used in the design matrix.

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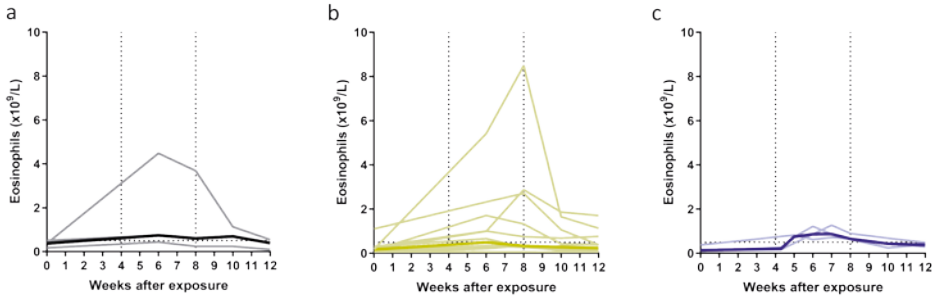
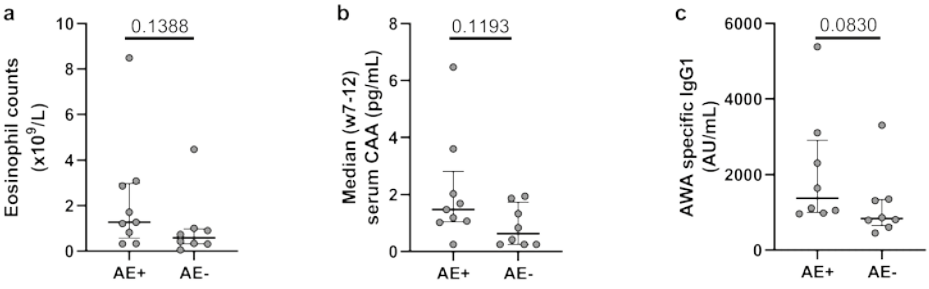
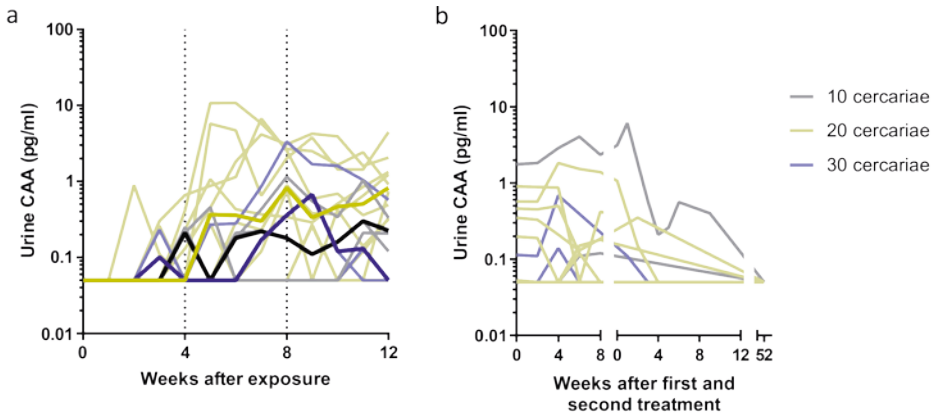
Extended data



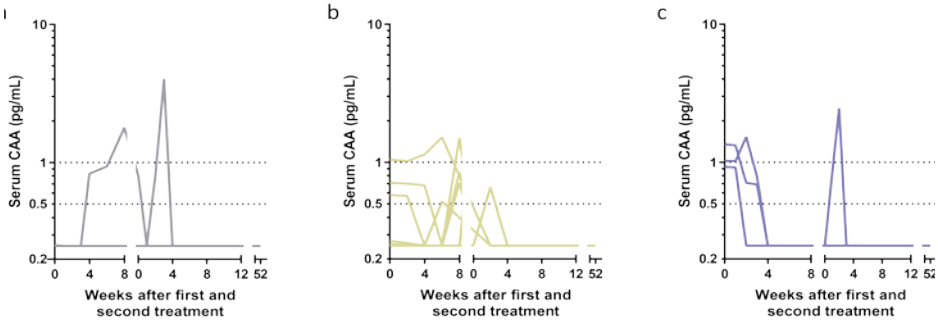
Extended Data Figure 1. Eosinophil counts. a-c. Eosinophil counts (x10^9/L) per volunteer and the median per group. The thin green, red, and blue lines represent data of individual volunteers infected with 10 (*n* = 3), 20 (*n* = 11) or 30 (*n* = 3) cercariae respectively, while thick lines represent the median of each group.



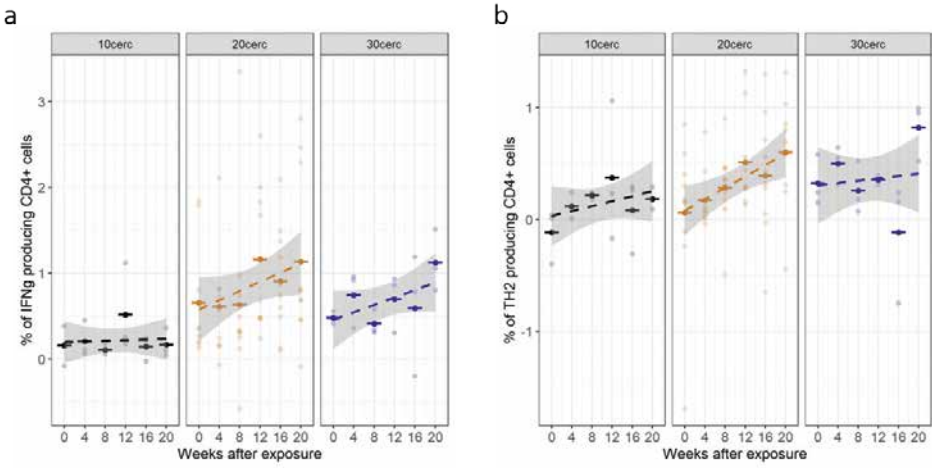
Extended Data Figure 2. Relation between symptoms of an acute schistosomiasis syndrome and immunological readouts. The relation between the presence of symptoms of an acute schistosomiasis infection and **a**. the highest eosinophil count (*n* = 17), **b**. the median serum CAA level from week 7 to 12 (*n* = 17), or **c**. the AWA specific IgG1 response at week 16 (*n* = 16). All using the two-sided Mann-Whitney *U* test. Individual data is presented as dots, the line represents the median, while the error bars represent the interquartile range of the groups.



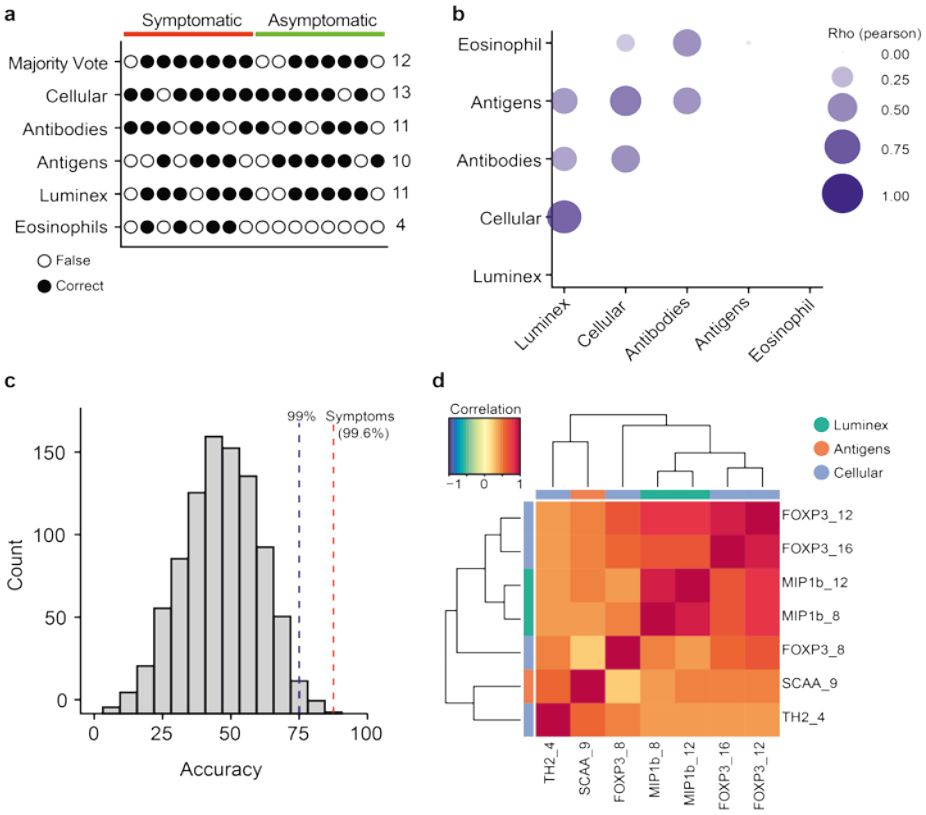
Extended Data Figure 3. Urine CAA levels. a. Urine CAA levels after exposure and **b**. after first and second praziquantel treatment (week 0) and at week 52 after exposure. The thin green, red, and blue lines represent of individual volunteers infected with 10 (*n* = 3), 20 (*n* = 11) or 30 (*n* = 3) cercariae respectively, while thick lines represent the median of each group before treatment.



Extended Data Figure 4. Serum CAA levels after treatment. a-c. Serum CAA levels in pg/ml after the first treatment, second treatment, and at week 52. All values below the detection threshold of 0.5 pg/mL, are plotted at 0.25 pg/mL. The gray, yellow and blue lines represent data of individual volunteers infected with 10 (*n* = 3), 20 (*n* = 11) or 30 (*n* = 3) cercariae respectively.

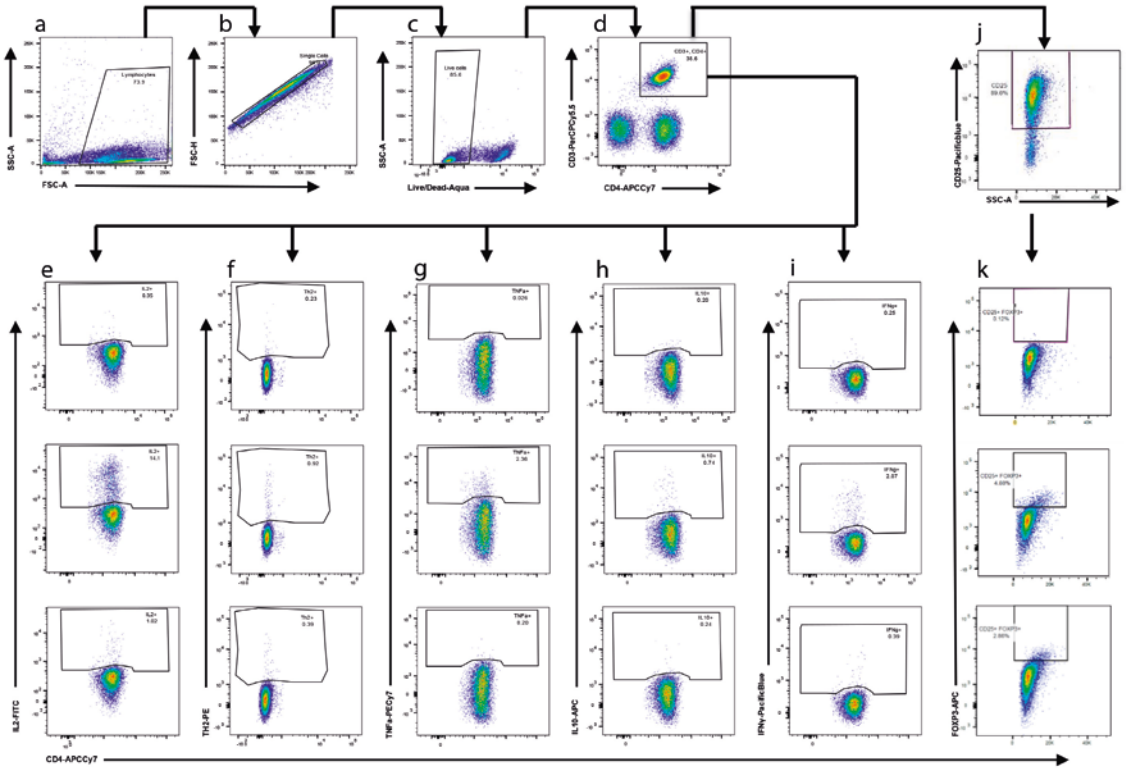


Extended Data Figure 5. IFN-γ and TH2 cytokine producing CD4⁺ T-cells over time. The percentage of **a**. IFN-γ and **b**. Th2-cytokine producing CD4⁺ T-cells over time in weeks after exposure in the 10 cercariae (gray, *n* = 3), 20 cercariae (yellow, *n* = 10) and 30 cercariae (blue, *n* = 3) groups. Dotted lines are linear regression lines, gray areas are confidence intervals, light dots are individual data, and horizontal lines with dots are the average values.



Extended Data Figure 6. Effect of eosinophil data and model performance. a. Individual predictions across folds for the full model per dataset including majority vote. Each symbol represents one prediction, with volunteers in columns and datasets in rows. Filled circles indicate a correct prediction and open circles a false prediction. Symptomatic and asymptomatic volunteers are indicated in red and the number of correct predictions per dataset is indicated. **b**. Mean Pearson correlation score between datasets using the first component of the projection onto the latent space across all folds from the model including all datasets. Size and color of circles reflect the mean rho value. **c**. Permutations analysis (n = 1000) with leave-one-out cross-validation on the full model using all subjects and including the four datasets without eosinophils. Blue and red dashed lines indicate the 99th percentile and the accuracy when comparing symptomatic and asymptomatic individuals (99.6%), respectively. **d**. Spearman correlation matrix of the seven consensus features selected in > 75% of folds in the leave-one-out cross-validation. Features were clustered using hierarchical clustering with complete linkage on Euclidean distance. All graphs are based on n = 16.

Supplementary



Supplementary Figure 1. Flow cytometry gating strategy. Gating of stimulated PBMCs. **a**. gating on lymphocytes. **b**. gating on single cells. **c**. gating of live cells **d**. gating on $CD4^+$ cells. **e-i**: gating on selection of IL2, Th2 (IL-4+IL-5+IL-13), TNF-α, IL10, or IFN-γ positive cells. **j**. gating on $CD25^+$ cells **k**. gating on FOXP3 positive cells. For cells stimulated with either medium, Staphylococcal Enterotoxin B (SEB) or adult worm antigen (AWA).

Supplementary table 1. Demographics and cercariae retrieved after exposure. Demographics of included volunteers, (median and range). The number of heads, tails and whole body cercariae retrieved from volunteers' skin after infection (median and range). The 10, 20 and 30 cercariae groups were compared using the two-sided Mann-Whitney U test (top) or the Spearman's rank correlation (bottom).

	10 cercariae (*n*=3)	20 cercariae (*n*=11)	30 cercariae (*n*=3)	All volunteers (*n*=17)	*P*-value
Demographics					
Gender					0.34
• Male	1	5	0	6	
• Female	2	6	3	11	
Age in years (median (range))	20 (19-26)	30 (18-45)	23 (21-25)	25 (18-45)	0.21
BMI in kg/m^2 (median (range))	21.7 (20.5-24.1)	25.1 (20.7-28.5)	20.9 (18.6-24.4)	24.1 (18.6-28)	0.12
Cercariae retrieved after exposure					
Heads (median (range))	0 (0-1)	0 (0-1)	1 (0-1)	0 (0-1)	0.37
Tails (median (range))	5 (4-6)	8 (4-12)	14 (10-19)	8 (4-19)	**0.002**
Whole body cercariae (median (range))	0 (0-1)	0 (0-3)	1 (0-3)	0 (0-3)	0.33

Supplementary table 2. Volunteer's age and sex. Listing of volunteer code, group allocation, cercarial exposure, sex and age.

Volunteer	Group	Cercarial exposure	Sex	Age
Cs1-501	group 1	10	F	20
Cs1-516	group 1	10	F	26
Cs1-753	group 1	10	M	19
Cs1-106	group 2	30	F	23
Cs1-495	group 2	30	F	21
Cs1-674	group 2	30	F	25
Cs1-069	group 3	20	F	22
Cs1-229	group 3	20	F	35
Cs1-425	group 3	20	M	20
Cs1-004	group 4	20	M	18
Cs1-102	group 4	20	F	30
Cs1-464	group 4	20	M	36
Cs1-759	group 4	20	M	25
Cs1-780	group 4	20	F	39
Cs1-832	group 4	20	F	23
Cs1-923	group 4	20	M	35
Cs1-967	group 4	20	F	45

Supplementary table 3. Adverse events. The number of volunteers developing a related (either possibly, probably or definitely related) solicited or unsolicited adverse event (AE) throughout the study period. Data is presented per cercarial dose group and in total. The maximum severity of an AE is presented in the table.

			10 cercariae (n=3)			20 cercariae (n=11)			30 cercariae (n=3)			Total (n=17)		
			Mild	**Moderate**	**Severe**	**Mild**	**Moderate**	**Severe**	**Mild**	**Moderate**	**Severe**	**Mild**	**Moderate**	**Severe**
Local	**solicited**	Pruritus	3			10			2			15 (88%)		
		Cercarial dermatitis	3			10			3			16 (94%)		
	unsolicited	Burning feeling				3						3 (18%)		
		Dysesthesia				1						1 (6%)		
Systemic	**solicited**	Fever	1				1	1			2	1 (6%)	1 (6%)	3 (18%)
		Headache				1	4	1			3	1 (6%)	4 (24%)	4 (24%)
		Fatigue					3						3 (18%)	
		Malaise					2			1			3 (18%)	
		Cough					1						1 (6%)	
		Myalgia				1			1			2 (12%)		
		Night sweats					1						1 (6%)	
		Nausea		1							1		1 (6%)	1 (6%)
		Abdominal pain	1						1			2 (12%)		
		Diarrhea		1						1			2 (12%)	
	unsolicited	Less ability to focus	1									1 (6%)		
		Elevated liver enzymes												1 (6%)
		Syncope									1			1 (6%)
		Periorbital edema				1				1		1 (6%)	1 (6%)	
		Pharyngitis/sore throat				2						2 (12%)		

None of the volunteers experienced vomiting, urticaria, backpain or anorexia

Supplementary table 4. The antibodies used for staining of the cells.

Antibody	Label	Dilution	Company	Cat.
IL-2	FITC	1:25	BD Biosciences	340448
IL-4	PE	1:20	BD Biosciences	340451
IL-5	PE	1:250	Biolegend	504303
IL-13	PE	1:100	Biolegend	501903
CD4	PerCP-ef710	1:400	eBioscience	46-0047
TNF	PE-Cy7	1:1000	eBioscience	25-7349
IL-10	APC	1:250	BD Biosciences	554707
CD3	APC ef780	1:800	eBioscience	47-0038
IFN-γ	BV421	1:1000	Biolegend	502531
CD3	FITC	1:100	BD Biosciences	555339
CD4	Pe-Cy7	1:150	BD Biosciences	557852
CD25	BV421	1:200	BD Biosciences	562442
CD127	APCe780	1:200	eBioscience	47-1278-42
FOXP3	APC	1:100	eBioscience	17-4776-42
Human FC block	-	1:100	eBioscience	14-9161
Live/Dead Aqua	AmCyan	1:400	ThermoFisher	L34957

III

Improvement of controlled human infection models

CHAPTER 8

Are placebo controls necessary in controlled human infection trials for vaccines?

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Abstract

Controlled human infection trials, whereby a small group of healthy participants is deliberately exposed to a pathogen under controlled circumstances, can provide preliminary data for vaccine efficacy and for the selection of the most promising candidate vaccines for field trials. Because of the potential harm to participants through the deliberate exposure to a pathogen, the use of smaller groups minimises the cumulative risk. As such, a control group that receives a placebo vaccine followed by controlled exposure to a pathogen should be scientifically well justified. As these types of trials are designed to generate consistent infection rates and thus comparable outcomes across populations and trial sites, data from past studies (historical data) could be used as a valid alternative to placebo groups. In this Personal View, we review this option and highlight the considerations for choosing historical data as a suitable control. For the widespread application of this method, responsibility for the centralisation and sharing of data from controlled human infection trials lies with the scientific community.

Introduction

Between 2001 and 2003, eight women with type 1 diabetes were enrolled in a trial to assess the safety of a single-donor islet transplantation and all achieved insulin independence in the first year after the procedure.[1] Even though this study was not designed as a randomised controlled trial with a placebo group, the outcome was convincing because the treatment effect was clearly evident. The clinical course of type 1 diabetes in the absence of islet transplantation is known with certainty: patients will continue to need exogenous insulin. Although health-care professionals are often wary of evidence from sources other than randomised controlled trials to infer treatment effects, there are multiple examples of convincing results without this randomised design. Examples in the field of infectious diseases include treatment with phototherapy for skin tuberculosis in the 1890s[2–4] and treatment with streptomycin for tuberculous meningitis in the 1940s.[5] Both studies had a large effect size (eg, an 80% reduction in deaths using sulphamidochrysoidin for puerperal sepsis),[6] showing that treatment effectiveness can sometimes be measured reliably even in the absence of a control group.

In controlled human infection trials, susceptibility to infection after vaccination or clearance of the infection after taking a drug are tested in healthy individuals by exposing them to pathogenic microorganisms. In this Personal View, we will focus only on the design of vaccine trials. Most of these studies are done as randomised controlled trials in which participants are randomly assigned to either a vaccine or placebo group. The treatment allocation is commonly blinded to both the investigators and participants, and individuals in both the vaccine and placebo group are challenged with the infectious agent to compare the attack rates (figure 1A). Less frequently, an infectivity control group is used in an open-label design to establish if the pathogen is infectious during the procedure, rather than to measure the infection rate (figure 1B). In the historical control approach, data are based on results from a placebo or non-intervention group from previous controlled human infection studies (figure 1C).

Controlled human infection trials to test the efficacy of new interventions have increased in number from 15 studies in the 1950s to 140 studies from 2011 to 2017.[7] The advantages are that they require small sample sizes (10 to 50 participants) and are designed to detect large effects in an homogeneous population with 100% exposure. By contrast, clinical trials in endemic areas are large (hundreds to thousands of participants) and have heterogeneity in pre-exposure, infection dose, exposure timing, and incidence of exposure, resulting in lower rates of infection and decreased power. Controlled human infection trials can also select for products with the highest potential for efficacy in field studies.[7,8] As a result, they will lower overall costs by reducing the number of products progressing to field studies. Additionally, this type of trial allows multiple products to be evaluated in parallel and reduces unnecessary exposure of participants in field trials to ineffective products. As such, controlled human infection trials are generally accepted as a tool to minimise the risk of late clinical failure.[7]

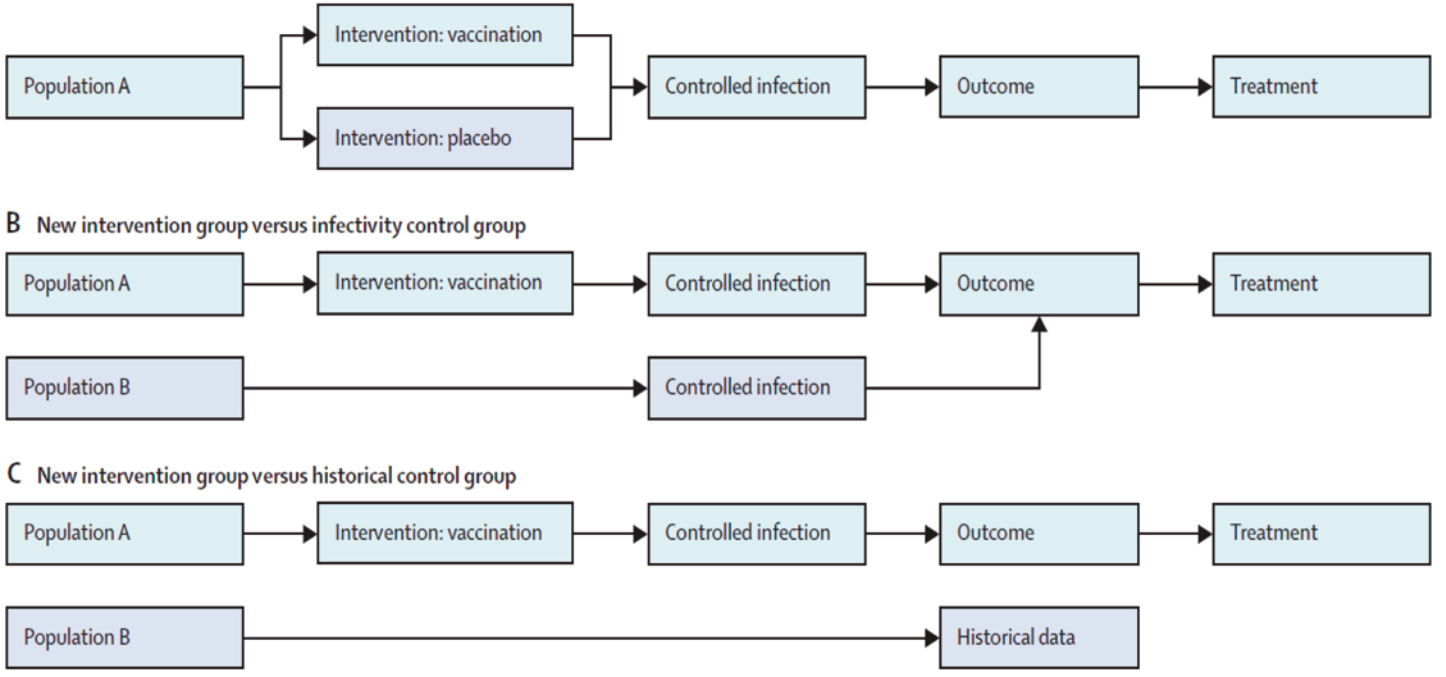

Figure 1. Controlled human infection trial designs in vaccine research. Rows show different trial designs using a placebo control group, infectivity control group, and historical control group. (A) In a randomised controlled trial, participants are given an inactive intervention (placebo) or the vaccine. Both groups are subsequently exposed to an infectious agent during the controlled infection. (B) In an open-label trial, participants in the infectivity control group do not receive the vaccine or placebo, but are exposed to an infectious agent during the controlled infection. (C) Historical data from previous human infection studies can be used as a control group. Columns represent the different trial stages. (1) Population: volunteers are screened and selected to participate in a controlled human infection trial. (2) Intervention: participants are given a vaccine or placebo, or are left untreated. (3) Controlled infection: after a predefined period, participants are deliberately exposed to a pathogen. (4) Outcome: volunteers are monitored to see if they have reached the infection endpoint; at this stage historical data can serve as a control group. (5) Treatment: all volunteers receive pathogen specific treatment.

Similarly to phase 1 trials (dose-finding studies in healthy participants), controlled human infection studies are subject to ethical debate as they seemingly breach the so-called do no harm principle by exposing healthy people to risks (ie, a pathogen), with no direct benefit to the individual.[8] Participants might be subjected to symptomatic infection, a high frequency of blood, urine, or stool sampling, or invasive procedures, such as bronchoscopy. Moreover, periods of quarantine might be required, and, in the case of a transmissible agent, there might be potential for third-party exposure. Although the risks to volunteers are very small, serious events have occurred previously.[9–12] As such, the total sum of risks and the burden on participants and third parties should be carefully weighed against the value of science and the expected benefits to society.[13] This concept is especially important for high risk or high burden studies, where the number of individuals exposed to a pathogen should be minimised. A framework has been proposed to ensure proper ethical justification for the inclusion of healthy volunteers;[8,13,14] risk and burden should be reduced as much as possible and the scientific rationale should be carefully articulated.[15] Importantly, we will argue that placebo control groups might not be necessary to obtain valid study results in some controlled human infection models and their inclusion should therefore be scientifically scrutinised and ethically justified.

Healthy volunteer controls

A healthy volunteer control group can be scientifically justified in four different scenarios: first, to show that the infection procedure was successful; second, if one of the secondary outcome measurements (eg, immunological response) is unknown and these data add indispensable scientific value; third, the infection rate (primary outcome measure) is unknown; and finally, differences between an intervention group testing a new vaccine and a placebo control group are expected to be small.

In the first scenario, infectivity controls provide information on the quality and procedure of infection. For example, clinical malaria trials used between four and six volunteers in the infectivity control group to prove that the exposure procedure resulted in infection.[16–18] In these situations, the size of the control group depends on the expected infection rate, requiring information based on previous studies. The goal is to show that at least one healthy volunteer can be successfully infected. As such, the infectivity control group is usually smaller than the placebo control group, which will also determine the frequency of infections.

In the second scenario, when the expected infection rate (the primary outcome) is known from historical data, the use of a placebo control group can still be essential to determine secondary outcomes. For instance, to compare immune responses between people that are colonised and non-colonised with pneumococcus, along with vaccine efficacy, placebo control samples might be needed.[19] In this case, previously obtained specific samples (eg, freshly processed samples) or data might be absent and the size of the placebo group should then be based on the sample size calculation of the secondary endpoint.

In the third scenario, when the infection rate is unknown, there is no other way to determine the efficacy of a vaccine other than to include a placebo control group. However, in established infection models, the procedures are typically standardised and the expected infection rate for placebo control groups is well known. Because of this consistency, an historical control

group could serve as a benchmark. As an example, over 2650 volunteers worldwide have been experimentally exposed to malaria in controlled human infection trials, with more than 99% of participants developing patent parasitaemia after five bites from mosquitoes infected with *Plasmodium falciparum*.[20–22] As the number of successful infections is consistent between trials, these data can be used for comparison as a historical control group.

In the final scenario, when the expected efficacy of a vaccine, which is often based on animal studies or target-product profiles, is low, the uncertainty of historical data is generally too large and a placebo group is needed to act as a comparator.[23] This control is also required in human infection trials where the magnitude or precision of the treatment effect needs to be established with certainty. However, the group sizes are often too small to reliably determine the treatment effect size, and the purpose of these trials is to instead search for signals of vaccine efficacy.

Historical controls

To examine the suitability and validity of historical data for a specific controlled human infection model it is important that the data are based on comparable populations, the method is comparable, the infection rate from previous studies is reproducible, and that there are suitable data to provide a reliable outcome estimate.[24] Although these models are designed to mimic naturally occurring infections, data from non-controlled epidemiological studies are generally unsuitable to use as historical controls because of differences and uncertainties in infection dose, route of infection, and population. These criteria are equivalent for the selection of historical data for non-controlled human infection trials.[25]

Population

The first variable to consider is the study population. Immunological responses to a specific pathogen might vary according to race, age, health status, and previous exposure. For consistent data and comparisons, the differences in demographics should have no effect on the infection rate between the historical control group and the intervention group. Demographic variation is often already reduced as controlled human infection studies generally have strict inclusion criteria, selecting for only healthy participants. Population differences were clearly shown in a controlled human malaria infection study in which semi-immune African participants showed a reduced infection rate of 64% compared with nonimmune European participants (100%),[26] underlining the need for similar population characteristics when used for comparison.

Methods

When the population is considered similar, variations in method that could influence the infection rate should be examined. For controlled human infection models, differences between species can affect outcome measures. For example, about 65% of volunteers were infected when given *Salmonella enterica* serovar Typhi at a dose of 1×10^4 colony forming units (CFU) but for

Salmonella enterica serovar Paratyphi a dose of 1×10^3 CFU was needed.[27,28] However, with the *Cryptosporidium* model, infection with 1×10^5 *Cryptosporidium muris* oocysts or 1×10^5 *Cryptosporidium meleagridis* oocysts resulted in the same infection rate (100%).[29,30] Differences in strains can also be important; for instance, *Neisseria gonorrhoeae* MS11mkC is more infectious than the FA1090 strain, with the estimated dose needed for 50% infection being $1{\cdot}8 \times 10^3$ CFU (MS11mkC) and $1{\cdot}0 \times 10^5$ CFU (FA1090).[31] Other models use one strain only, such as for *Haemophilus ducreyi*.[32]

Centre clustering (eg, controlled human infection studies for one pathogen that are all done in one centre), between centre differences (eg, related to variations in protocols between study centres), seasonal variation, batch-to-batch variability, and operator effects (eg, variation in adverse event registration by operators) can all result in inconsistent outcomes. For controlled human infections of *Necator americanus* (hookworm), the percentage of infected volunteers was similar (90–100%) after controlled exposure to the same hookworm dose, but substantial differences in egg counts were observed (125 eggs per g *vs* 2400 eggs per g) and depended on the batch, study centre, or operator effects.[33,34] In the influenza A model, infection with 1×10^7 of the A/Kawasaki/8/86 H1N1 strain resulted in variable proportions of viral shedding from 70–100%.[35,36] This variety could be due to seasonal variation, but might also be related to the amount of pre-exposure among volunteers (population). These factors should be taken into account when a study is done in another geographical region or during a different season.

The infection dose is directly related to the primary study outcome. For example, increasing the dose of *S.* Typhi from 1×10^5 to 1×10^9 CFU raised the percentage of infected volunteers from 28% to 95%.[37] For many models, the pathogen dose has been standardised and is generally based on an initial dose-escalation study whereby infection rates, and the risks and burden to the participants, are carefully balanced.

The route of infection also affects the study outcome, as each method requires a specific dose to obtain 100% infection rates. For example, *the Plasmodium falciparum* model uses controlled exposure through mosquito bites, intramuscular or intradermal injections, or by direct venous inoculation.[38–40] Only one route of infection is used in most models, such as with the dengue virus, respiratory syncytial virus, rhinovirus, and influenza virus.[41–44]

The infection rate can be based on microbiological (infection model) or clinical parameters (disease model). Both can be used as historical data as long as the criteria to determine infection rate between studies are similar. A well-described disease model for *Vibrio cholerae* states that participants producing more than 3 L of diarrhoea are considered positive for cholera;[45] however, if this cut-off point varies between studies it will affect the measured infection rate. This difference can be seen in the enterotoxigenic *Escherichia coli* model, where diarrhoea is listed as three watery stools per 24 h or one to two loose stools in 24–48 h.[46] Distinct microbiological outcome measures might also lead to variation, such as with the influenza model that uses titration on canine kidney cells, PCR, or the inoculation of embryonated eggs followed by the haemagglutination inhibition assay.[44] However, if the sensitivity of these tests are similar they can be pooled together to generate historical data.

Outcome consistency

In addition to population and methods, the infection rates should be compared and must be comparable between studies. A model with consistent outcomes was shown in the *S.* Typhi

model with 65%–67% of volunteers developing infection according to predefined criteria.[47–49] This finding contrasts with the enterotoxigenic *E.coli* model which resulted in infection rates of between 50% and 100% when the same infection dose is being used.[46] Consistent outcomes with low variability are suitable data for historical controls.

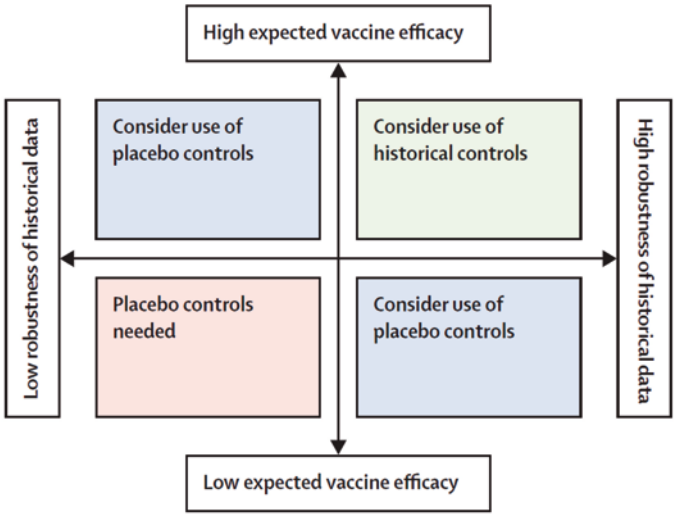

Figure 2. Considerations for choosing the control group in controlled human infection trials.

Suitable data

The variability of the data should be offset by the size of the dataset, and large numbers of participants, such as with the rhinovirus model (5760 individuals),[7] are more likely to generate suitable historical evidence than models that have recently been established (eg, *Schistosoma* spp infections with only 17 participants).[50,51]

As with any sample size calculation, the estimated group size needed depends on the expected efficacy of the vaccine and the variability in infection rates between vaccinated and control groups. Historical controls will probably have a high variability in infection rates because of the heterogeneous nature of the data. Therefore, for studies that are testing a vaccine with a low expected efficacy, the use of a placebo control group (with a lower variability in infection rates) is likely to lead to more robust results. In general, historical data are suitable to use when they are robust and the expected vaccine efficacy is high (figure 2). Realising that historical controls have uncertainties and cannot be used as fixed-effect estimators is important.[52,53] However, there are sophisticated modelling techniques that can analyse different datasets separately and together to better assess the effects of data pooling and corresponding sample size calculations to more accurately estimate infection rates and the variability around these estimates.[53] Although caution should be taken when pooling historical data—for example, if there is variability in the outcome between centres—one could envision selecting historical data from the trial site where the intervention was performed, instead of pooling all available data.

In conclusion, the use of historical data needs to be carefully considered and must take into account the population, method, outcome, and the amount of available data. The suitability to use historical data depends on the controlled human infection model and the design of the study. All of these factors should be evaluated before the start of a new controlled human infection trial.

Future directions

Given the public and research community's perception regarding the deliberate infection of healthy volunteers,[8,13] participants should be included with the utmost care and through solid scientific justification. The design of controlled human infection studies should be scrutinised so that the output is scientifically impactful, yet minimises the cumulative risk with proper justification of placebo controls.

Controlled human infection studies are designed to generate uniform outcomes (eg, infection rates). When these data are sufficiently consistent, they can be used to generate an historical control group as an alternative to a placebo control, reducing the number of participants exposed to a pathogen. To facilitate data pooling, study designs should be harmonised between centres. Agreement on the route and dose of infection, the use of similar inclusion and exclusion criteria to reduce the variability of study populations, harmonisation of endpoints by using similar microbiological techniques or clinical criteria, and optimisation of pathogen production according to good manufacturing practice guidelines are likely to optimise controlled human infection models, generating better quality historical data in the future. Such efforts have been undertaken for the malaria model, resulting in guidelines for the "Standardization and conduct of *P. falciparum* sporozoite controlled human malaria infection trials".[54] This could also be done for other controlled human infection models by establishing consensus groups that will outline methods aimed to harmonise between centres.

Moreover, several open access data repositories are available, such as ImmPort (https://immport.niaid.nih.gov/home) from the National Institutes of Health and Zenodo (https://zenodo.org/) from OpenAIRE, where controlled human infection trial data can be deposited.[55,56] These repositories improve access to trial results, making it easier to pool historical data, analyse variability, and ultimately work with larger and more exhaustive datasets. In addition, a newly established digital platform funded by the Wellcome Trust (https://tghn.org/) aims to promote sharing of protocols and data, complementing the existing network from the Bill and Melinda Gates Foundation (see https://chimstudies.org). As these initiatives show, funders can actively promote and support open data sharing, creating opportunities for data pooling, data reuse, and the identification of consistent endpoints.

Whether the use of historical controls will be considered acceptable to the regulators for licensure depends on the balance between the risk of controls, whether alternative investment is required, and the position of the trial in the development pathway. The considerations presented here are meant to ignite and guide the discussion around the use of historical controls in controlled human infection studies.

Are placebo controls necessary in controlled human infection trials for vaccines? The answer depends on the pathogen-specific model, the expected vaccine efficacy, and the quality of the

available historical data. In our opinion, each researcher has the moral obligation to consider alternatives to the randomised controlled study design. Alternative approaches, such as the use of an historical control group, should be explored and prespecified. If the use of a placebo group is deemed absolutely necessary, this decision should be justified in the protocol, trial register, and in the research paper.

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CHAPTER 9

Summarising discussion

The importance of this thesis

In this thesis we describe the use of controlled human infection models for various primary outcome measures. We show the importance of the established controlled human malaria infection (CHMI) model to test the kinetics of new strains, NF135.C10 and NF166.C8. In addition, the CHMI model was used to evaluate the efficacy of the first vaccine based on genetically attenuated parasites (GAP) administered by direct venous inoculation, PfSPZ-GA1 vaccine. We also describe how we developed a new model, the controlled human *Schistosoma* infection (CoHSI) model. We describe a dose-finding study, to determine the lowest pathogen dose resulting in a high infection rate while still being safe. The results of this study show that this model is a useful tool to test new vaccines, drugs and diagnostic tests in the future. Finally, we describe how some well-established controlled human infection models can be designed to result in a lower cumulative risk of the whole study. This study design involves the use of historical controls instead of placebo controls.

Malaria

Genetic diversity

1. Variation between strains

Whole sporozoite vaccines are immunisation strategies based on the administration of attenuated whole sporozoites to prevent blood stage parasitaemia. Currently these whole sporozoite vaccines are based on the *Plasmodium falciparum* strain NF54 or its daughter strain 3D7.[1] Immunisations are followed by controlled human malaria infection (CHMI) to determine the protective efficacy of the whole sporozoite vaccine. This CHMI is either homologous, same strain, or heterologous, another strain. A protective efficacy up to 100% can be reached by homologous CHMI after whole sporozoite immunisations. Where the protective efficacy depends on the immunisation dose, interval and route of administration.[2-5]

These high efficacy rates in homologous CHMIs do not reflect the efficacy in field studies. As there is a large genetic diversity of *P. falciparum* between geographical regions,[6] it is likely that data of heterologous CHMIs are more representative of what will happen in field trials. Unfortunately, so far the results of heterologous CHMIs are poor compared to the high protective efficacy rates of homologous CHMIs. After whole sporozoite immunisations 11-80% efficacy was shown after heterologous CHMI.[3,7-10] Hence, it is important to improve the vaccine efficacy in heterologous CHMIs and to investigate whether study results from heterologous CHMIs are comparable to the field.

To be able to perform heterologous CHMIs new *P. falciparum* strains are being investigated. The strain NF135.C10, originating from Cambodia, was first introduced a few years ago. This strain shows a shorter prepatent period after infection compared to NF54, but comparable clinical signs and symptoms.[11] To expand the number of strains for vaccine research we introduced

the new *P. falciparum* strain NF166.C8, originating from Guinea (West Africa), and performed additional tests on NF135.C10 as described in **Chapter 2**. We showed that infection with one of these two strains, compared to NF54 infection, resulted in shorter pre-patent periods and higher first peak of parasitaemia. The latter can be used to determine the number of parasites released from the liver. This data was supported by our *in vitro* data showing more infected hepatocytes and a higher number of nuclei per schizont with the strains NF135.C10 and NF166. C8 compared to NF54.

We performed additional research, which was described in **Chapter 3**, to determine whether these differences in kinetics of NF135.C10 and NF166.C8 should change the CHMI. We hypothesised that a lower number of bites from NF135.C10 or NF166.C8-infected mosquitoes should be sufficient to reach comparable infection rates and parasitaemia compared to NF54. The standard number of mosquito bites in NF54-CHMIs are five, which results in >99% infection rates,[12] while lower number of infected mosquito bites result in lower infection rates.[13,14] We found comparable data with NF135.C10 and NF166.C8, as five infected mosquito bites resulted in an 100% infection rate, while one or two infected mosquito bites resulted in an infection rate of 75%. In conclusion, as the same number of infected mosquito bites were needed to reach comparable infection rates, the results of our research did not support our hypothesis that due to kinetic differences a lower number of NF135.C10 or NF166.C6-infected mosquito bites will result in 100% infection. As infection with less than 5 mosquito bites with either of the three strains results in an infection rate below 100%, there appears to be a strain-independent threshold that should be overcome in order to develop blood stage malaria. The cause of this threshold is unclear and may occur at the stage of probing by the mosquito, the number of sporozoites entering the blood stream, or the number of sporozoites that invade the hepatocytes. The differences in parasite multiplication after hepatocyte invasion (Chapter 3) result in higher first peak of parasitaemia in these new strains. For future research this means that infection with five NF135.C10 or NF166.C6-infected mosquito bites results in higher levels of parasitaemia. In addition, this might lead to an increased immunogenicity of these strains during liver stage as more parasites are present.

Both of our studies described in **Chapter 2** and **Chapter 3** showed differences in the kinetics of various strains. It is possible that these variations will lead to a suboptimal vaccine efficacy when tested in heterologous CHMI.

2. Future perspective

Although the whole sporozoite vaccines show high efficacy rates when used in homologous CHMIs,[2-5] the efficacy of these vaccines is low with heterologous CHMIs. Several study designs might lead to an increased protection against heterologous strains, making the vaccine more suitable to use in the field: 1. Increase immunisation dose, 2. Other immunisation strain, or 3. Combine strains for immunisation.

1. Increase immunisation dose

Increasing the immunisation dose of a vaccine could result in better protection rates. Several homologous and heterologous studies were performed with PfSPZ vaccine, which is based on radiation attenuated sporozoites (RAS). Overall, these studies show a higher immunisation dose results in higher protection rates.[2,3,9] However, when PfSPZ Vaccine was administered with a

dose higher than one million sporozoites, this seems to result in a reduced efficacy compared to a dose lower than one million sporozoites.[15]

2. Other immunisation strain

As was shown in **Chapter 2** some strains result in a higher first peak of parasitaemia than others. This higher first peak of parasitaemia is present in strains NF135.C10 and NF166.C8, and it reflects a higher number of parasites released from the liver. It is possible that this parasite load in the liver results in a better immune response of the host during liver stage. This could ultimately lead to higher antigenic loads. To test this hypothesis, a whole sporozoite vaccine should be developed with either NF135.C10 or NF166.C8. Based on **Chapter 2** and **Chapter 3** this strain could be NF166.C8. If this hypothesis holds, this would result in a higher protective efficacy after heterologous CHMI with vaccines based on NF166.C8, compared to heterologous CHMIs with vaccines based on NF54. This hypothesis is being investigated with NF135.C10, where NF135.C10 CPS immunisations are followed by homologous and heterologous (NF166.C8) CHMI (clinicaltrials.gov NCT03813108).

3. Combine strains for immunisation

A recent study shows the genetic differences between the *P. falciparum* strains that are most commonly used in whole sporozoite vaccine studies; NF54, 3D7, NF135.C10, NF166.C8 and 7G8.[16] These include immunologically important pre-erythrocytic antigens. These differences could be an explanation why vaccines tested by heterologous CHMIs show less protective efficacy as compared to homologous CHMIs. As an immune response against a specific antigen in NF54 might not fully protect against another strain which lacks the exact same antigen.

One way to optimise whole sporozoite vaccines could be to generate a vaccine with multiple *P. falciparum* strains. To design a multiple strain vaccine it is important to include strains that are genetically diverse. The genetical differences between geographical regions are larger than the genetical differences found within geographical regions. Based on the genetic differences there are four geographical regions, which are largely covered by the most commonly used strains; NF54, NF7G8, NF135, and NF166.[16] It remains to be seen whether this combination of strains is sufficient to generate a potent whole sporozoite vaccine. However, since all genetic regions are covered, it could have great potential.

Two types of multiple strain vaccines can be distinguished. The first option is one vaccine with multiple strains that is administered several times, while the second is a combination of several vaccines, each with one strain, that are applied sequentially. The first vaccination strategy is based on the idea that an immune response against multiple epitopes of all strains will be generated, which are boosted each immunisation. The disadvantage is that it could lead to the dominance of an immune response against some strains over other strains as was seen with the Dengue vaccine.[17,18] The second vaccination strategy is based on the idea that an immune response against conserved and strain specific epitopes is being generated. Where each new vaccine induces an immune response against the new strain combined with a boost against conserved epitopes of the parasite. The main disadvantage is that this boost might not be sufficient when there is only a mild immune response against conserved epitopes.

Another option would be to generate four separate vaccines for various regions. Based on their genetic differences, these regions could be Africa, South America, Asia and Papua-New Guinea.[16] All these regions, except Papua-New Guinea are largely covered by the most commonly used strains; NF54, NF7G8, NF135, and NF166.

3. Study design

Most of the CHMI studies are performed using a randomised placebo-controlled study design. However, as we discussed in **Chapter 8** this is not always the study design with lowest cumulative risk and burden while reaching reliable outcomes. The infection rate after a CHMI with five infected mosquito bites is well known and is >99%.[1,12] As such, the use of a placebo control group to determine the primary outcome "efficacy" is not necessary, and could even be considered unethical. Before studies with new malaria strains are performed it should be investigated what number of mosquitoes are needed in CHMI to reach 100% infection rates, as we did in **Chapter 3**. When this number is repeatedly similar, the use of a placebo group to determine the vaccine efficacy is no longer needed. In those situations infectivity controls can be used instead to ensure an adequate infection procedure and guarantee lower cumulative risk and burden of the study. In **Chapter 2** we used placebo controls, since historical data on all strains were not yet sufficient. In **Chapter 4** we used placebo controls to be able to study the secondary outcome, the immunological response to a CHMI with or without PfSPZ-GA1 Vaccine immunisations.

Whole sporozoite vaccines

1. Genetically attenuated vaccines

There are several types of whole sporozoite immunisation strategies which have been developed. The first is immunisation with radiation attenuated sporozoites (RAS). The US- based biotech Sanaria® produced aseptic, purified, cryopreserved *P. falciparum* RAS, PfSPZ Vaccine, for direct venous inoculation (DVI).[19] The second is immunisation by the administration of sporozoites under chemoprophylaxis (CPS; chemoprophylactic sporozoites).[5] Sanaria® developed 'PfSPZ-CVAC' (aseptic purified cryopreserved PfSPZ sporozoites under chemoprophylaxis) as immunisation strategy.[20] And the third is altering the parasite genetically to generate a specific phenotype, the genetically attenuated parasites (GAP).

When we compare these three immunisation strategies, most studies are being performed with RAS. RAS are able to enter hepatocytes where they will only partially develop.[21,22] The degree of development depends on the irradiation dose, with the standard dose of 15,000RAD.[23] Remarkably, sporozoites exposed to a higher irradiation dose do not enter hepatocytes and immunisations with these sporozoites does not result in protection. This implies that the invasion of hepatocytes is crucial to generate an immune response.[8,22,24] It is thought that a longer duration of parasite exposure during liver stage, e.g. late arrester, will result in a better immune response of the host.[25] Unfortunately late arrest during liver stage cannot be reached with irradiated sporozoites, as irradiation with less than 15,000RAD results in incomplete attenuation and as consequence blood stage malaria develops.[22]

In contrast to RAS, CPS enter the liver where they develop until they are released in the blood stream. As chloroquine acts on the asexual blood stage parasite only, the parasites will develop normally throughout the entire liver stage, but they do not develop within the blood. As a consequence volunteers do not develop clinical disease.[26] CPS immunisation results in a longer exposure time to parasites in liver stage compared to RAS, which leads to a better immune response.[5] However, this immunisation strategy is very difficult to introduce to millions of people in the field due to the main safety risk, the chance of developing malaria under inadequate

chloroquine levels. RAS, especially PfSPZ Vaccine which can be administrated by DVI instead of mosquito bites, is easier to introduce in the field and without the risk of developing blood stage malaria.[22]

Comparable to CPS, a GAP would ideally replicate throughout most of the liver stage. The advantage over CPS would be that it does not carry the risk of developing malarial disease. GAPs rely on the deletion of genes that are crucial for the parasite to continue development within liver stage. Depending on the genes that are deleted, the parasite will arrest early or late during liver stage development. At first, several GAPs were tested in animal studies, showing the potential of GAP vaccines.[27,28] Recently the first *P. falciparum* GAPs were developed for human testing, all based on the strain NF54. The first GAP tested in humans was GAP2KO with deletion of P52 (also known as P36p) and P36.[29] Both P52 and P36 are 6-CYS proteins. This family of proteins has various roles in the establishment and maintenance of the parasitophorous vacuole during liver stage development.[30] The deletion of either P52 or P36 in *Plasmodium berghei* did not result in complete attenuation.[31,32] Deletion of both P52 and P36 in *P. berghei* did not result in compete attenuation *in vivo*,[33] while deletion of both P52 and P36 in *P. falciparum* resulted in full arrest during liver stage development in humanised mice.[34] In line with these pre-clinical data, this GAP was not fully attenuated and blood stage parasitaemia did develop in a trial with human volunteers. It is possible that this GAP did not result in full attenuation as deletion of either one of this paralogue genes did not result in complete attenuation either.[25] Hereafter GAPs were developed that included the deletion of genes that were considered key developmental factors, combined with another gene.[25] This resulted in the development of the first GAP tested in humans with a triple deletion, the *P. falciparum* GAP3KO with deletion of *P53*, *P36* and *slarp* (sporozoite and liver stage asparagine-rich protein). In addition to the deletions of *P53* and *P26* in GAP2KO, *slarp* was deleted. *Slarp* is a differentiation factor which down-regulates liver stage specific proteins, such as *UIS3* and *UIS4*.[35,36] *In vivo* experiments showed that deletion of *slarp* resulted in complete arrest early in liver stage.[36] Similar results were found with *P. falciparum* GAP3KO in human volunteers as none developed blood stage malaria after exposure by mosquito bites.[37] A study on the protective efficacy of *P. falciparum* GAP3KO was performed recently (clinicaltrials.gov NCT03168854).

In addition to these studies we performed the first study using direct venous inoculation (DVI) to administer a GAP vaccine, PfSPZ-GA1 Vaccine of which the results are described in **Chapter 4**. PfSPZ-GA1 Vaccine has a deletion of the *P. falciparum* genes *b9* and *slarp*.[38] *B9* is also member of the 6-CYS protein family. Pre-clinical data showed that deletion of both genes resulted in complete arrest *in vitro* and *in vivo* in humanised mice. Exposure to various dosages of PfSPZ-GA1 Vaccine in human volunteers did not result in blood stage parasitaemia. Unfortunately the efficacy of the PfSPZ-GA1 Vaccine, was lower than expected, with a sterile protection in 12% of volunteers and a delayed time to patency in 68% of the volunteers. In addition, immunisation with $4.5x10^5$ sporozoites of PfSPZ Vaccine, the active control group, showed no protective efficacy. This was remarkably and unexpectedly lower than the 86.7% sterile protection shown with the same dose in a prior study.[3] The specific cause of this reduced efficacy of PfSPZ Vaccine remains unclear, but lies either in the difference in immunogenicity of the PfSPZ Vaccine or the difference in stringency of the CHMI compared to other studies. In case the stringency of CHMI caused the reduced efficacy of PfSPZ Vaccine, this could also have affected the efficacy of PfSPZ-GA1 Vaccine. However, it seems to be unlikely that there were differences in stringency between CHMIs. Both studies immunised with mosquito bites three weeks after the last immunisation. Our study used NF54 as CHMI, while the other study used 3D7, its daughter strain, which could have

small genetic differences. As the NF54 CHMI is more homologous to this NF54-based vaccine it is unlikely that this explains the differences in vaccine efficacy, as homologous CHMIs show better protection than heterologous CHMIs.[2-5,7-10] As such, it is more likely that there was a difference in immunogenicity of the PfSPZ Vaccine between both studies.

2. Future perspective

All three GAP vaccines that were tested in humans, GAP2KO, GAP3KO and PfSPZ-GA1 vaccine, are early arresters.[25] This means that they arrest early during liver stage development, only forming a single-cell liver stage trophozoite. So far, no successful late arrester GAPs have been developed for *P. falciparum*. In rodent studies not only early arrester GAPs, but also late arrester GAPs were developed.[25] Several late arresters remain without breakthrough infections, e.g. double deletion of *PlasMei2* and *LISP2*.[39-41] These rodent studies show that successful development of fully attenuated late arrester GAPs is possible. Unfortunately it has been shown that the deleted genes used in the full late liver stage arresting rodent GAPs do not guarantee similar results in *P. falciparum* GAPs with orthologue gene(s) deleted. For example, deletion of *FabI* from the type two fatty acid biosynthesis pathway (FAS II) results is complete arrest during liver stage in a rodent GAP, while FabI deletion in *P. falciparum* results in severely attenuated development of salivary gland sporozoites.[42] For future studies it remains interesting to further explore the possibilities for the development of *P. falciparum* late arrester GAPs. The advantage of a late arrester GAP is thought to be a stronger immune response due to longer time of exposure with a higher number of parasites. This is what can be seen in vaccines based on RAS with limited liver stage development, compared to CPS with full liver stage development. CPS immunisations result in a better immune response.[5,24]

3. Clinical development pathway

The potency of a vaccine is generally tested in animal models before testing these in humans. There are two main options available to test GAP vaccines in an animal model. One option is to develop and test a rodent *Plasmodium spp.* with deletion of orthologue genes. However, as discussed before, it is possible that deletion of genes in rodent *Plasmodium spp* lead to a different phenotype than deletion of orthologue genes in *P. falciparum*.[40-42] As a consequence it is preferred to test the GAP in humanised mice.[34] After testing vaccines in animal models, promising vaccine candidates will enter the clinical development pathway (figure).

The first step in the clinical development pathway is focussed on testing the safety of a vaccine, a phase I trial. Hereafter, safe vaccines can be put to test in a CHMI trial. The use of CHMI trials is common in RAS and CPS, but can also be used for GAPs. In these trials healthy volunteers are immunised several times followed by a CHMI. The CHMI trial can be divided into two separate trials. At first, as a proof of principle, the GAP can be administered by mosquito bites. In such studies the phenotype and immunogenicity will be investigated. The disadvantage is that this can only be used as an immunisation strategy and not as a vaccine. The advantage is that it is much cheaper than the next step, the development of a GAP-vaccine that can be administered by DVI. It is possible to skip the proof of principle step and directly develop the vaccine for administration by DVI. For both of these CHMI trials the primary outcome is the vaccine efficacy. As groups are small the estimate of the vaccine efficacy will not be very reliable, but it gives a hint on the potency of the vaccine. This potency could be divided in not potent (<25% of volunteers

protected against malaria) and mild, moderate or very potent (≥25-50%, ≥50-75%, ≥75% of volunteers protected against malaria, respectively). These percentages are suggested based on the malaria vaccine technology roadmap. In the initial version of the roadmap the goal was a malaria vaccine with a protective efficacy of ≥50% by 2015, while the new goal is a malaria vaccine with a protective efficacy of ≥75% by 2030.[43] As groups in CHMI trials are small and only give an estimation of the efficacy it is suggested to proceed to field studies with vaccines that are moderate or very potent (≥50% efficacy in CHMI) immediately, while mild potent vaccines (≥25-50% efficacy in CHMI) should be further improved or tested, if possible, before testing in field studies.

If the vaccine passes the proof-of-concept CHMI trials, the first step is another CMHI trial in an endemic area. Results of this CHMI trial can be different from the clinical CHMI studies, as the population in the field CHMI trial will likely have been exposed to malaria before. This extra step gives another hint on the potency of the vaccine.

Vaccines which pass the field study CHMI may move to phase II studies in endemic areas. Based on the new vaccine goals, reaching an efficacy of ≥75%, only vaccines that are moderate or very potent in phase II studies should be introduced to a large phase III field study in an endemic area. Vaccines with an efficacy of ≥75% in a phase III study could be registered, while vaccines that are mild or moderate potent should first be further improved. This is in contrast to the study results previously found with Mosquirix (RTS,S), the only malaria vaccine currently tested in large phase IV field studies. Mosquirix showed a vaccine efficacy of 56% before 2015,[44] these results were in line with the previous goal; ≥50% efficacy by 2015. New vaccines should, however, meet the new, higher, efficacy standards.

Although this clinical development pathway appears to be clear, there are some marginal issues to be addressed. Administration of whole sporozoite vaccines by DVI on a large scale is difficult, as the transport and preparation of the vaccine requires strict conditions. At first the transportation and storage of the vaccine should be in nitrogen. Hereafter the vaccine should be prepared and administered within 30 minutes after start of the preparation. Lastly, the vaccine should be injected directly into the veins, which requires additional technical skills of the people administering vaccines. So preferably, for practical use, there should be some adjustments, such as being able to keep the vaccine in high temperature, room temperature or in the refrigerator, extend the time the vaccine is stable and being able to administer the vaccine intramuscular.

In **Chapter 8** another important issue was addressed, namely the use of placebo controls in controlled human infection studies. In CHMI studies the infection rate of the placebo group after CHMI is >99% based on the data of studies performed the last decades.[1,12] For future studies there is no need to include a placebo group to determine the efficacy of a vaccine since a small group with infectivity controls should be sufficient. However, there may be a need for a placebo control group to determine secondary outcomes, such as the variation in immune response.

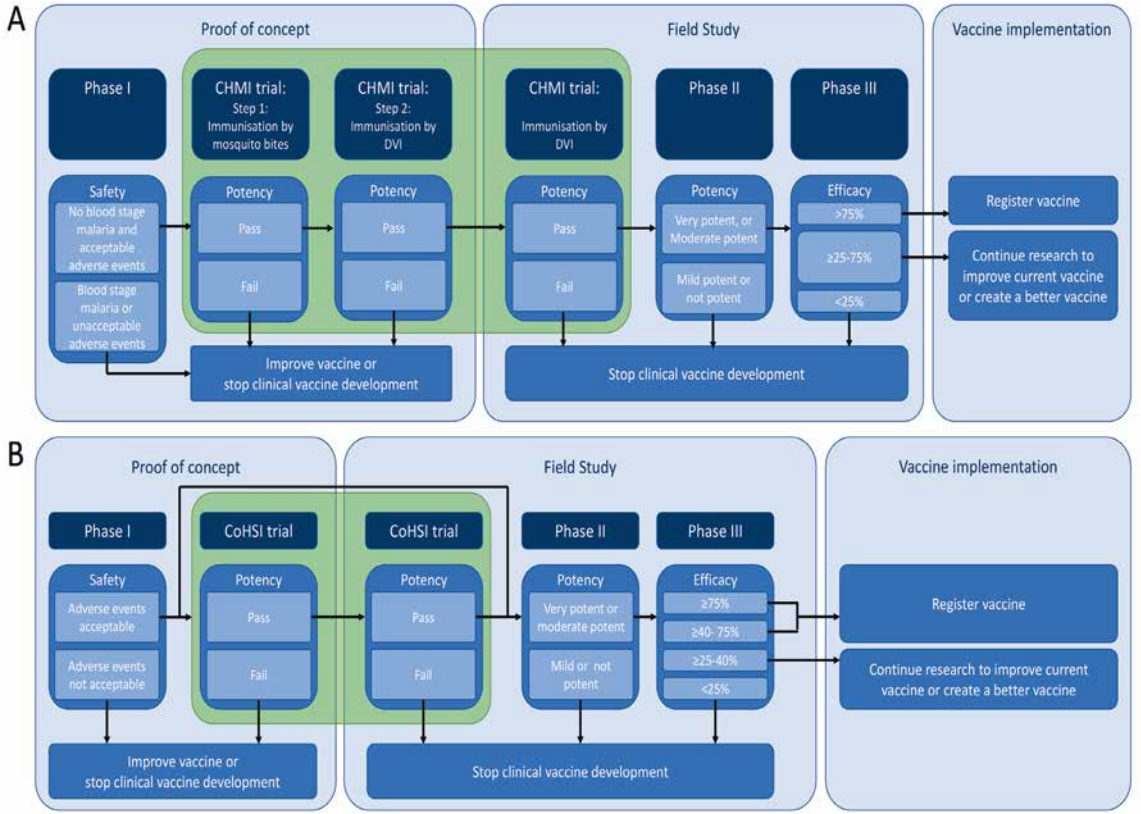

Figure. Suggested clinical vaccine development pathways.
A. Clinical development pathway for a malaria vaccine. **B.** Clinical development pathway for a schistosomiasis vaccine. In green the trials performed with the use of a controlled human infection model.

Schistosomiasis

A new controlled human infection model

1. The importance of a controlled human schistosome infection model

Schistosomiasis can be treated with the drug Praziquantel, which has a cure rate of only 42-91%.[45,46] This cure rate could be higher, as it is impossible to distinguish between uncured infections and reinfections in an endemic setting. Also, praziquantel has no effect on the immature worms, so it will not cure people that were recently (re-)infected. In addition, it is likely that in some patients praziquantel treatment leads to a reduced worm- and egg burden and not to complete eradication.

If schistosomiasis is left untreated this can lead to severe pathology. Since it takes years before this pathology develops it is important to detect and treat the disease at an early stage. This is difficult as people often have no symptoms until severe pathology develops. In areas with known high endemicity, preventive chemotherapy is given. Depending on the schistosomiasis prevalence this mass drug administration (MDA) can be administered up to once a year.[47] The prevalence of schistosomiasis is reduced with 37% in school children one year after administration, and the intensity of infection with 41%. Based on modelling a substantial reduction of prevalence can be reached with MDA when administered yearly with at least 70% community participation.[48] However, this data might be too optimistic as there are indications that repetitive MDA reduces the efficacy of praziquantel.[49] If MDA would be discontinued, schistosomiasis will likely return to pre-control levels within 30 years.[48] In conclusion, since MDA does not prevent from reinfection and results in modest prevalence reduction, its effect is limited and temporary.

A more sustainable method to achieve schistosomiasis control would be to prevent infection. One way would be to prevent exposure to cercariae, which are present in infected water. Since the infected water is often used by the endemic population to wash laundry and wash themselves, to cook or clean and to collect fish, it is often impossible to prevent exposure. However, this preventive advice can be given at travellers to endemic areas. Another way to prevent infection is through vaccination. Unfortunately no vaccine against schistosomiasis is registered yet, but there are several vaccines against *Schistosoma mansoni* in pre-clinical testing; rSm-TSP-2/Alhydrogel, rSm14/GLA-SE, and rSm-p80/GLA-SE.[50-53] Testing these vaccines in field trials is expensive and takes a long time. To reduce the time to test a vaccine, reduce the costs and prevent exposure of a vulnerable population to a vaccine that might not work,[54] we developed a controlled human schistosome infection (CoHSI) model as described in **Chapter 5**.

2. How to design and optimize a new model

We think there are several steps to be taken before a new controlled human infection (CHI) model can be developed and tested: 1. Is there a need to design a model for this pathogen?; 2. Is the expected burden/risk for volunteers after exposure to the pathogen acceptable?; 3. Can we produce this pathogen safe and according to all regulations so it can be used to infect volunteers?; and 4. What pathogen dose should be used?

1. Is there a need to design a model for this pathogen?
CHI models should only be used if there is additional value to be expected in addition to the current research.[55] The use of a CHI model to test vaccines or drugs would reduce the time and costs to test a vaccine, compared to field studies.[54,56,57] Also the number of volunteers needed is less, as only non pre-exposed volunteers, without any immunity to or presence of the pathogen, are used. Also the conditions are more controlled as the time of infection and the pathogen dose are similar. This is in contrast to field studies where larger groups are needed as not all volunteers will be exposed to the pathogen in the months after vaccination or, in drug research, the time of exposure and pre-exposed dose is not standardised.[54] In addition, it could prevent exposing a vulnerable population to a vaccine or drug that might not work. The use of CHI studies reduce late clinical failure as only potent candidates will be tested in large field studies.[54,56]

We concluded that the use of a schistosomiasis CHI model would be beneficial based on the advantages of CHI models just described, combined with insufficient disease control with MDA, and inadequate preventive options. A schistosomiasis CHI model could be of additional value, not only to accelerate vaccine research, but also to speed up drug research, to gain a better understanding of the host-pathogen interaction, and to investigate the host immune-response to the pathogen.[58]

2. Is the expected burden/risk for volunteers after exposure to the pathogen acceptable?
Before establishing a model it should be clear that no harm and low risks are expected in volunteers. For cercarial exposure and schistosomiasis there are some adverse events that are likely to develop in some volunteers. Directly after cercarial invasion in the skin a cercarial dermatitis can develop which can last for days.[59] In the acute phase of schistosomiasis one could expect symptoms related to an acute schistosomiasis syndrome (Katayama fever), which are likely to be acceptable, temporary and do not result in permanent harm or death [60,61] In addition, angio-oedema could develop.[62-65] After years of chronical infection various symptoms can develop which are related to the formation of granulomas around *Schistosoma* eggs that get trapped in tissue. These symptoms depend on the location and extent of the granulomas, e.g. liver cirrhosis, portal hypertension, or malignant transformation of tissue.[59,60]

We considered all of the above risks to be acceptable, except those related to long term infection. To eliminate these long-term risks, a model was designed which we described in **Chapter 5**, with exposure to single-sex male cercariae only, as these do not deposit eggs.

3. Can we produce this pathogen safe and according to all regulations so it can be used to infect volunteers.
When developing a new CHI model it is important to adhere to all regulations and determine the safest way to use the pathogen. In **Chapter 5** we described how we were able to develop the pathogen according to all European Union (EU) regulations and how we were able to select male cercariae only, to ensure safest exposure to the pathogen. There are, however, no clear guidelines on the development and manufacture of human CHI agents. The International Alliance for Biological Standardization (IABS) recently recommended the development of these guidelines, ideally coordinated by WHO officials.[66]

4. What infection dose should be used
Once a CHI model is designed it is important to establish a pathogen dose. A dose-escalation study can be used to determine the optimal dose, which is the lowest pathogen dose resulting

in a high infection rate, with acceptable side effects for volunteers. For example, in malaria trials the dose was standardised to five infected mosquito bites resulting in an infection rate of >99% with limited side effects.[12]

We started with a low dose of 10 cercariae and planned to increase to a higher dose of 30, 60 and eventually 100 cercariae until 10 volunteers were infected with the same dose. In **Chapter 7** we describe that exposure to 10 cercariae did not lead to an 100% infection rate and symptoms of volunteers were mild. Although 30 cercariae did lead to an 100% infection rate, the adverse events were too severe to continue with this dose. These adverse events, described in **Chapter 6**, mainly existed of long-term fever and headache. Two out of three volunteers exposed to 30 cercariae experienced multiple severe adverse events. Hereafter the dose was reduced to 20 cercariae, resulting in acceptable side effects with a high infection rate of 82%. We concluded that future vaccine and drug research using this single-sex male-only CoHSI model could be performed safely with a dose of 20 cercariae per volunteer.

3. Clinical development pathway and future perspective.

With the establishment of the CoHSI model a new tool to test schistosomiasis vaccines is available. This model gives the option to test vaccines as a proof of concept without having to proceed directly to the field. This is a great advantage as limited resources are available for this neglected tropical disease.[67,68] Testing vaccines with the CoHSI model is expected to be cheaper than directly performing large field trials. This is related to the controlled conditions, which result in a shorter period of time needed for a trial, and a reduced number of participants.[56] CoHSI trials last only several months, as several immunisations with a vaccine or placebo are followed by CoHSI a few weeks later. Hereafter there are weekly visits until 12 weeks after cercarial exposure, to determine the development of schistosomiasis and the intensity of infection, based on serum CAA levels, as we described in **Chapter 7**. Only a small group of *Schistosoma*-naïve volunteers, selected on stringent criteria, is included in CoHSI trials in non-endemic areas. As a consequence the vaccine efficacy, which is determined as the reduced intensity of infection, cannot be directly translated to the efficacy in the field, but refers to the potency of the vaccine. It is suggested to group vaccines as not potent (efficacy <25%), mild potent (25-40%), moderate potent (40-75%), or very potent (≥75%). This suggested classification is based on schistosomiasis vaccine targets put forward by the WHO and experts in schistosomiasis, with targets of ≥40% or ≥75% vaccine efficacy respectively.[69,70]

With the advantages described, the CoHSI model could be implemented in a clinical development pathway (figure). This proposed clinical development pathway for a schistosomiasis vaccine is similar to the clinical development pathway for a malaria vaccine that was described before. However, the CHMI model is well-established and its position in the clinical development pathway is clear, while the importance of and the exact position of the CoHSI model in the clinical development pathway still needs to be determined.

The proposed clinical development pathway of a schistosomiasis vaccine also starts with a clinical phase I trial to determine the safety of a vaccine. If safe, a CoHSI trial in a clinical setting in *Schistosoma*-naïve volunteers could follow. When the vaccine passes, a CoHSI trial in an endemic area will follow. The outcome of this study is the vaccine efficacy in the target population. Depending on the outcome, a large field study could follow. It is likely that the vaccine efficacy will differ because variations in immune responses are to be expected in non-pre-exposed volunteers from a non-endemic area and pre-exposed volunteers from an endemic

area, as we discussed in **Chapter 7**. For this purpose, efforts are made to implement the CoHSI model in the schistosomiasis endemic country Uganda.[71]

The next step of the clinical development pathway depends on the outcome of the CoHSI studies. Moderate and very potent vaccines may be put forward to phase II field trials. Mild potent vaccines could be further developed to improve efficacy, e.g. by combining vaccines and/or changing the adjuvants. If the vaccine passes towards phase II studies, the results of this phase II study determines whether a phase III field study may follow. It is suggested to stop further research on vaccines that are not potent in phase III, continue research on vaccines that are mild potent and register vaccines that are moderate or very potent (expected efficacy ≥40%) in phase III studies. When a vaccine is implemented, additional research may continue to improve the efficacy.

However, the CoHSI model also faces limitations. So far, the vaccine based on Sm-p80 showed promising efficacy data in baboons and a phase I study will follow soon.[52,72] For both vaccines based on Sm14 and Sm-TSP-2, phase I studies have been performed in non-endemic areas and showed good safety profiles and immune responses.[51,73] A phase Ib dose-escalation study on rSm-TSP-2/Alydrogel +/- AP 10-701 started recently (clinicaltrials.gov NCT03910972). And a phase II trial has been performed with the rSm14/GLA-SE vaccine in 30 adult males in a high endemic area in Senegal (clinicaltrials.gov NCT03041766). As this study confirmed the safety and long-lasting immunogenicity of the vaccine, a phase IIb study in Senegalese school children was performed (clinicaltrials.gov NCT03799510).[74,75] For the vaccine rSm14/GLA-SE there is no additional value to perform a CoHSI trial as the vaccine is already in phase II testing. It would be interesting to perform CoHSI trials on rSm-p80/GLA-SE and on rSm-TSP-2/Alydrogel before introducing these in large field studies.

Before using the CoHSI models for these vaccines, it is good to realise that there are some limitations to the CoHSI model in its current form. The major limitation is that the current CoHSI model only uses male cercariae. As a consequence the effect of vaccines on worm pairs, female worms and eggs cannot be evaluated. This is important when determining the efficacy of a vaccine based on an antigen that is expressed more on female than male worm, such as the vaccine Sm-p80. Research in baboons with Sm-p80/GLA-SE showed a 82-93% reduction of female worms, tissue egg load and hatching of eggs into miracidia, while only a 43% reduction of male worms was seen.[52] Determining the potency of this specific vaccine in a CoHSI trial would be more interesting using a single-sex female CoHSI model instead of the single-sex male CoHSI model we described in **Chapter 7**. Before using a single-sex female model a new dose-finding study should be performed, where the number of cercariae needed to safely establish an infection, determined by serum CAA levels, is tested. It is possible that female worms produce other amounts of CAA than males, as female worms do not fully mature without the presence of males, and female adult worms remain smaller than males.[76] Also female worms can lay non-viable eggs without the presence of males,[77] although the number of eggs deposited are lower than in the presence of males.[78] Despite the deposition of eggs in a single-sex female model this could still be safe as there is exposure to a limited number of cercariae, which will result in a limited number of non-viable eggs that will be deposited. Is addition, volunteers will be treated after 2-3 months so there will be egg deposition during a very limited time. As such, it is thought that a single- sex female CoHSI model could be safe and there are plans to perform a dose-finding single-sex female CoHSI study this year.

Concluding remarks

This thesis contributed to several steps of vaccine development pathways for both malaria and schistosomiasis by the use of controlled human infection models. Two new malaria strains were shown to be safe for vaccine testing, with a similar risk profile as the generally used strain. These strains can now be used for vaccine testing with heterologous CHMIs, which is likely to give more comparable results to field studies. The first in human GAP vaccine administered by DVI was shown to be safe, but resulted in limited less protective efficacy against malaria. For schistosomiasis a controlled human infection model was developed. The dose-finding study showed that exposure to a dose of 20 cercariae is safe and results in a high infection rate. Finally, suggestions were made to further improve the safety of controlled human infection studies, with the use of historical controls. Overall, this thesis shows the importance on how controlled human infection models contribute to vaccine development.

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APPENDIX:

Gecontroleerde humane infectiemodellen als hulpmiddel bij malaria en schistosomiasis vaccinatie onderzoek

In dit proefschrift is het gebruik van gecontroleerde humane infecties (CHI) voor diverse doeleinden beschreven. Als eerste komt het belang van het bestaande malaria CHI-model aan bod. Om malariavaccin onderzoek te kunnen verbeteren zijn nieuwe malaria stammen getest (**Hoofstuk 2 en 3**). Daarnaast is middels het gebruik van het malaria CHI-model een nieuw vaccin getest (**Hoofdstuk 4**). Als tweede is beschreven hoe de ontwikkeling van het nieuwe schistosomiasis CHI model tot stand is gekomen (**Hoofdstuk 5**). Waarna de resultaten van het eerste onderzoek met behulp van dit *schistosoma* CHI-model worden beschreven (**Hoofdstuk 6 en 7**). Als laatste wordt besproken hoe deze CHI-studies veiliger gemaakt kunnen worden (**Hoofdstuk 8**).

Parasieten

Parasieten zijn een groep ziekteverwekkers die gedurende hun leven in of op minimaal één gastheer verblijven. Een gastheer is een levend wezen, bijvoorbeeld mens, slak of mug, die een parasiet nodig heeft om te kunnen ontwikkelen en overleven. Iedere parasiet heeft zijn eigen levenscyclus met specifieke gastheer. Vaak zijn er meerdere parasiet-stadia per gastheer. De kleinste parasieten zijn eencellig, zoals malaria, terwijl wormen de grootste parasieten zijn. Deze wormen variëren enorm in lengte, van kleiner dan een centimeter tot enkele meters lang. De meeste parasitaire infecties zijn goed te behandelen met medicijnen. Diverse infecties kunnen voorkomen worden met behulp van een vaccin, maar deze zijn er voor parasitaire infecties nauwelijks.

Gecontroleerde humane infecties (CHI)

Er wordt op grote schaal onderzoek verricht naar het ontwikkelen van vaccins tegen parasieten die dodelijk kunnen zijn of veel ziektelast kunnen veroorzaken. Als een vaccin geschikt lijkt te zijn wordt eerst de veiligheid van een vaccin onderzocht door deze te testen op vrijwilligers. Dit heet fase I onderzoek. Hierbij worden vrijwilligers eenmalig blootgesteld aan het vaccin en wordt gekeken welke klachten zij in de weken hierna ontwikkelen.

Om na te gaan of een vaccin ook werkt, dat wil zeggen ziekte voorkomt, wordt de laatste tientallen jaren veel onderzoek verricht middels gecontroleerde humane infectie (CHI) studies. Dit zijn studies waarbij een groep vrijwilligers enkele keren een vaccin toegediend krijgen (immunisaties) waarbij meestal gelijktijdig een andere groep een placebo toegediend krijgt. Een placebo is een middel dat er fysiek uit ziet als het vaccin, maar geen werkzame stof bevat.

Hierna worden beide groepen blootgesteld aan de parasiet. Deze manier van infecteren is de daadwerkelijke CHI. Vervolgens worden alle vrijwilligers goed onder controle gehouden en wordt gekeken wie de ziekte ontwikkelen. Aan de hand van het aantal zieken in de vaccinatie- en de placebogroep is te berekenen hoe goed het vaccin lijkt te werken.

Bij CHI-studies wordt over het algemeen getest op gezonde vrijwilligers die niet eerder een infectie met de specifieke ziekteverwekker hebben gehad. Hierdoor hebben CHI-studies diverse voordelen boven het onderzoeken van vaccins in gebieden waar de ziekte veel voor komt, de endemische gebieden. De grootste voordelen zijn dat meerdere vaccins tegelijk getest kunnen worden, dat er minder vrijwilligers nodig zijn door de gecontroleerde omstandigheden (dat wil zeggen dezelfde hoeveelheid van een ziekteverwekker op hetzelfde moment in gezonde vrijwilligers) en dat het onderzoek goedkoper is om te verrichten. Daarnaast worden uiteindelijk alleen de vaccins die erg potent lijken te zijn onderzocht in grote groepen, soms kwetsbare, mensen in endemische gebieden. Waardoor zij niet onnodig worden blootgesteld aan vaccins die mogelijk niet werken.

Malaria

Malaria is de ziekte die veroorzaakt wordt door de parasiet *Plasmodium*. Er zijn verschillende soorten *Plasmodium* die ziekte kunnen veroorzaken in mensen. *Plasmodium falciparum* is de soort die malaria tropica veroorzaakt. Dit is de dodelijkste soort malaria. In totaal ontwikkelen jaarlijks ruim 200 miljoen mensen malaria en overlijden ruim 400.000 mensen aan deze ziekte. Het merendeel hiervan zijn kinderen onder de vijf jaar. Daarnaast lopen zwangeren een groter risico op het ontwikkelen van de ziekte.

Levenscyclus en klachten

De parasiet heeft verschillende levensstadia in zowel de mens als de mug, welke ieder een andere naam hebben. Als je wordt gestoken door een geïnfecteerde mug kunnen malaria-sporozoieten vanuit de speekselklieren van de mug de huid binnendringen. Vervolgens migreren ze naar de lever, waar ze een levercel binnengaan en zich ontwikkelen tot malaria-schizonten. Als deze volledig ontwikkeld zijn, barsten deze open en komen er malaria-merozoieten vrij in de bloedbaan. Deze infecteren vervolgens rode bloedcellen waarin ze zich verder kunnen vermenigvuldigen. Na verloop van tijd ontstaan er vrouwtjes en mannetjes malaria-gametocyten. Als patiënten op dat moment gestoken worden door een mug, kunnen de gametocyten door de mug opgezogen worden en kunnen zij in de mug paren. Vervolgens zal de parasiet diverse stadia in de mug doorlopen. Na enkele weken kan de mug een nieuw persoon infecteren.

Mensen die geïnfecteerd zijn met malaria worden pas ziek als de parasieten vanuit de lever in de bloedbaan terecht komen. Hier gaat, in het geval van malaria tropica, minimaal een week overheen. Mensen hebben vaak last van koorts, hoofdpijn en algehele malaise. Als de ziekte onbehandeld wordt gelaten nemen klachten toe en kan dit leiden tot neurologische klachten, uitval van organen, coma en uiteindelijk de dood.

Behandelen of voorkomen

Malaria is goed te behandelen met medicijnen, mits deze op tijd worden toegediend. Het probleem is dat het overgrote deel van patiënten in ontwikkelingslanden woont waar zij vaak slechte toegang tot zorg hebben. Hierdoor komen patiënten vaak pas in het ziekenhuis aan als zij al erg ziek zijn. Een behandeling komt dan niet altijd meer op tijd.

Door muggenbeten te voorkomen kan de ziekte malaria ook voorkomen worden. In de praktijk is dit lastig en maatregelen om beten te voorkomen helpen dan ook onvoldoende. Een malariavaccin kan voorkomen dat mensen de ziekte ontwikkelen. Op het moment is er één vaccin beschikbaar tegen malaria, Mosquirix. Dit vaccin wordt momenteel op grote schaal getest in sub-Sahara Afrika en geeft goede resultaten. Zo komen er minder gevallen van ernstige malaria voor en overlijden minder kinderen aan de ziekte. Het grootste probleem is echter dat het vaccin maar bij ruim de helft van de kinderen werkt en dat het in de loop van de tijd steeds minder goed werkt in dezelfde patiënt. Om deze reden blijft onderzoek naar een beter vaccin in volle gang.

Vaccins van hele parasieten (Hoofdstuk 4)

De laatste jaren wordt veel onderzoek verricht naar vaccins op basis van de volledige parasiet en dan met name de sporozoiet. Deze vaccins zijn onder te verdelen in drie verschillende soorten. 1. Bestraalde parasieten (RAS). 2. Parasieten met gelijktijdig toedienen van een anti-malaria medicijn (CPS). 3. Parasieten waarvan de genetische code, het DNA, is aangepast (GAP).

1. RAS: als malaria-sporozoieten bestraald worden kunnen ze nog wel een levercel binnen gaan, maar zich hier vervolgens niet volledig ontwikkelen. Hierdoor zal de parasiet overlijden voordat deze in de bloedbaan terecht kan komen en zal je er ook niet ziek van worden. Tijdens deze kortdurende leverfase zal het menselijk lichaam wel een afweerreactie maken tegen de parasiet.
2. CPS: chloroquine is een anti-malaria medicijn dat malariaparasieten doodt zodra deze in het bloed terecht komen. Als malaria sporozoieten gelijktijdig met deze behandeling worden gegeven zullen ze zich volledig ontwikkelen in de lever, waarna ze zullen overlijden zodra ze in het bloed terecht komen. Het voordeel hiervan is dat de parasieten zich volledig ontwikkelen in de lever, waardoor zich gedurende een langere tijd een afweerreactie kan ontwikkelen.
3. GAP: het is mogelijk het DNA van malariaparasieten aan te passen; genetische modificatie. Hierdoor is het mogelijk de parasiet zo aan te passen dat deze zich niet volledig ontwikkelt in de lever. Door deze ontwikkeling in de lever kan wel een afweerreactie plaatsvinden.

Zowel bij RAS als bij CPS kan volledige bescherming tegen malaria optreden nadat deze meerdere keren zijn gegeven. In beide gevallen neemt deze bescherming af in de loop van de tijd. Voor een GAP is dit nog onvoldoende onderzocht. De reden om hier onderzoek naar te verrichten is omdat GAP twee voordelen heeft. Afhankelijk van het type GAP kan deze zich mogelijk langer ontwikkelen in de lever dan RAS. In dat geval wordt er een beter afweerreactie gemaakt, net als bij CPS. Daarbij kan zich geen malaria ontwikkelen. Dat is wel het risico van CPS als de medicatie niet goed gebruikt wordt.

In **Hoofdstuk 4** beschrijven we het testen van een GAP-vaccin, PfSPZ-GA1 Vaccine, met behulp van een malaria CHI-onderzoek. Bij deze GAP zijn twee genen verwijderd uit de parasiet, waardoor deze zich in een vroeg leverstadium niet verder kan ontwikkelen. We toonden eerst aan dat het vaccin veilig was om te gebruiken. Hierna immuniseerden we vrijwilligers drie keer waarna ze een gecontroleerde humane malaria infectie (CHMI) kregen. Van alle vrijwilligers die PfSPZ-GA1 Vaccine kregen, was 12% volledig beschermd tegen malaria. Uit de groep vrijwilligers die een placebo hadden ontvangen was niemand beschermd. De overige vrijwilligers die PfSPZ-GA1 Vaccine hadden gekregen ontwikkelden pas enkele dagen later malaria dan de vrijwilliger in de placebogroep. Dit wijst erop dat deze vrijwilligers wel deels beschermd waren.

Het gebruik van verschillende stammen (Hoofdstuk 2 & 3)

In ieder gebied waar de malariaparasiet *Plasmodium falciparum* voorkomt zijn er geringe verschillen in het DNA van de parasiet. Dit worden verschillende stammen genoemd. De vaccins op basis van hele parasieten zijn allemaal ontwikkeld met de stam NF54. Deze stam komt van een patiënt die malaria opliep in de omgeving van een Nederlands vliegveld. Sinds 30 jaar wordt deze gekweekt in een laboratorium en gebruikt voor onderzoeksdoeleinden. Bovenstaande vaccins, RAS, CPS en GAP, zijn op dit moment allemaal op basis van deze stam. De CHMI die plaats vindt om te beoordelen of een vaccin goed werkt is ook op basis van deze stam NF54. Deze CHMI vindt meestal plaats met behulp van muggenbeten. De beten van vijf geïnfecteerde muggen zorgen voor het ontwikkelen van malaria in >99% van alle vrijwilligers die geen vaccin kregen toegediend.

Om te weten of vaccins op basis van deze ene stam ook werken tegen parasieten van een andere stam is het van belang om met meerdere stammen onderzoek te verrichtten. Hiervoor zijn naast NF54 ook de stammen 7G8 en NF135.C10 beschikbaar. In **Hoofdstuk 2** beschrijven we voor het eerst onderzoek met de nieuwe stam NF166.C8. We laten zien dat deze stam veilig is om te gebruiken. Daarnaast laat ons onderzoek zien dat een infectie met de stammen NF135.C10 of NF166.C8 leidt tot meer parasieten die uit de lever in het bloed terecht komen dan bij NF54. Om een CHMI met NF135.C10 of NF166.C8 mogelijk te maken hebben we gekeken hoeveel geïnfecteerde muggenbeten er nodig zijn om een infectie met een van deze stammen te ontwikkelen, wat we in **Hoofdstuk 3** hebben beschreven. Dit aantal blijkt hetzelfde te zijn als bij NF54, namelijk de beten van vijf geïnfecteerde muggen. Als vrijwilligers aan minder muggenbeten worden blootgesteld, ontwikkelt 75% van de vrijwilligers malaria.

Schistosomiasis

Schistosomiasis is de ziekte die veroorzaakt wordt door de parasiet *Schistosoma*. Er zijn verschillende soorten *Schistosoma* die de ziekte kunnen veroorzaken in mensen, waarvan *Schistosoma mansoni* en *Schistosoma haematobium* het vaakst voorkomen. Wereldwijd hebben 290 miljoen mensen schistosomiasis, waarvan ruim de helft van de mensen klachten heeft. Het is lastig vast te stellen hoeveel mensen jaarlijks aan de ziekte overlijden, maar naar schatting zijn dit er tussen de 24.000 en 150.000.

Levenscyclus en klachten

De parasiet *Schistosoma* heeft verschillende levensstadia in zowel de mens als de zoetwaterslak. Infectie vindt plaats door te zwemmen in water waar geïnfecteerde slakken voorkomen. De slak laat *Schistosoma* larven vrij, de cercariën, welke door de huid van de mens naar binnen kunnen dringen. Ze ontwikkelen zich in de mens tot volwassen wormen die zich uiteindelijk als paar zullen gaan nestelen in een van de grote vaten rondom de blaas (*S. haematobium*) of rondom de darmen (*S. mansoni*). Als de wormen hun eindbestemming bereiken zal het vrouwtje eitjes uitscheiden welke door het weefsel van de darm of de blaas in de ontlasting of urine terecht komen. Een deel van de eitjes blijft vast zitten in het weefsel waardoor ontstekingen kunnen ontstaan. Als de eitjes uit de ontlasting of urine terecht komen in zoet water kan zich in het ei het stadium miracidium ontwikkelen. Als deze miracidia uit de eitjes komen kunnen deze slakken infecteren. In de slak vinden opnieuw diverse ontwikkelingsstadia plaats. Uiteindelijk leidt dit tot de ontwikkeling van cercariën welke weer door de slak kunnen worden vrijgelaten.

Tijdens deze cyclus in de mens kunnen verschillende klachten optreden. De eerste weken na infectie kan zwemmersjeuk optreden op de plaatsen waar de cercariën de huid binnen zijn gedrongen. Hierna kunnen in de acute fase, de eerste maanden, hevige klachten optreden, met name bij mensen die voor de eerste keer geïnfecteerd zijn. Dit wordt Katayama syndroom genoemd, waarbij klachten zoals koorts, hoofdpijn en algehele malaise op de voorgrond staan. Vervolgens kan de ontsteking rond de eitjes die vast zitten in het weefsel zich in de loop van de jaren uitbreiden, naarmate er meer eitjes vast komen te zitten. Het duurt lange tijd voor mensen hier klachten van ontwikkelen en het kan na jaren van infectie zelfs leiden tot kanker.

Behandelen of voorkomen

Schistosomiasis is te behandelen met het medicijn praziquantel. Het probleem is dat praziquantel niet bij iedereen werkt. Er wordt geschat dat het middel in gebieden waar de ziekte veel voor komt, endemische gebieden, in 42-91% van de gevallen werkt. Praziquantel werkt alleen tegen volwassen wormen en voorkomt niet dat mensen opnieuw besmet raken. Omdat mensen vaak niet weten dat ze de infectie hebben totdat klachten optreden, kan er lange tijd overheen gaan voordat behandeling plaats vindt. In endemische gebieden wordt jaarlijks praziquantel uitgedeeld, mass drugs administration (MDA), om de ziekte zoveel mogelijk beperkt te houden. Dit is echter onvoldoende om de ziekte uit te kunnen roeien.

De infectie is te voorkomen door contact met zoet water te vermijden. Dit advies is geschikt voor reizigers, maar is lastig voor mensen die in endemische gebieden wonen. Zij zijn vaak afhankelijk van het water wat gebruikt wordt om hun kleding en zichzelf te wassen, te koken en om vis te vangen. Een andere manier om infectie te voorkomen is het gebruik van een vaccin. Echter een vaccin tegen schistosomiasis is nog niet beschikbaar. Er zijn momenteel drie vaccins tegen *Schistosoma mansoni* in verschillende stadia van ontwikkeling. Of deze voldoende effectief zijn zal verder onderzoek uit moeten wijzen.

Gecontroleerd humaan *Schistosoma* infectie onderzoek (Hoofdstuk 5, 6 & 7)

Vanwege de bovengenoemde voordelen van CHI-studies samen met het belang van het snel ontwikkelen van een vaccin voor schistosomiasis, hebben we een gecontroleerd humaan schistosomiasis infectie (CoHSI) model opgezet. Het opzetten van dit model werd beschreven in **Hoofdstuk 5**. Het belangrijkste was om dit nieuwe model zo veilig mogelijk te maken voor vrijwilligers. Aangezien de eitjes van *Schistosoma* kunnen zorgen voor klachten op lange termijn is besloten een model op te zetten waarbij vrijwilligers alleen aan mannelijke cercariën blootgesteld worden. De eerste studie die we met het model hebben verricht was om te beoordelen aan hoeveel cercariën vrijwilligers blootgesteld moeten worden om een aantoonbare infectie te ontwikkelen. De eerste groep is blootgesteld aan 10 cercariën, waarna niet alle vrijwilligers geïnfecteerd waren. Vervolgens is een groep blootgesteld aan 30 cercariën. Van deze groep waren alle drie de vrijwilligers geïnfecteerd, maar ontwikkelde twee vrijwilligers klachten die passen bij het Katayama syndroom, zoals staat beschreven in **Hoofdstuk 6**. Vanwege de hevige klachten is besloten om de volgende groep aan 20 cercariën bloot te stellen. In totaal zijn 11 vrijwilligers aan deze dosis blootgesteld. Een deel van de vrijwilligers heeft klachten ontwikkeld, maar niet van dezelfde hevigheid als bij de vrijwilligers blootgesteld aan 30 cercariën. In **Hoofdstuk 7** beschrijven we de uitkomsten van dit eerste CoHSI onderzoek. Van de 11 vrijwilligers blootgesteld aan 20 cercariën heeft uiteindelijk 82% een *Schistosoma*-infectie opgelopen. Ondanks dat dit niet resulteert in 100% van de vrijwilligers met een infectie, is toch besloten om bij een vervolgonderzoek deze dosis van 20 cercariën te gebruiken. De bijwerkingen zijn bij deze dosis acceptabel en vergelijkbaar met andere modellen, terwijl dat bij een hogere dosis niet het geval is.

Optimaliseren CHI-modellen (Hoofdstuk 8)

Zoals hierboven beschreven, zijn sommige CHI-modellen reeds uitgebreid getest. Hierdoor zijn deze in de loop van de jaren gestandaardiseerd, waardoor wereldwijd dezelfde opzet gebruikt wordt. Het voordeel hiervan is dat resultaten van verschillende onderzoekscentra vergeleken kunnen worden. Daarnaast kunnen deze resultaten gebruikt worden om CHI-modellen veiliger te maken, waarbij het geringe risico voor vrijwilligers nog verder afneemt. De meest gebruikte studie opzet is een vaccin groep vergelijken met een controlegroep. In **Hoofdstuk 8** beschrijven we dat er naast deze studie opzet ook andere mogelijkheden zijn. Een mogelijkheid is het gebruik van historische data. Hierbij worden resultaten uit andere studies gebruikt, de historische controlegroep, in plaats van een placebogroep. Een goed voorbeeld hiervoor is het malariamodel. Zoals eerder beschreven weten we dat >99% van de gezonde vrijwilligers in de placebogroep malaria ontwikkelen als ze worden blootgesteld aan vijf malaria geïnfecteerde muggen. Deze historische data kan voor een vervolgstudie gebruikt worden in plaats van een placebogroep. Om zeker te weten dat er niks mis gaat met de CHMI-procedure, kan ervoor gekozen worden om een kleine groep vrijwilligers een CHMI te laten ondergaan. Het gebruik van historische data is vooralsnog niet mogelijk voor het schistosomiasis model, omdat er nog maar één studie met dit model uitgevoerd. De toekomst zal uitwijzen of blootstelling aan 20 cercariën altijd leidt tot hetzelfde percentage geïnfecteerde vrijwilligers.

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Curriculum vitae

Marijke Langenberg was born in Tholen, the Netherlands, on the 15th of January 1985. After completing her higher education in Bergen op Zoom she went to Leiden to study medicine. During the second year of study she performed research on psychosocial care for HIV/AIDS patients in Surinam. During her scientific internship at the department of parasitology she investigated the use of different laboratory techniques for nematodes in Beira, Mozambique. Her last clinical internship was at the tropical diseases outpatient clinic of the Harbour hospital in Rotterdam. In February 2010 she got her medical degree.

After receiving her medical degree she worked on a voluntary base as a doctor in Mongolia for several weeks. In May 2010 she started working at the department of internal medicine at the Maasstad hospital in Rotterdam. Here she started her residency to become a specialist in internal medicine in 2011. In 2014 she continued her residency at the department of internal medicine at the Leiden University Medical Center (LUMC) in Leiden. Later that year she worked as an investigator in a controlled human malaria infection study at the Harbour hospital in Rotterdam. Hereafter she started her PhD project at the parasitology group at the LUMC. During her PhD she mainly focused on malaria and schistomiasis controlled human infection studies.

In April 2019 she continued with her residency to become a doctor in internal medicine specialising in infectious diseases. In July 2020 she decided to switch career and started to work as a doctor in infectious disease control at the GGD Rotterdam-Rijnmond.

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