Controlled human infection models as a tool for malaria and schistosomiasis vaccine research

Langenberg, M.C.C.

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.

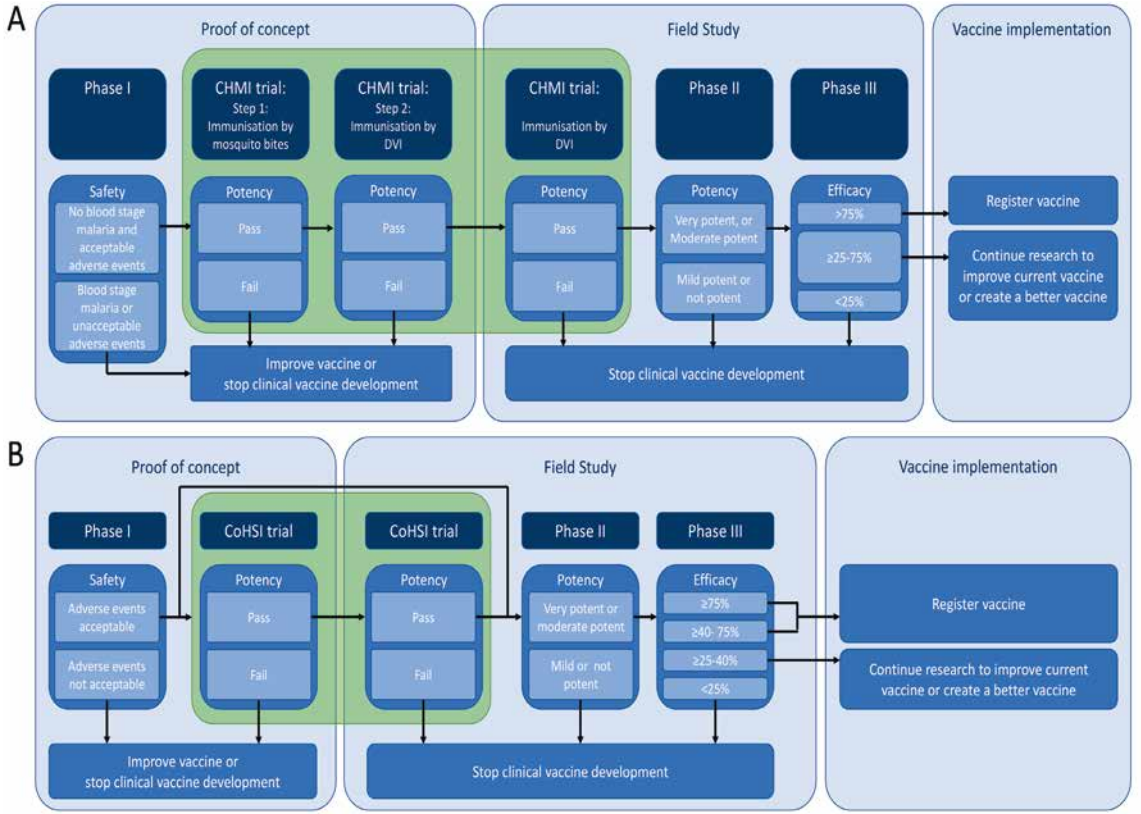

Figure. Suggested clinical vaccine development pathways.
A. Clinical development pathway for a malaria vaccine. **B.** Clinical development pathway for a schistosomiasis vaccine. In green the trials performed with the use of a controlled human infection model.

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.

References

1. Sauerwein RW, Roestenberg M, Moorthy VS. Experimental human challenge infections can accelerate clinical malaria vaccine development. *Nat Rev Immunol* 2011; **11**(1): 57-64.

2. Seder RA, Chang LJ, Enama ME, et al. Protection against malaria by intravenous immunization with a nonreplicating sporozoite vaccine. *Science* 2013; **341**(6152): 1359-65.

3. Epstein JE, Paolino KM, Richie TL, et al. Protection against Plasmodium falciparum malaria by PfSPZ Vaccine. *JCI Insight* 2017; **2**(1): e89154.

4. Epstein JE, Tewari K, Lyke KE, et al. Live attenuated malaria vaccine designed to protect through hepatic CD8(+) T cell immunity. *Science* 2011; **334**(6055): 475-80.

5. Roestenberg M, McCall M, Hopman J, et al. Protection against a malaria challenge by sporozoite inoculation. *N Engl J Med* 2009; **361**(5): 468-77.

6. Mita T, Jombart T. Patterns and dynamics of genetic diversity in Plasmodium falciparum: what past human migrations tell us about malaria. *Parasitol Int* 2015; **64**(3): 238-43.

7. Walk J, Reuling IJ, Behet MC, et al. Modest heterologous protection after Plasmodium falciparum sporozoite immunization: a double-blind randomized controlled clinical trial. *BMC Med* 2017; **15**(1): 168.

8. Hoffman SL, Goh LM, Luke TC, et al. Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites. *J Infect Dis* 2002; **185**(8): 1155-64.

9. Lyke KE, Ishizuka AS, Berry AA, et al. Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection. *Proc Natl Acad Sci U S A* 2017; **114**(10): 2711-6.

10. Schats R, Bijker EM, van Gemert GJ, et al. Heterologous Protection against Malaria after Immunization with Plasmodium falciparum Sporozoites. *PLoS One* 2015; **10**(5): e0124243.

11. Teirlinck AC, Roestenberg M, van de Vegte-Bolmer M, et al. NF135.C10: a new Plasmodium falciparum clone for controlled human malaria infections. *J Infect Dis* 2013; **207**(4): 656-60.

12. Epstein JE, Rao S, Williams F, et al. Safety and clinical outcome of experimental challenge of human volunteers with Plasmodium falciparum-infected mosquitoes: an update. *J Infect Dis* 2007; **196**(1): 145-54.

13. Rickman LS, Jones TR, Long GW, et al. Plasmodium falciparum-infected Anopheles stephensi inconsistently transmit malaria to humans. *Am J Trop Med Hyg* 1990; **43**(5): 441-5.

14. Verhage DF, Telgt DS, Bousema JT, et al. Clinical outcome of experimental human malaria induced by Plasmodium falciparum-infected mosquitoes. *Neth J Med* 2005; **63**(2): 52-8.

15. Jongo SA, Church LWP, Mtoro AT, et al. Increase of dose associated with decrease in protection against controlled human malaria infection by PfSPZ Vaccine in Tanzanian adults. *Clin Infect Dis* 2019.

16. Moser KA, Drabek EF, Dwivedi A, et al. Strains used in whole organism Plasmodium falciparum vaccine trials differ in genome structure, sequence, and immunogenic potential. *Genome Med* 2020; **12**(1): 6.

17. Rothman AL, Kanesa-thasan N, West K, Janus J, Saluzzo JF, Ennis FA. Induction of T lymphocyte responses to dengue virus by a candidate tetravalent live attenuated dengue virus vaccine. *Vaccine* 2001; **19**(32): 4694-9.

18. Zhou H, Deem MW. Sculpting the immunological response to dengue fever by polytopic vaccination. *Vaccine* 2006; **24**(14): 2451-9.

19. Hoffman SL, Billingsley PF, James E, et al. Development of a metabolically active, non-replicating sporozoite vaccine to prevent Plasmodium falciparum malaria. *Hum Vaccin* 2010; **6**(1): 97-106.

20. Mordmuller B, Surat G, Lagler H, et al. Sterile protection against human malaria by chemoattenuated PfSPZ vaccine. *Nature* 2017; **542**(7642): 445-9.

21. Itsara LS, Zhou Y, Do J, Grieser AM, Vaughan AM, Ghosh AK. The Development of Whole Sporozoite Vaccines for Plasmodium falciparum Malaria. *Front Immunol* 2018; **9**: 2748.

22. Mellouk S, Lunel F, Sedegah M, Beaudoin RL, Druilhe P. Protection against malaria induced by irradiated sporozoites. *Lancet* 1990; **335**(8691): 721.

23. Clyde DF, Most H, McCarthy VC, Vanderberg JP. Immunization of man against sporozite-induced falciparum malaria. *Am J Med Sci* 1973; **266**(3): 169-77.

24. Friesen J, Matuschewski K. Comparative efficacy of pre-erythrocytic whole organism vaccine strategies against the malaria parasite. *Vaccine* 2011; **29**(40): 7002-8.

25. Kreutzfeld O, Muller K, Matuschewski K. Engineering of Genetically Arrested Parasites (GAPs) For a Precision Malaria Vaccine. *Front Cell Infect Microbiol* 2017; **7**: 198.

26. Slater AF. Chloroquine: mechanism of drug action and resistance in Plasmodium falciparum. *Pharmacol Ther* 1993; **57**(2-3): 203-35.

27. Mueller AK, Labaied M, Kappe SH, Matuschewski K. Genetically modified Plasmodium parasites as a protective experimental malaria vaccine. *Nature* 2005; **433**(7022): 164-7.

28. Mueller AK, Camargo N, Kaiser K, et al. Plasmodium liver stage developmental arrest by depletion of a protein at the parasite-host interface. *Proc Natl Acad Sci U S A* 2005; **102**(8): 3022-7.

29. Spring M, Murphy J, Nielsen R, et al. First-in-human evaluation of genetically attenuated Plasmodium falciparum sporozoites administered by bite of Anopheles mosquitoes to adult volunteers. *Vaccine* 2013; **31**(43): 4975-83.

30. Annoura T, van Schaijk BC, Ploemen IH, et al. Two Plasmodium 6-Cys family-related proteins have distinct and critical roles in liver-stage development. *FASEB J* 2014; **28**(5): 2158-70.

31. Ishino T, Chinzei Y, Yuda M. Two proteins with 6-cys motifs are required for malarial parasites to commit to infection of the hepatocyte. *Mol Microbiol* 2005; **58**(5): 1264-75.

32. van Dijk MR, Douradinha B, Franke-Fayard B, et al. Genetically attenuated, P36p-deficient malarial sporozoites induce protective immunity and apoptosis of infected liver cells. *Proc Natl Acad Sci U S A* 2005; **102**(34): 12194-9.

33. Annoura T, Ploemen IH, van Schaijk BC, et al. Assessing the adequacy of attenuation of genetically modified malaria parasite vaccine candidates. *Vaccine* 2012; **30**(16): 2662-70.

34. VanBuskirk KM, O'Neill MT, De La Vega P, et al. Preerythrocytic, live-attenuated Plasmodium falciparum vaccine candidates by design. *Proc Natl Acad Sci U S A* 2009; **106**(31): 13004-9.

35. Aly AS, Mikolajczak SA, Rivera HS, et al. Targeted deletion of SAP1 abolishes the expression of infectivity factors necessary for successful malaria parasite liver infection. *Mol Microbiol* 2008; **69**(1): 152-63.

36. Silvie O, Goetz K, Matuschewski K. A sporozoite asparagine-rich protein controls initiation of Plasmodium liver stage development. *PLoS Pathog* 2008; **4**(6): e1000086.

37. Kublin JG, Mikolajczak SA, Sack BK, et al. Complete attenuation of genetically engineered Plasmodium falciparum sporozoites in human subjects. *Sci Transl Med* 2017; **9**(371).

38. van Schaijk BC, Ploemen IH, Annoura T, et al. A genetically attenuated malaria vaccine candidate based on P. falciparum b9/slarp gene-deficient sporozoites. *Elife* 2014; **3**.

39. Vaughan AM, Sack BK, Dankwa D, et al. A Plasmodium Parasite with Complete Late Liver Stage Arrest Protects against Preerythrocytic and Erythrocytic Stage Infection in Mice. *Infect Immun* 2018; **86**(5).

40. Vaughan AM, O'Neill MT, Tarun AS, et al. Type II fatty acid synthesis is essential only for malaria parasite late liver stage development. *Cell Microbiol* 2009; **11**(3): 506-20.

41. Yu M, Kumar TR, Nkrumah LJ, et al. The fatty acid biosynthesis enzyme FabI plays a key role in the development of liver-stage malarial parasites. *Cell Host Microbe* 2008; **4**(6): 567-78.

42. van Schaijk BC, Kumar TR, Vos MW, et al. Type II fatty acid biosynthesis is essential for Plasmodium falciparum sporozoite development in the midgut of Anopheles mosquitoes. *Eukaryot Cell* 2014; **13**(5): 550-9.

43. Malaria Vaccine Funders Group. Malaria Vaccine Technology Roadmap. 2013.

44. Wilby KJ, Lau TT, Gilchrist SE, Ensom MH. Mosquirix (RTS,S): a novel vaccine for the prevention of Plasmodium falciparum malaria. *Ann Pharmacother* 2012; **46**(3): 384-93.

45. King CH, Olbrych SK, Soon M, Singer ME, Carter J, Colley DG. Utility of repeated praziquantel dosing in the treatment of schistosomiasis in high-risk communities in Africa: a systematic review. *PLoS Negl Trop Dis* 2011; **5**(9): e1321.

46. WHO: Schistosomiasis, Stategy: control and preventive chemotherapy (http://www.who.int/schistosomiasis/strategy/en/).

47. https://www.who.int/schistosomiasis/strategy/p41_sch.pdf?ua=1 (accessed June 24, 2019).

48. Gurarie D, Yoon N, Li E, et al. Modelling control of Schistosoma haematobium infection: predictions of the long-term impact of mass drug administration in Africa. *Parasit Vectors* 2015; **8**: 529.

49. Crellen T, Walker M, Lamberton PH, et al. Reduced Efficacy of Praziquantel Against Schistosoma mansoni Is Associated With Multiple Rounds of Mass Drug Administration. *Clin Infect Dis* 2016; **63**(9): 1151-9.

50. Merrifield M, Hotez PJ, Beaumier CM, et al. Advancing a vaccine to prevent human schistosomiasis. *Vaccine* 2016; **34**(26): 2988-91.

51. Santini-Oliveira M, Coler RN, Parra J, et al. Schistosomiasis vaccine candidate Sm14/GLA-SE: Phase 1 safety and immunogenicity clinical trial in healthy, male adults. *Vaccine* 2016; **34**(4): 586-94.

52. Zhang W, Molehin AJ, Rojo JU, et al. Sm-p80-based schistosomiasis vaccine: double-blind preclinical trial in baboons demonstrates comprehensive prophylactic and parasite transmission-blocking efficacy. *Ann N Y Acad Sci* 2018; **1425**(1): 38-51.

53. Molehin AJ. Schistosomiasis vaccine development: update on human clinical trials. *J Biomed Sci* 2020; **27**(1): 28.

54. Bambery B, Selgelid M, Weijer C, Savulescu J, Pollard AJ. Ethical Criteria for Human Challenge Studies in Infectious Diseases. *Public Health Ethics* 2016; **9**(1): 92-103.

55. Miller FG, Grady C. The ethical challenge of infection-inducing challenge experiments. *Clin Infect Dis* 2001; **33**(7): 1028-33.

56. Roestenberg M, Hoogerwerf MA, Ferreira DM, Mordmuller B, Yazdanbakhsh M. Experimental infection of human volunteers. *Lancet Infect Dis* 2018.

57. Hoffman SL. Experimental challenge of volunteers with malaria. *Ann Intern Med* 1997; **127**(3): 233-5.

58. Darton TC, Blohmke CJ, Moorthy VS, et al. Design, recruitment, and microbiological considerations in human challenge studies. *Lancet Infect Dis* 2015; **15**(7): 840-51.

59. Gryseels B, Polman K, Clerinx J, Kestens L. Human schistosomiasis. *Lancet* 2006; **368**(9541): 1106-18.

60. Ross AG, Vickers D, Olds GR, Shah SM, McManus DP. Katayama syndrome. *Lancet Infect Dis* 2007; **7**(3): 218-24.

61. Bottieau E, Clerinx J, de Vega MR, et al. Imported Katayama fever: clinical and biological features at presentation and during treatment. *J Infect* 2006; **52**(5): 339-45.

62. Rocha MO, Greco DB, Pedroso ER, et al. Secondary cutaneous manifestations of acute schistosomiasis mansoni. *Ann Trop Med Parasitol* 1995; **89**(4): 425-30.

63. Olson BG, Domachowske JB. Mazzotti reaction after presumptive treatment for schistosomiasis and strongyloidiasis in a Liberian refugee. *Pediatr Infect Dis J* 2006; **25**(5): 466-8.

64. Bachmeyer C, Cabbanes A, Eouzan D, Gauthier M, Steichen O. [Eyelid swelling in a patient with acute schistosomiasis]. *Presse Med* 2011; **40**(5): 556-7.

65. Stenstad T. A man with fever, urticaria, periorbital oedema and dry cough. *Tidsskr Nor Laegeforen* 2015; **135**(20): 1860-4.

66. https://media.tghn.org/articles/Conclusion_of_CMC_for_challenge_agents_meeting.PNG (accessed March 04, 2020)

67. Regmi S, Callender T, Knox AF, Bhopal A. Research and development funding for 13 neglected tropical diseases: an observational economic analysis. *Lancet* 2014; **384**: S20-S.

68. Reed SL, McKerrow JH. Why Funding for Neglected Tropical Diseases Should Be a Global Priority. *Clinical Infectious Diseases* 2018; **67**(3): 323-6.

69. Mo AX, Colley DG. Workshop report: Schistosomiasis vaccine clinical development and product characteristics. *Vaccine* 2016; **34**(8): 995-1001.

70. WHO: Status of Vaccine Research and Development of Vaccines for Schistosomiasis Prepared for WHO PD-VAC (http://who.int/immunization/research/meetings_workshops/Schistosomiasis_VaccineRD_Sept2014.pdf).

71. Koopman JP, Egesa M, Wajja A, et al. Risk assessment for the implementation of controlled human Schistosoma mansoni infection trials in Uganda. *AAS Open Res* 2019; **2**: 17.

72. Siddiqui AJ, Molehin AJ, Zhang W, et al. Sm-p80-based vaccine trial in baboons: efficacy when mimicking natural conditions of chronic disease, praziquantel therapy, immunization, and Schistosoma mansoni re-encounter. *Ann N Y Acad Sci* 2018.

73. Keitel WA, Potter GE, Diemert D, et al. A phase 1 study of the safety, reactogenicity, and immunogenicity of a Schistosoma mansoni vaccine with or without glucopyranosyl lipid A aqueous formulation (GLA-AF) in healthy adults from a non-endemic area. *Vaccine* 2019; **37**(43): 6500-9.

74. Tendler M, Almeida MS, Vilar MM, Pinto PM, Limaverde-Sousa G. Current Status of the Sm14/GLA-SE Schistosomiasis Vaccine: Overcoming Barriers and Paradigms towards the First Anti-Parasitic Human(itarian) Vaccine. *Trop Med Infect Dis* 2018; **3**(4).

75. McManus DP, Bergquist R, Cai P, Ranasinghe S, Tebeje BM, You H. Schistosomiasis-from immunopathology to vaccines. *Semin Immunopathol* 2020.

76. Erasmus DA. A comparative study of the reproductive system of mature, immature and "unisexual" female Schistosoma mansoni. *Parasitology* 1973; **67**(2): 165-83.

77. Shaw MK. Schistosoma mansoni: vitelline gland development in females from single sex infections. *J Helminthol* 1987; **61**(3): 253-9.

78. Michaels RM. Mating of Schistosoma mansoni in vitro. *Exp Parasitol* 1969; **25**(1): 58-71.