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Building bridges: a multidisciplinary approach to controlled human hookworm infection

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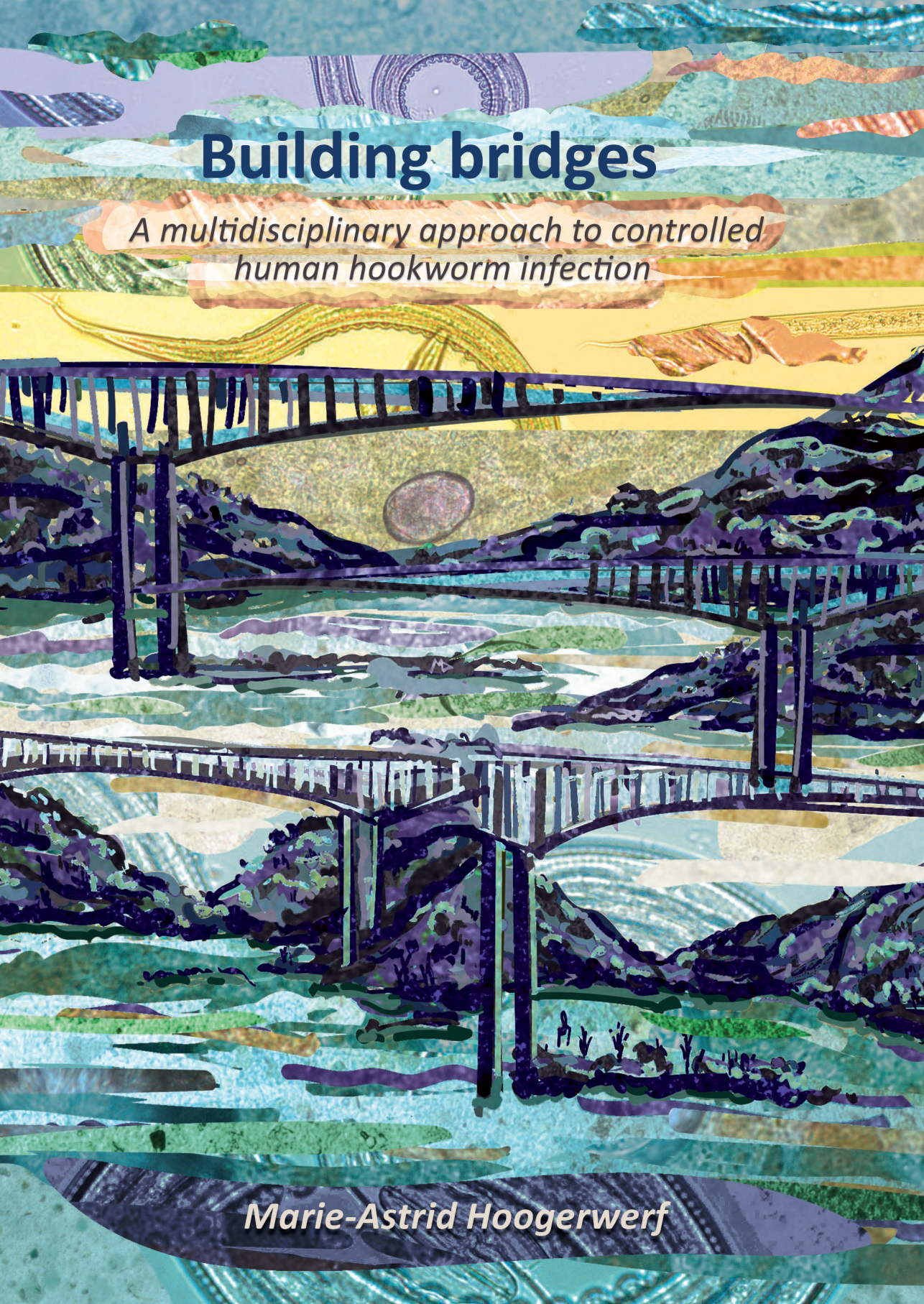
Building bridges

A multidisciplinary approach to controlled human hookworm infection



Marie-Astrid Hoogerwerf

Building bridges

a multidisciplinary approach to controlled human hookworm infection

Marie-Astrid Hoogerwerf

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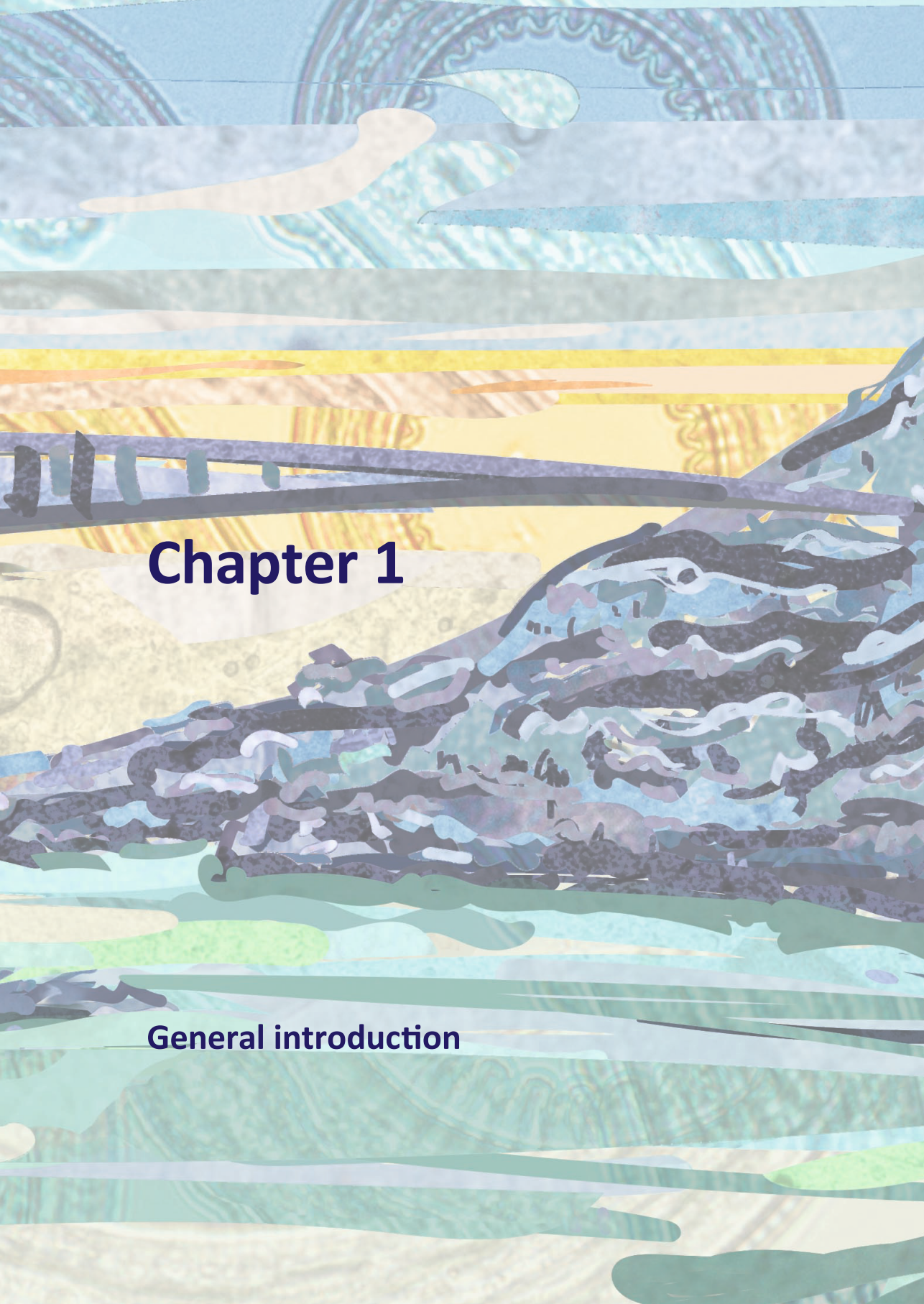

Chapter 1

General introduction

Controlled human infections

The deliberate exposure of humans with an infectious agent dates back to the 18th century, when Jenner inoculated his gardener's son with cow pox to prevent him from developing small pox and thereby laid the foundation for the concept of vaccination. Since then, deliberate infections have led to enormous developments in the preventive field of vaccination, have been used as therapeutic agents, as prove of Koch's postulates and to study pathogenicity factors. Controlled infections are furthermore increasingly applied as an effective and quick method of assessing vaccine- and drug efficacy.[1] Experimental infection of volunteers is also called 'controlled human infection', shortened to CHI. CHI-models are now being used in a range of diseases, including models in bacteria, viruses and parasites and range from colonisation models that generally do not cause symptoms (such as pneumococcus, lactobacillus) to models with overt symptomatology that is even used a primary outcome measure, such as the cholera and typhoid fever models.

Data generated in CHI-studies have led to several major milestones in knowledge of pathogenicity, host-pathogen interactions and vaccine development. CHI-studies in respiratory viruses have generated important knowledge on viral transmission,[2] trials in norovirus identified susceptibility factors of infection related to blood type[3] and CHI-studies have determined the importance of the typhoid toxin in developing typhoid fever.[4] Data from controlled malaria infection studies have played an important role in the development of the first malaria vaccine RTS'S.[5 6] The cholera challenge model demonstrates how CHI-studies into pathogenicity and then into vaccine efficacy can lead to a licensed vaccine. Observations on the development of protection after repeated exposure in controlled infections have first led to the development of an oral live-attenuated cholera vaccine, which in turn has now been licensed based on the convincing data from a human challenge study. [7-9]

Due to their efficiency and potential for quick screening of drug- and vaccine candidates, CHI-studies are particularly valuable for research into pathogens affecting the world's poorest population for which research funding and opportunities are scarce. These include among others schistosomiasis, for which recently a controlled infection model has been developed[10] and soil-transmitted helminths.

The controlled human hookworm infection model

Hookworms are a soil-transmitted helminth affecting around 300 million people worldwide.[11] The chronic blood- and protein loss caused by the attachment and subsequent blood feeding of the hookworm to the intestinal wall results in iron-deficiency anaemia and malnutrition, particularly in children and women of child-bearing age.[12] This significantly impacts children's development, and the resulting impairments have been estimated to generate a loss of productivity of between $7.5 and $138.9 billion annually.[13] As hookworm infection itself is strongly correlated with poor economic circumstances,[14] this reduction in wage-earning potential perpetuates the poverty cycle.

Current hookworm control efforts rely on mass drug administration programs. However, these have not yet succeeded in eliminating hookworm due to high rates of re-infection and the exclusion of adults in treatment programs.[15] In addition, treatment coverage rates still do not meet the standards set by the WHO and fall short of those projected to be necessary to achieve hookworm transmission control.[16 17]

A vaccine would greatly contribute to efforts to control hookworm.[13] Currently two hookworm vaccine candidates are being researched.[18 19] However, development and efficacy testing of vaccines is hampered by a lack of preclinical models, since hookworm has uniquely adapted to the human host and results in animal models cannot be easily translated to humans.[20] This also hinders the understanding of possible mechanisms of protection and human immunological responses against hookworm infections. Field studies in endemic areas can generate more knowledge, however studying immune responses is confounded by co-infections and unknown circumstances of infection intensity and duration. Vaccine field studies would require large sample sizes and impractically long follow-up.[21] The controlled human hookworm infection model (CHHI) could therefore greatly contribute to both effective vaccine-testing and to knowledge of hookworm infection immunology.

The projected hookworm vaccine does not need to induce sterile immunity to be effective, as a reduction in infection intensity will already have a significant clinical effect.[21] Reliable detection of infection is therefore a key issue in developing the CHHI model. Currently the only practical endpoint for a vaccine efficacy trial is egg excretion in faeces, by microscopic techniques or PCR. This however is a highly variable outcome,[22] which reduces study power and necessitates large sample sizes for vaccine efficacy trials. Finding ways to reduce this variation and obtaining egg counts that are comparable to field settings would greatly improve the reliability of the CHHI model. This thesis aims to find improvements to the challenge model and to outcome measures through repeated infection and statistical modelling, and will then apply this model to an immunisation study.

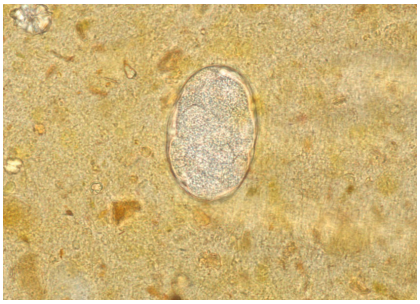

Figure 1 A. Hookworm egg (*Necator americanus*). B. L3 hookwom larvae (*Necator americanus*)

Studying microbiome using controlled human infections

It has been hypothesized that the immunomodulatory effect observed in hookworm infection is mediated through an effect on the gut microbiome.[23] It is therefore of interest to study the changes in gut microbiome after hookworm infection. Field studies have shown an increased richness and species diversity in infected individuals,[24] although clearance of the infection did not show an effect on relative species abundance.[25] However, there are many inconsistencies between results from field studies, due to the presence of confounders such as co-infections, malnutrition and limitations of the usually cross-sectional study designs.[26] Here, studying the microbiome in the controlled setting of the CHHI-model could generate clearer insights into the relation between infection and gut microbiome. In for example pneumococcal colonisation studies the CHI model has already been successfully applied to study susceptibility to colonization in relation to microbiome factors, changes in microbiome following pneumococcal colonization and the relation with viral co-infection.[27] [28] For hookworm, small scale studies with small infectious inoculae[29] [30] have found no major impact on microbiome community structure, although a trend towards an increase in species richness comparable to findings in field studies was described. However, the small infectious doses and limited number of participants reduces generalizability of these results and leaves room for further investigations.

Ethical aspects of controlled human infection studies

Despite the clear practical and scientific arguments for the use of CHI-studies, their conduct is an ongoing source of ethical debate. There have indeed been historical examples of infection studies that breach ethical standards, such as the Tuskegee syphilis study[31] and the Guatemala STD[32] inoculation study. However, these studies are considered unacceptable not because of the deliberate infection itself, but because they breached basic ethical principles, such as informed consent.[32] CHI-studies must adhere to the same guidelines as all other research, although CHI-study specific questions may apply.

The first ethical framework for the evaluation of challenge studies was suggested by Miller and Grady in 2001,[33] posing the questions and considerations that should be taken into account when reviewing a CHI study. The authors conclude that if these questions and considerations are met, CHI-studies may be ethically justifiable. Hope and MacMillan[34] have stated that the central issue regarding challenge studies lies in the risk of harm to participants and argue why in their opinion the concept of deliberate infection itself is no different than other research in healthy volunteers. Bambery et al have expanded the framework suggested by Miller and Grady by incorporating protection of the public, independent ethical review and compensation for harm to the critical questions.[35] When taking into account these criteria and with proper oversight, the authors argue that challenge studies should not only be considered ethically acceptable but may even sometimes be ethically required.[35]

A common factor in current ethical literature is a particular focus on the balance of risks versus benefits, importance of informed consent and the issue of deliberate infection

itself. Another issue that is frequently raised not only regarding CHI-studies, but in all studies involving healthy volunteers, is the motivation of participants and the influence of the financial compensation on the decision to take part.[36] Although this has been the subject of academic debate between researchers and regulatory authorities, the opinions of participants themselves have largely been lacking. This is a gap in knowledge that this thesis will aim to address.

Thesis outline

This thesis starts to provide an overview of controlled human infections in **chapter 2**. We then first focus on the controlled human hookworm infection model.

In **chapter 3** we describe the results of the pilot study to establish the CHHI model in Leiden and show through a prolonged follow-up phase that egg excretion develops a plateau level that can be used as an outcome measure in further studies.

Chapter 4 aims to further improve the CHHI model by investigating the effect of repeated infections on egg output and variability. We show that repeated infection does decrease variability without increasing adverse events and therefore propose an improved challenge model.

Chapter 5 shows another application of the hookworm controlled infection model in the investigation of changes in the gut microbiome after infection. This chapter describes the analysis of gut microbiome composition of the participants in the repeated infection trial from chapter 4 and shows that increased gastro-intestinal symptoms are associated with increased microbiome instability during acute infection which recovers in the immunotolerant phase.

The repeated infection challenge model described from chapter 4 is then implemented in **chapter 6** where it is used as the challenge following immunization using short-term infection. Here, we demonstrate for the first time that protection against hookworm infection can be elicited by repeated short-term infection and describe the importance of the skin in developing an immune response.

In **chapter 7** we investigate motivations and experiences of participants in CHI trials and describe why in our view CHI-participants could be described as 'deliberate decision-makers'.

In the summarizing discussion in **chapter 8** we aim to bring together all research described in this thesis and show how a multidisciplinary approach is needed to enhance improvements in controlled human infection models.

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Chapter 2

Experimental infection of human volunteers

Meta Roestenberg, Marie-Astrid Hoogerwerf, Daniela M. Ferreira, Benjamin Mordmüller, Maria Yazdanbakhsh

Lancet Inf Dis 2018 Oct;18(10):e312-e322

Abstract

Controlled human infection trials (CHI), in which healthy volunteers are experimentally infected, can accelerate the development of novel drugs and vaccines for infectious diseases of global importance. The exploitation of CHI models is expanding exponentially from around 60 studies per decade in the 1970's to more than 120 studies already published in the current decade. Influenza, rhinovirus and malaria are the most frequently practiced CHI models. CHI trials have provided historical landmark data for several registered drugs and vaccines and provided unprecedented mechanistic insight into host-pathogen interaction. Because of their often invasive nature and the use of healthy volunteers, CHI studies contradict the "do not harm" principle and as such demand critical ethical review according to established frameworks which are similar to phase 1 clinical testing. Advances such as the principle of controlled colonisation and the expansion of models to other sites will further broaden the horizon for CHI. Here, we review the use of CHI trials and provide an outlook for this dynamic field in the future.

Introduction

Controlled human infections (CHI), through the transfer of body fluid such as serum,[1] respiratory secretions[2] or faecal filtrates[3] laid the foundation for infectious disease research starting in the 17th century. Unparalleled human experimentation led to the identification of causative organisms (norovirus,[3] influenza,[2] dengue,[4] sarcocystis[5]), not only proving Koch's postulates, but also providing an opportunity to study incubation periods and clinical disease. Important discoveries were made through CHI trials, such as the identification of toxins as causative agents in diarrhoeal disease following instillation of a filtrate of *Vibrio cholera* culture broth in volunteers in 1966.[6] Whilst the ethical circumstances of these initial studies were often questionable, the realization that they provided a core platform for the study of infectious diseases, has resulted in the increased use of CHI models in the past decades. As CHI models have moved into the 20th century, ethical frameworks have been developed and rigorous independent review of risks and benefits of the study are carried out. The aims of the studies involving CHI have shifted from exploratory and descriptive studies to trials that take a central position in the vaccine and drug development pipeline. [7] CHI trials often act as a gatekeeper for proceeding to field efficacy trials, although in exceptional cases, may be accepted as proof-of-efficacy in Phase 3 clinical development.[8] The development of novel CHI models and the exploitation of existing CHI models in the product development pipeline has been propelled recently by the investments of large funders such as the Bill and Melinda Gates Foundation, the Wellcome Trust and the UK Medical Research Council. CHI studies, by allowing preliminary efficacy testing amongst 10-100 participants, are cheaper compared to phase 2 and 3 clinical trials in endemic areas with samples sized ranging from hundreds to 100,000 participants. In addition, CHI studies allow for a large number of candidate products to be tested, increasing the chances of identifying highly potent products and minimizing the risk of late clinical failure and reducing exposure of (vulnerable) populations to inactive interventions (figure 1). The resulting diversity in CHI models is impressive, with considerable heterogeneity in inoculum, endpoint and clinical procedures (table 1). In CHI trials biomarkers, protective responses and mechanisms of disease can be studied more precisely, which ultimately feed back into the product development pipeline to improve the next generation of medicines. Novel technological advances such as –omics tools are applied to identify risk factors such as diet, microbiome, co-infections or genetic background in complex multiparametric analysis. Pathogens are altered by genetic modification in order to identify key virulence genes (NCT03067961) or provide less virulent challenge inocula which will allow for clinically less severe CHI models.[9] In this review, we provide an overview of the active CHI models, discuss their contribution to biomedical science and risk some predictions of what can be expected in this dynamic field in the future.

Table 1. Summary of characteristics per CHI model based on published data. Most commonly used strains are reported, number of volunteers is estimated from publications.

Pathogen	Route	Dose	Strain	End points	Est. # volunteers	In/outpatient/ isolation	Ref
Rhinovirus intranasal		10.000 TCID50	HRV-16, HRV-39	viral replication, clinical symptoms	5760	outpatient	[10]
Influenza virus	intranasal	10^3-10^7 TCID50	*	viral shedding in nasal lavage, clinical symptoms	3540	in patient quarantine	[2]
Plasmodium falciparum	mosquitos, intravenous	5 mosquitos, 3200pfSPZ	NF135.10, NF54	parasitemia	2650	outpatient	[11 12]
ETEC	oral	≥ 5x10^8 CFU	B7A, H10407, E24377A	diarrhoea	1215	outpatient	[13]
Vibrio cholerae	oral	10^5 CFU	El Tor Inaba N16961, O139	diarrhoea	1210	inpatient	[8 14]
***Salmonella* Typhi**	oral	1-5x10^4 CFU	Quailes	fever or bacteraemia	1000	outpatient	[15]
Respiratory syncytial virus	intranasal	4log10 PFU/ml	M37, A2	viral load in nasal lavage, respiratory symptoms	1000	in patient quarantine	[16]
Shigella spp	oral	10 CFU – 10^{10} CFU	*S. flexneri* 2457T, *S. sonnei* 53G	diarrhoea, antibody response	850	inpatient	[17]
Norovirus	oral	48 RT-PCR U	8FIIa, GI.1, GII.4	gastro-enteritis, PCR faeces, ELISA	810	inpatient	[18 19]
Lactobacillus spp	oral, vaginal	oraal 10^9 CFU 1dd, 7.5x10^8 CFU subs	*L. rhamnosis* GR-1, *L. reuteri* RC-14, *L. crispatus* CTV05	clinical UTI	800	outpatient	[20]

Table 1. Summary of characteristics per CHI model based on published data. Most commonly used strains are reported, number of volunteers is estimated from publications. *(continued)*

Pathogen	Route	Dose	Strain	End points	Est. # volunteers	In/outpatient/ isolation	Ref
Streptococcus pneumoniae	intranasal	10^5-10^6 CFU	6B, 23F	colonisation	790	outpatient	[21]
Haemophilus ducreyi	Intraepidermal and intradermal	10-150 CFU	35000HP	pustule formation	550	outpatient	[22]
Dengue virus	subcutaneously	10^3 PFU	DEN2Δ30	viremia, rash, neutropenia	520	outpatient/ inpatient	[4]
Francisella tularensis	aerosol	10^4-10^8 organisms	SCHU S4	systemic symptoms	500	inpatient	[23]
Neisseria lactamica	intranasal	10^4 CFU	Y92-1009	colonisation	310	outpatient	[24]
Plasmodium vivax	mosquitos, intravenous	5 mosquitos, 3200 pFSPZ	wild-type	parasitemia	300	outpatient	[25]
Campylobacter jejuni	oral	10^6-10^9 CFU	initially 81-176, now CG8421	diarrhoea	260	inpatient	[26]
Cryptosporidium spp	oral	10-10^5 oocysts	**	stool oocysts	260	outpatient	[27 28]
Necator americanus	Transdermal	10-50L3 larvae	Papua New Guinea	Eggs in stool	250	outpatient	[29]
***Escherichia coli* (UTI)**	Urethral catheter	10^5-10^6/ml	83792, HU2117	clinical UTI	200	outpatient	[30]
BCG	intradermal	1-4x10^5 CFU	BCG	immune response	140	outpatient	[31]

Table 1. Summary of characteristics per CHI model based on published data. Most commonly used strains are reported, number of volunteers is estimated from publications. *(continued)*

Pathogen	Route	Dose	Strain	End points	Est. # volunteers	In/outpatient/ isolation	Ref
Neisseria gonorrhoea	urethral catheter	$1{,}8 \times 10^3$ Ms11mkC, 1.0×10^5 FA1090	FA1090, MS11mkC	colonisation	140	outpatient	[32]
Giardia lamblia	oral	$5\text{-}10^4$ trophozoites	GS-M83/85	cysts in stool, antibody response	120	inpatient	[33]
Helicobacter pylori	oral	10^4 CFU	Baylor 100	urea breath test, histology	80	outpatient	[34]
***Salmonella* Paratyphi**	oral	$1\text{-}5 \times 10^3$ CFU	NVGH308 strain	fever or bacteraemia	40	outpatient	[35]
Parvovirus B19	nasal	Up to 5^{10} viral genomes	Wild-type	viremia	12	Inpatient isolation	[36]

ETEC= Enterotoxicogenic Escherichia choli, Salmonella Typhi/Paratyphi = *Salmonella enterica* subsp. *enterica* serovar Typhi
* A/Texas/39/91 (H1N1), A/California/2009 (H1N1), A/Winsonsin/67/2005 (H3N2)
** *C. muris*: RN66, *C. meleagridis*: TU1867, *C. hominis*: Iowa strain, *C. parvum*: Iowa strain

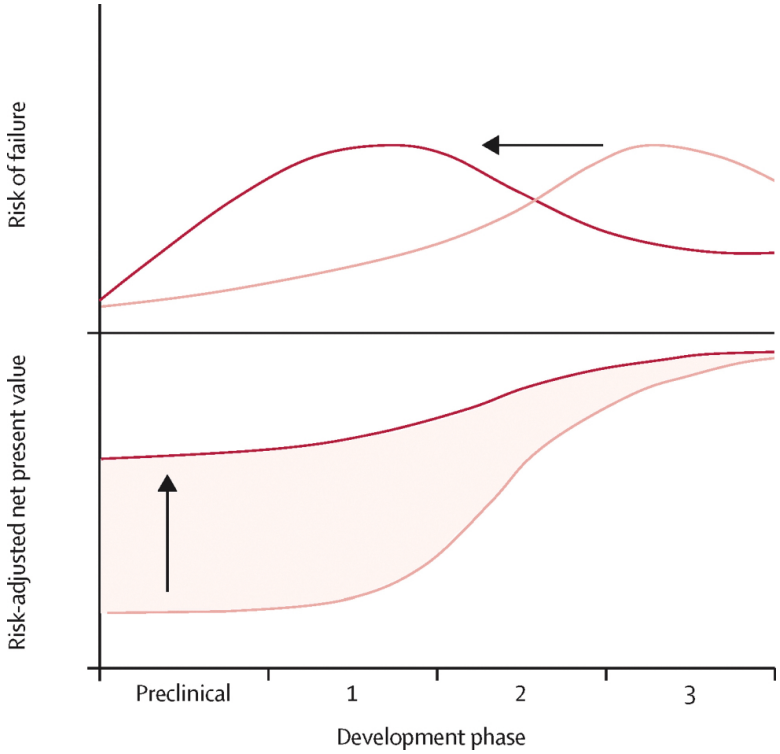

Figure 1. Graphic representation of the risk of failure and the risk-adjusted net present value of a product before (light red) and after (dark red) introduction of a controlled human infection (CHI) model

The CHI model will increase the risk of failure in the early stage of clinical development, but reduce it at a later stage. Because the risk distribution shifts towards higher risks in the early stages of development, the risk-adjusted net present value of the product will increase. As such, the increased initial investments in the CHI models are returned through increased risk-adjusted net present value.

Ethical considerations

The son of Edward Jenner's gardener has become the historic symbol of CHI when he was inoculated with cowpox in 1796. Other famous examples are infection of Macdonalds' children with pertussis, mentally retarded children with hepatitis virus at Willowbrook State school in New York[37] and malaria infections in Nazi Germany.[38] Following these experiments, the Nuremberg Code (1946) and later the Declaration of Helsinki (1964) provided guidelines for the conduct of medical research involving humans including informed consent procedures. CHI studies raise ethical debate because they seemingly breach the "do no harm" principle. However, the purpose of the CHI trial is not to do harm but to ultimately benefit global health.[40] Nonetheless, CHI trials inherently carry a risk for participants. They

can only be performed in treatable or self-limiting diseases where no irreversible pathology occurs.[39] The risk of a serious adverse event (SAE) should be assessed independently from the risk of discomfort. CHI studies may target a certain degree of discomfort (e.g. cholera, malaria, typhoid), but this may not necessarily be serious. In essence, the ethical principles in CHI are similar to those applied in phase I trials where healthy volunteers put themselves at risk without the possibility of deriving direct benefit. [39 40 41] Justification for these trials lies in the potential value of the foreseeable knowledge for society. The degree of risk which is believed acceptable thus depends on the benefits. [34] Formal limits to these risks have not been established and some argue that they should be equal to the risks people would normally take in many areas of life.[40] CHI trials may raise debate on the appropriate compensation of the trial subject, protecting public confidence and the risk of spread of infections, an ethical framework for which is provided by Bambery et al.[42] Using quarantine to minimizing the risk of spread of challenge agents should be carefully considered as it substantially increases discomfort to the trial subjects as well as costs of the trial.

Considering the body of literature on CHI, SAEs seem to be rarely reported. In influenza and malaria CHI, four possibly related SAEs have been reported in an estimated 6000 volunteers. An episode of elevated serum transaminase and dilated cardiomyopathy was recorded in influenza CHI,[43 44] while two cardiac SAEs were reported in a *Plasmodium falciparum* CHI trial.[45 46] The latter episodes might have been myocarditis, an known immunological complication in vaccinology.[47] *P. vivax* CHI experienced a set-back when malaria relapses occurred in two volunteers, due to a previously unrecognized genetic polymorphism that hinders bioactivation of the curative drug primaquine.[48] However, five years of follow-up showed that none had further relapses.[49]

The dynamic scientific context of CHI trials continues to fuel regulatory and ethical discussions. Current ethical debate involves the use of genetically modified organisms (GMOs) and in particular its containment, as well as the use of CHI in populations with increased risks or resource poor environments. For example, pneumococcus colonisation has been performed in elderly and asthmatics (DF, personal comm.), whereas rhinovirus CHI has been performed in mild-to-moderate asthma and COPD patients. [10 50] In this case, the resulting rhinovirus infection was well tolerated despite enhanced respiratory symptoms and secondary bacterial infections requiring increased vigilance.[51]

The transfer of CHI studies to areas where infections are endemic (e.g. malaria, typhoid, pneumococcal disease), will raise specific ethical issues such as cultural acceptance, appropriate remuneration and consent procedures. A recent workshop in Blantyre, Malawi addressed these issues.[52] The ability to study the infection in a population with different disease incidence, co-infections, environmental exposures, nutrition status and immune responses has obvious benefits for the product development pipeline. Needless to say, thorough capacity building of infrastructure, clinical expertise, institutional review boards, pharmacists and ethicists will be needed.

CHI in product development

The contribution of CHI studies to the development of novel vaccines has been championed by the Live Oral Cholera Vaccine CVD 103-HgR study in 197 healthy volunteers.[8] CVD 103-HgR was licenced in several countries since 1993, but only recently in the US.[53] Volunteers were challenged by ingestion of wild-type *V. cholera* and were monitored for the occurrence of moderate or severe diarrhoea. The vaccine showed 90% efficacy, which, together with a good safety profile, led to licensure by the FDA.[8] CHI models have accelerated the development of vaccines or drugs for a number of infections and are increasingly being used as proof in principle by product developers and as gatekeepers for further investment by funders (figure 1). CHI trials are generally small, including between 20-100 volunteers (figure 2B). Influenza, rhinovirus and malaria are the most practiced CHI trials (figure 2A+C).

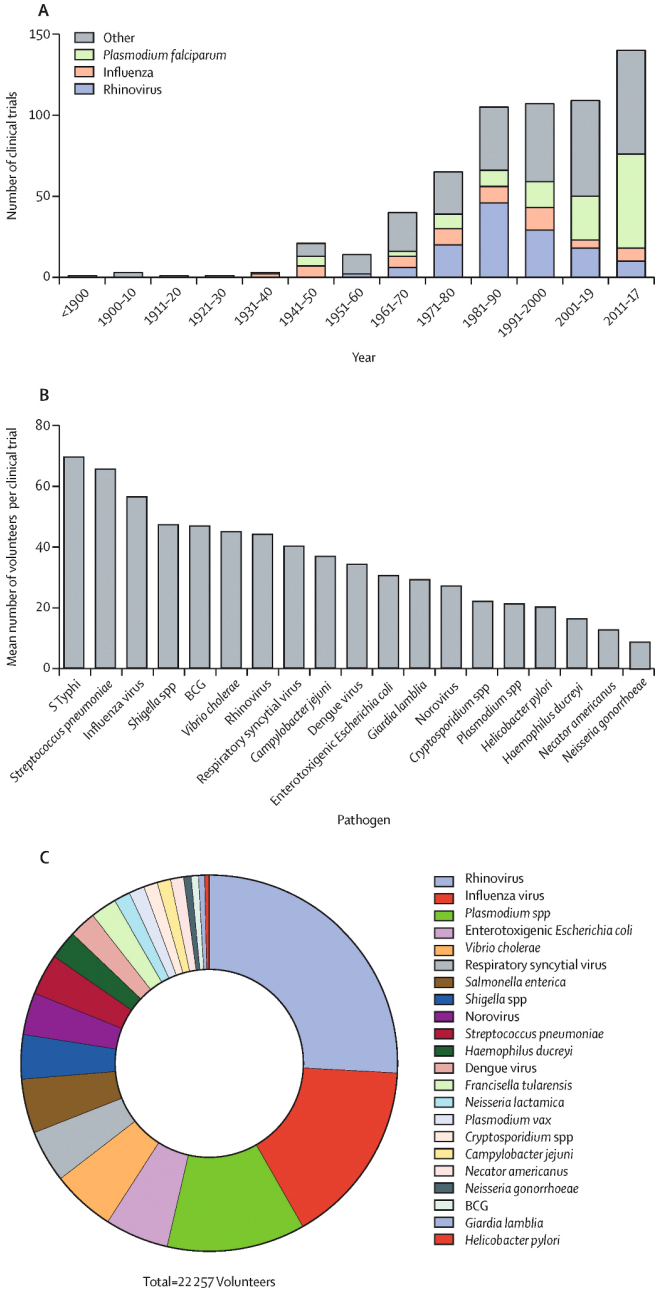

Figure 2. Numbers of volunteers in controlled human infection (CHI) trials
(A) Estimated number of CHI trials reported per decade for rhinovirus (blue), influenza (red), Plasmodium falciparum (green), and other infections (grey). (B) Estimated mean number of volunteers per trial for CHI models with different pathogens. (C) Estimated cumulative number of volunteers previously experimentally infected in a CHI trial per pathogen reported in the literature from 1900. S Typhi=Salmonella enterica serotype Typhi.

An important milestone achieved through CHI has been the licensure of the world's first malaria vaccine based on the subunit circumsporozoite protein (CSP) from *Plasmodium falciparum (Pf)*, which has recently gained EMA approval.[54] Pivotal proof of concept data for this vaccine were generated almost 10 years ago in a series of CHI trials showing cumulatively ~40% protective efficacy after challenge with *Pf* in adults.[55] The CHI results were confirmed in a phase 3 trial, which showed a similar partial (~30%) efficacy in children in Africa.[56] Malaria CHI also proved to be instrumental in identifying candidates with poor efficacy, saving time and efforts by halting their clinical development.[57]

In the field of malaria, CHI has been driven by the development of continuous *in vitro* culture techniques for *P. falciparum* and the rearing of laboratory-infected Anopheles mosquitoes.[58] [59] The salivary-gland parasites were attenuated by radiation and used to inoculate replication-deficient parasites into volunteers. Ground breaking results showed that full protective immunity to *Pf* could be induced by exposing volunteers to the bites of >1000 of these mosquitoes carrying radiation attenuated parasites.[60] The next level in technological advance was the ability to produce aseptic, purified, cryopreserved sporozoites.[61] This work has formed the foundation for the clinical development program of the live attenuated malaria vaccine (PfSPZ Vaccine), which is now given by intravenous injection of radiation-attenuated extracted, purified and cryopreserved sporozoites.[62] In parallel, an even more potent vaccine has been developed based on the exposure of volunteers to live sporozoites under chloroquine prophylaxis (chemoattenuation) resulting in sterile immunity or 100% protection.[63] [64]

The genetic diversity of the malaria parasite poses a major obstacle for vaccine and drug development. The availability of genetically diverse strains of *Pf* for CHI allows for an accelerated assessment of potential cross-strain immunity.[12] [65] In addition, the availability of clinical grade blood stage parasites[66] or purified, vialed and cryopreserved sporozoites for injection (PfSPZ Challenge, Sanaria Inc.)[11] means that the malaria CHI model no longer relies on the production of mosquitoes at the clinical site. This facilitated the transfer of the malaria CHI model to novel sites, an important step to enable phase 2a trials in endemic areas.[67] [68] To increase the array of available strains, controlled production of infected mosquitoes is currently being set up in several centres in Africa.

In malaria drug development, CHI provided the first proof for efficacy of "old" antimalarial drugs such as paludrine[69] but also novel antimalarials such as atovaquone/proguanil[70], ferroquine,[71] artefenomel,[72] griseofulvin,[73] or more recently DSM265.[74] [75] With the advent of molecular methods for detection of parasites as low as 5-20 per mL blood[76] it is possible to carefully dissect parasite growth rates to determine drug and vaccine mechanisms of action. Recently, treatment with piperaquine during CHI blood stage malaria was shown to induce gametocytes, potentially adding a sexual stage *Pf* CHI model to the current portfolio.[77] This may be an important platform to accelerate the development of transmission blocking vaccines, recently identified as a priority in the Malaria Vaccine Technology Roadmap.[78]

The other frequently used model has been controlled influenza infection. Influenza CHI enabled the clinical testing of the first generation of influenza vaccines, which were based on infected and formalin inactivated allantoic fluid.[79] Later, CHI trials led to the first registration of a live attenuated vaccine for influenza A.[80] Immunological analysis showed that pre-inoculation hemagglutinin inhibition titre and particularly neuraminidase inhibition titres in healthy, unvaccinated volunteers, as well as pre-challenge CD4+ T-cell responses (not CD8+ cells) predict clinical outcome after CHI.[81 82] Building upon these immunological findings, a human monoclonal antibody targeting an influenza conserved epitope,[83] a trivalent DNA vaccine[84] and a viral vectored vaccine against conserved influenza antigens[85] were all proven efficacious in influenza CHI, providing hopeful prospects for the development of cross-strain and long-lasting influenza vaccines.

Influenza CHI have also played a central role in the FDA registration of the first influenza antiviral drug amantadine in 1966.[86] Thereafter, studies showed efficacy of the amantadine analogue rimantadine[87] (FDA approved in 1994) and a range of antivirals such as zanamivir, oseltamivir and preamivir.[44, 88-90] Influenza CHI is now applied to study the development of strains resistant to these novel antivirals as people are treated with increasing concentrations of the drug.[91] In terms of respiratory infections, CHI has facilitated the development of drugs for other respiratory infections, such as respiratory syncytial virus.[92]

Impressive progress has been booked in dengue vaccine development. The early down-selection of dengue vaccine candidates is imperative because antibody dependent enhancement of viral replication may pose vaccinees at risk of more severe disease, as was seen in a phase 3 study.[93] Recently, a live attenuated recombinant dengue vaccine (TV0003) showed complete protection in CHI[94] and is now undergoing phase 3 evaluation in Brazil and Thailand (NCT02406729, NCT02332733). Remarkably, the dengue CHI model was developed as a result of the live attenuated dengue vaccine programme, where an insufficiently attenuated dengue strain (rDEN2Δ30) failed as vaccine candidate because it led to viremia and rash, this provided an opportunity for use as CHI.[4]

CHI models have also been instrumental for a number of gastrointestinal infections. For example, the most advanced Norwalk virus vaccine, an intranasal VLP formulation, proved to be efficacious in two separate CHI studies.[18 95] In *Salmonella enterica* serovar Typhi research, CHI allowed for the early benchmarking of novel vaccine candidates against the licenced Ty21a oral live attenuated vaccine.[96] In enterotoxicogenic *E. coli* studies, the therapeutic effect of trimethoprim-sulfamethoxazole was first documented in CHI studies.[97] Multiple prophylactic and therapeutic medicines have been tested in the ETEC CHI model, in which bismuth subsalicylate and an oral colicin E2 treated whole-cell vaccine, showed potential as effective prophylactics. [98 99]

Despite the fact that CHI studies take a central role in the clinical development pipeline, formal statements of regulators are needed to endorse such trials to support the use of

CHI by developers on the path to licensure. Last year, the WHO produced a statement on the regulatory considerations for the use of controlled human infection trials for vaccine development.[100] Such position statements can also highlight the limitation of CHI studies. For example, inoculation routes may differ between natural infections and CHI trials,[2] the trial population may not be similar to the population at risk, challenge strains may differ from natural infections, protective immune mechanism may not be universally applicable and the selection of susceptible adults without pre-existing immunity might reflect intrinsic vulnerability which may not hold true for the whole population. Despite these differences, the results obtained in CHI trials are generally confirmed in phase 2 efficacy trials. To our knowledge, there is no example of a vaccine or drug which failed in CHI and was found efficacious in later phase 2 or 3 field trials.

The predictive value and reproducibility of CHI studies is highly dependent on the quality of the challenge material. The regulatory requirements for the production of this material may vary in different continents. Current regulatory environments have shown that increased control may not always be beneficial to the CHI model, which should preferably remain low cost and be flexible to changes in circulating strains (e.g. influenza) in order to be clinically relevant. Therefore, consistent unifying quality control and assurance measures for challenge material are needed in order to balance safety and costs of production.

Novel CHI models in poverty-related and neglected diseases

Because of the potential to reduce costs and time to registration, CHI models are particularly appealing for the development of products for resource-poor countries where infectious diseases are still responsible for considerable morbidity and mortality. Among the infections that fall in this category, aside from malaria, *Mycobacterium tuberculosis* (MTB) would be obvious. A MTB CHI model could provide a critical platform for the downselection of potential novel antibiotics as well as vaccine candidates. CHI studies with MTB are problematic because diagnosis is not straightforward, there is a potential of spread and treatment is associated with a significant side-effects. As a replacement for MTB infection, intradermal injections with the vaccine bacille Calmette-Guérin (BCG, attenuated *Mycobacterium bovis*) are tested as a surrogate CHI, followed by a punch biopsy 14 days after injection to investigate bacterial persistence and immune parameters.[101] The low recovery of bacteria after CHI limits sensitivity of the model.[102] However, prior vaccination with BCG did result in a decreased recovery rate of BCG after challenge, suggesting that the model is able to reveal protective immune effects.[31] Besides the nature of the pathogen, another important limitation of the model is the dermal inoculation route as opposed to the natural inhalation of mycobacteria and therefore administration of aerosolized BCG or other (attenuated) mycobacterial strains are currently being investigated.[135]

Hookworm infections are one of the most prevalent neglected diseases for which only very limited number of anthelminthic drugs are available.[104] These drugs are widely used and the

concern for the development of resistance is growing. High reinfection rates indicate that vaccines with long-term action are needed to effectively control or eventually eliminate these parasites.[105] A number of vaccine candidates are undergoing early clinical testing.[106] *Necator americanus* hookworm CHI can potentially contribute to go-no-go decisions for these vaccines. As animal models are lacking, hookworm larvae are cultured from faeces of chronically infected donors extensively screened for transmissible diseases such as HIV, HBV and HCV. Hookworm CHI has been performed in ~250 volunteers in order to test whether hookworms, through induction of regulatory responses, can have therapeutic effects on inflammatory diseases such as celiac disease, IBD and allergic rhinosinusitis.[29, 107-109] Standardising the model, through achieving a stable egg output to serve as a reliable quantitative endpoint for vaccine efficacy testing, is the focus of current efforts and will prepare the model for evaluating candidate vaccine efficacy (NCT01940757).

Following the example of hookworm, CHI models are being developed for two other parasitic diseases of global importance: schistosomiasis and cryptosporidium. Because parasites generally have a complex life cycle and may depend on multiple hosts for their maturation and development, the production of challenge material in compliance with all regulatory norms can be a daunting task.

For *Schistosoma mansoni* an important conceptual step to ensure safety of CHI volunteers has been the propagation of single sex cercariae which can infect humans and mature to adult stage without mating. In single sex infections no eggs are produced, circumventing the pathology associated with chronic schistosomiasis caused by egg-induced granuloma formation and fibrosis. A highly sensitive diagnostic test based on circulating anodic antigen was crucial for the development of the model as it allows for accurate quantification of worm loads despite the lack of eggs.[110] The first results of a *Schistosoma mansoni* CHI is expected soon (NCT02755324). The model will be suitable for testing new drugs and currently available vaccines such as Sm14, TSP2 and Smp80.[111] However, anti-fecundity vaccines or drugs that target egg laying, cannot be tested in these single sex infections.

A recent evaluation of the causes of moderate-severe diarrhoea in children <2 years of age revealed Cryptosporidium as being the second or third leading pathogen. It is associated with malnutrition and enteropathy.[112] This is why recent efforts have been put into reviving the pre-existing cryptosporidium CHI model[27] to comply with 21st century regulations and serve the vaccine and drug development pipeline. Unfortunately, cryptosporidium cannot be cultured *in vitro* and is difficult to maintain in animal models. *C. hominis* cysts can only be produced by infection of gnotobiotic neonatal piglets. Considerable investments are now underway to allow purification of this material. However, culture of cysts without the need of animal models would be an important step forward for this model.

Colonisation models

Culture-independent technologies have revealed the diversity of the human microbiome.[113] In an era of increasing antimicrobial resistance, the study of controlled colonisation in healthy volunteers has proven to be instrumental to dissect the dynamics of mucosal carriage of bacteria which precedes invasive bacterial infections. With regards to controlled colonisation, the upper respiratory tract microbiome has been the most studied.

The most frequently used model for colonisation is nasal instillation of *S. pneumoniae*, which leads to a roughly 50% colonization in healthy volunteers and lasts 2-5 weeks as confirmed by nasal washes.[21, 114 115] Interestingly, invasive pneumococcal disease has never been reported in studies of more than 800 inoculations performed so far. Despite the lack of clinical invasive disease, the model successfully predicted the efficacy of the 13-valent conjugate vaccine.[116] This has paved the way for the use of the *S. pneumoniae* colonization model in testing new protein-based vaccines (NCT02116998). The model has also been instrumental in studying natural protection and the dynamics of the nasal microbiome. [117-119] Strain specific immunity was induced by the controlled colonisation procedure, which was illustrated by a second challenge of volunteers with the same pneumococcal strain after 11 months.[120] Analysing the immune responses indicated that high levels of memory B cells and antibodies directed to capsular pneumococcal polysaccharide seem to be key to protection against pneumococcal acquistion.[120 121] Through the use of this model it was also possible to show that asymptomatic upper respiratory viral infections increase the risk of becoming colonized.[123] The effect of the paediatric live attenuated influenza vaccine on pneumococcal carriage is subject of an ongoing controlled colonisation trial in adults (ISRCTN16993271). Nasal mucosa and lung investigations during this co-infection study might provide important insights into how influenza predisposes to secondary pneumococcus infections and lead to better interventions. These studies have also directly assessed for the first time the impact of a viral vaccine on an entirely unrelated human pathogen and have highlighted the need to consider off-target beneficial (or detrimental) effects of vaccines on other important human pathogens. Given the scientific advances and the favourable safety profile of the pneumococcal colonization model, the model has been expanded to explore the susceptibility of at-risk populations including people with asthma (ISRCTN16755478) and the elderly (ISRCTN10948363).

In analogy to colonisation with pathogenic bacteria, it has been possible to deliberately colonize volunteers with non-pathogenic bacteria to investigate the effects on pathogen carriage.[24 30] As an example, intranasal *N. lactamica* colonisation protects from colonisation by *N. meningitides.* The efficacy of this approach was shown to be superior to the commonly used quadrivalent ACWY glycoconjugate vaccine.[24] Similarly, active colonisation of the bladder with non-pathogenic *E. coli* prevents urinary tract infections (UTI) in patients with recurrent UTIs.[124-126] These trials were designed based on studies demonstrating that untreated asymptomatic bacteriuria prevents symptomatic urinary tract infection in young

girls.[127] The concept was further extended to vaginal instillation of lactobacilli in women with recurrent UTI, but results are much less convincing when compared to the *E. coli* colonisation model of the bladder.[20]

More recent efforts include the development of colonization models with Nontypable *Haemophilus influenzae* and *Bordetella pertussis.* The *H. influenzae* model uses a challenge strain genetically modified to be streptomycin resistant which allows the investigators to recover the organism from the nasopharyngeal samples.[128] The pertussis model aims at achieving colonisation in 70% of the exposed volunteers without causing disease. The colonisation period, shedding and exploratory immunology are being assessed during a 17-day inpatient stay and follow-up over 1 year.[129] Very similarly, efforts to develop a Group A *Streptococcus pyogenes* controlled infection model are being undertaken. Because this models is aimed at inducing pharyngitis, it is formally not a model of colonisation but of disease.[130]

Colonisation models in principle do not reflect the pathophysiology of invasive disease, the colonisation phase is increasingly recognised as an important target for vaccination. For example in pneumococcus, colonisation drives transmission primarily amongst children. Protection against colonization, the reservoir of bacteria in the population, interrupts transmission of bacteria and provides herd protection against the disease. Despite their non-invasive design, the risk of invasive disease in these colonisation models cannot be completely averted. Controlled colonisation and infection models share many similarities such as the preparation of challenge material, inoculation routes and ethical considerations including the risk of dissemination of the challenge strain.

Scientific advances

CHI trials offer unprecedented opportunities to study host-pathogen interaction by taking multiple longitudinal samples before, during and after infection. Profiling immune parameters and linking those to the clinical outcome has shown to be extremely valuable in assessing correlates of protection. The availability of these validated correlates of protection will accelerate the development of novel vaccines by providing an easier surrogate endpoint for phase 2 field trials. In addition, they may guide the refinement of vaccine products, for example through rational selection of adjuvants which are known skew the immune response towards the preferred correlate. Also, the comparison of different populations in controlled infection trials may provide insight into population heterogeneity, which may impact the overall efficacy of the product when distributed widely in the target populations. In addition, studying the biology of CHI trials may lead to the discovery of novel vaccine targets. For example, in CHI models for salmonella,[131] shigella,[132] and norovirus[133] antibodies were identified which, if present at baseline, correlated with protection from the challenge. Potentially, these antibodies could provide clues for novel monoclonal antibody-based therapy or lead to the identification of functional antigens. Similarly, a study of peripheral

mononuclear blood cells of volunteers in a rhinovirus CHI study using MHC class II tetramers has led to the identification of specific memory T-cell populations that rapidly respond to infection and target conserved epitopes of the rhinovirus capsid proteins.[134] These epitopes will be subject of further research into their potential use as novel peptide vaccines.

Repeated CHI in the same individuals contributes to understanding the induction of natural immunity. A gradual decrease in the number of people reaching the endpoint is generally a sign of slowly acquired natural immunity, as was seen for shigella,[17] BCG,[31] cholera,[135] norovirus,[136] pneumococcus,[120] and enterotoxigenic E. coli.[137] This was not the case in RSV CHI, where previously protected individuals can again be susceptible after the next inoculation, indicating that naturally acquired immunity in RSV is transient.[138] Dissection of the humoral responses in these subjects revealed a defect in virus-specific IgA memory B-cells.[139] In *H. ducreyi* re-infection, a subgroup of participants maintains consistent susceptibility to clinical disease (pustule formation), whereas others do not. Probably, host factors such as gender, genetic background, immune responses and skin microbiome play a central role.[22 139]

The importance of host factors was also shown convincingly in other models. For example, individuals with an O blood group have increased susceptibility for norovirus while those with a B histo-blood group show a decreased risk of infection.[140] Interestingly, in infection with V. cholera volunteers with O blood group also suffered from more severe symptoms.[141] Because blood group and other related carbohydrate antigens are highly expressed on gut epithelial cells, their involvement in viral or bacterial docking is suspected. Indeed the H type-1 oligosaccharide ligand (a member of the ABH blood group family) was found to be critical for Norwalk virus binding.[140]

In order to fully understand the complex interplay between genetic background, diet, microbiome, co-infections and previous exposure in determining clinical outcome after CHI, comprehensive system biology approaches are required. Recently orthogonal datasets including transcriptomics, immunologic parameters as well as metabolomics signatures to zostavax, a live attenuated vaccine, were integrated and showed reactivation of networks that are tightly coupled with T- and B-cell responses.[142] Interestingly, such network analysis generated novel insights into the endocrine system as well as metabolomics playing a role in vaccine responses. These tools can now be applied to CHI in the context of both vaccine and drug development.

Future challenges and opportunities

The increasing costs for clinical development of novel drugs and vaccines for infectious diseases calls for tools to select those candidates with highest probability of success. The concept of “fast failure”, in which there is an early stop for the development of unsuccessful candidates is extremely important as it will allow reallocation of resources. CHI studies

may be used as model for phase 2 clinical efficacy. As such they may reduce development risk, lower overall costs and increase risk-adjusted net present value. Especially in poverty-related infectious disease research, cost-effective development of novel interventions is imperative. Despite the advantages of CHI in clinical development, these studies also have disadvantages. Particularly the use of a "surrogate" inoculum or volunteers which are much different from the target population poses significant limitations. As such, CHI will not resolve all problems in clinical development but, whenever possible, should be put to use as an accelerating tool.

Because of their often invasive nature and the use of healthy volunteers CHI trials continue to raise ethical debate amongst public, Institutional Review Boards (IRBs) and investigators.[143] The fear of long-term adverse effects such as reactive arthritis or post-infectious irritable bowel syndrome in Shigella or enterotoxicogenic *E. coli* infections are well known examples.[17] Quantitative risk data is needed to facilitate objective risk assessments, which need to be tailored to individual models and research targets. Currently, the lack of standardized reporting of adverse events and in particular serious adverse events as well as inoculation route, dose and timing of events hamper the meta-analysis of available safety data. Similarly, data on possible spread or secondary infections with challenge strain should be made publicly available to indicate the need for quarantine of subjects with gastrointestinal or respiratory infections. Easily accessible standardized safety data on CHI studies will also facilitate the evidence-based establishment or adjustment of CHI regulations and increase the expertise of IRBs in this domain.

A major hurdle in the development of novel CHI models is often the production of challenge inoculum compliant with regulations, which may be difficult, expensive and time consuming. In addition, regulatory requirements may vary across different continents. Public-private partnerships, funders and consortia of CHI researchers should share the responsibility for investing in sustainable, widely available, well-characterized master banks of this material and define the quality control assays that are believed to be essential for volunteers safety. In addition, the open sharing of knowledge and infrastructure would support best practices and provide a knowledge base for CHI model transfer and capacity building.

In conclusion, CHI models are emerging as powerful tools to down select promising new vaccines or drugs on their increasingly complex and expensive path towards licensure. Despite their invaluable contribution to science and product development, the demanding nature of CHI trials and risks involved requires careful risk-benefit assessments in which the safety of participants should be a primary concern at all times.

Search strategy and selection criteria

References were identified in PubMed using the search terms ("experimental infection*" OR "human challenge" OR "challenge study" OR "challenge model" OR "human infection"

OR “infection model” OR “volunteer study” OR “infection in volunteers” OR “volunteer challenge” OR “controlled human infection”), separately combined with each pathogen listed in the table. For each pathogen the Mesh-term was combined with an [All Fields] search of common synonyms. We searched for articles published between Jan 1, 1900 and 1 October 2017. Only articles in English, French or Dutch were reviewed. The references of reviews and key publications were searched to identify any other references. Only studies using pathogens to experimentally infect humans were included. Studies using an attenuated pathogen for the sole purpose of vaccination were not included in the estimation of total volunteer numbers. Articles were the total number of volunteers in the study could not be identified were not included in estimate of total volunteer numbers but were included in the estimation of total studies.

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Chapter 3

New insights into the kinetics and variability of egg excretion in controlled human hookworm infections

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Abstract

Four healthy volunteers were infected with 50 *Necator americanus* infective larvae (L3) in a controlled human hookworm infection trial and followed for 52 weeks. Kinetics of fecal egg counts in volunteers was assessed with Bayesian multi-level analysis, revealing an increase between weeks 7 and 13 followed by a plateau at about 1000 eggs per gram feces. Variation in egg counts was minimal between same day measurements but varied considerably between days, particularly during the plateau phase. These analyses pave the way for the controlled human hookworm model to accelerate drug- and vaccine efficacy studies.

Introduction

Hookworms affect almost 500 million people worldwide, predominantly in developing countries. Pathology is caused by blood- and protein loss at the site of intestinal attachment, particularly in individuals with low iron and protein stores such as children or women of childbearing age. [1]

Currently, hookworm elimination rates fall behind those of other soil-transmitted helminths[2], especially in high prevalence areas. High re-infection rates and the exclusion of adult populations in mass drug administration (MDA) programmes targeting school-age children, hamper the goal to interrupt transmission.[3] A vaccine would be a crucial tool to aid current hookworm control programmes. Two vaccine candidates are in phase 1 clinical testing in volunteers in Gabon, Brazil and the United States.[4] However, testing of efficacy in field trials is a large-scale, costly endeavour, slowing down vaccine development. [5]

Efficacy in vaccine trials can be estimated using a binary outcome, however quantitative measures are preferable. This relies on fecal egg counting, a widely used way of measuring infection intensity in humans. [6] However, in field settings, fecal egg counts are highly variable due to differences in the host immune response, dietary intake, episodes of diarrhoea, transport and storage of samples, laboratory conditions, availability of highly trained technicians and other technical factors, thereby limiting the power of field trials to detect vaccine efficacy. [7]

The development and clinical implementation of hookworm vaccines could be accelerated by the implementation of controlled human hookworm infection (CHHI) models within the product pipeline, as has been shown for malaria and influenza vaccines.[8] The CHHI model has been applied for immunomodulatory purposes in for example celiac disease [9] using doses of 10 or 20 L3 larvae. A recent trial by Diemert *et al.* aimed at developing a model for vaccine testing showed that infection with 50 L3 larvae resulted in patent infection in 9 out of 10 volunteers and was well tolerated.[10] However, egg counts were lower than typically seen in field studies. [11] To obtain a better comparison with the field, higher egg counts would be preferable. Furthermore, an improved understanding of the kinetics and variability of egg excretion over a prolonged period of time could help dissect factors underlying variability of egg output. As a result, the most reliable time points with lowest variability can be identified and the power of vaccine trials can be improved.

In this study we investigated the kinetics of egg excretion over an extended time period in a CHHI model using an infective dose of 50 L3 larvae. Patterns and variability in egg excretion were quantified using Bayesian multi-level analysis, based on which recommendations for the improved use of CHHI for testing of novel vaccines or medicine are proposed.

Methods

Necator americanus (Na) L3 larvae were produced according to the principles of Good Manufacturing Practice (GMP) guidelines, but not in a GMP-licensed cleanroom. Material for culture was obtained from a chronically infected donor from James Cook University,[9] carrying a *Na* strain originating from Madang, Papua New Guinea previously maintained at the University of Nottingham. The donor was confirmed negative for blood borne infections (HIV, Hepatitis B and C) and feces was screened for any viral, bacterial and parasitic pathogens. The feces containing *Na* eggs was cultured for 7 days after which larvae were harvested.[12] Water containing the infectious Na L3 larvae was cultured for pathogenic bacteria, identity of the infectious larvae was confirmed by PCR. Viability of the larvae, as measured by a visual count of moving larvae varied between 88-92% for different batches. Larvae were used for infection within ten days of harvesting. A detailed production process of the larvae is described in supplementary material A.

Healthy male and female volunteers aged 18-45 years old were recruited in April 2017 and provided written informed consent. Exclusion criteria were body mass index (BMI) <18.0 or >30.0 kg/m^2, iron deficiency anaemia, positive PCR on feces for *Na* or *Ancylostoma duodenale* hookworm or *Strongyloides*, positive serology for Hepatitis B, C, HIV, contra-indications for the use of albendazole, planned travel to a hookworm-endemic area, incomplete understanding of the study procedures, or any medical condition which could interfere with participation in the trial.

For the preparation of each dose, individual motile larvae were selected from the prior released batch to ensure highest possible viability. Every dose of 50 *Na* L3 larvae was dispensed within 15 minutes after preparation onto four gauzes, divided in two doses of 10 and two doses of 15 larvae, which were applied to the dorsal side of volunteers' upper arms and calves, respectively, for 60 minutes.

Adverse events, total eosinophil count and haemoglobin were collected weekly during the first 12 weeks and 6 and 12 months after infection. For every adverse event, time and date of onset and end, severity and causality were recorded. Adverse events could be unrelated, unlikely, possibly, probably or definitely related, the latter three categories are regarded as "related" in dichotomous analyses. Relatedness was assessed by the clinical trial physician. Fecal samples were collected weekly from week 5 after infection onwards and checked for *Na* eggs by Kato-Katz. Slides with 25 milligram of stool were prepared from homogenised stool specimens.[12] Two slides per fecal sample were read by two different slide readers. After 12 weeks of follow-up, volunteers were asked to return at irregular intervals (not a pre-specified pattern) for on-demand feces donation during the course of a year, depending on the need for samples at the laboratory. These on-demand donations were used for additional egg counts and further culturing of the larvae.

Variability in egg counts between and within individuals over time was analyzed using Bayesian multi-level analysis. We assumed that egg counts initially increase and then stabilize over time, according to a scaled cumulative normal distribution function. We considered the following three levels to describe the variation of egg counts. Firstly, the level at which egg counts stabilize was allowed to vary between individuals assuming a log-normal distribution. Secondly, variation in daily averages was assumed to follow a gamma distribution. Lastly, the sampling error in repeated egg counts on the same day in the same individual was assumed to follow a Poisson (variance equals mean) or a negative binomial distribution (variance is greater than mean). Parameter values were estimated using the package *rstan* (version 2.16.2) [13] in R (version 3.4.3).

This trial was approved by the local IRB (P17.001) and is registered under NCT03126552 (clinicaltrials.gov).

Results

Four volunteers were included in the study, three female, one male, aged 19-23 years. Infection with 50 *Na* L3 larvae was well tolerated and resulted in patent hookworm infection in all four volunteers.

All volunteers reported rash at the sites of infection (figure 1A), lasting for 11, 22, 31 and 32 days respectively, and itching lasting for 1-2 days. There was no difference in intensity of rash or itching between arms and legs. The most common adverse event was 11 episodes of abdominal pain, reported in all volunteers. Nine episodes were classified as mild, one as moderate and one as severe, lasting five hours. Other abdominal events were nausea (2 volunteers) and flatulence (one volunteer), all mild (figure 1B) starting at week three, increasing in frequency until week 6 and then declining until all abdominal complaints disappeared at week 9. None of the volunteers reported related adverse events after week 12. (Supplementary material B)

A

B			Number of volunteers (n=4)	Mild	Moderate	Severe
Solicited	*Local*	Rash	4	4	-	-
		Itching	4	4	-	-
	Systemic	Abdominal pain	4	2	1	1
		Nausea	2	2	-	-
		Flatulence	1	1	-	-
		Cough	2	1	1	-
Unsolicited	*Systemic*	Headache	3	1	1	1
		Sore throath	2	1	1	-

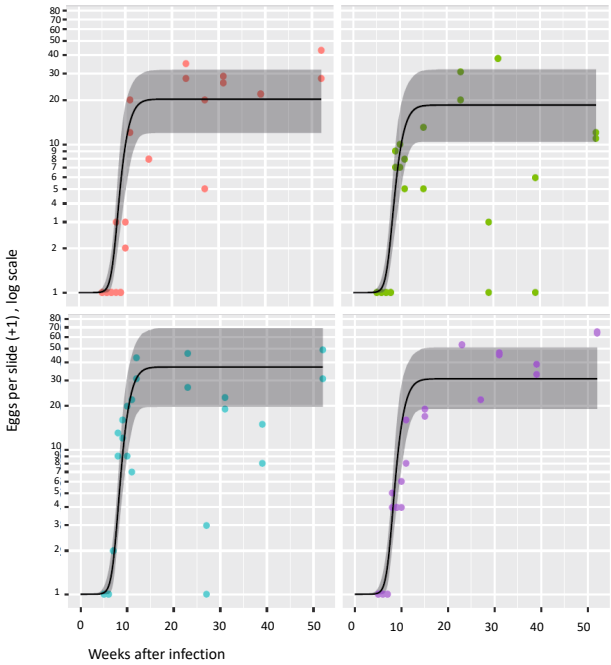
Figure 1. Overview of adverse events.

Representative rash at week 1 after infection. (left: arm, 10 L3 *Na* larvae; right: leg, 15 L3 *Na* larvae). A. All reported adverse events considered related to the study.

None of the volunteers developed anaemia. Eosinophil counts increased steeply in all volunteers after infection, peaking at week 6 (range 2.02-6.96x10^9/L). Eosinophils declined afterwards but remained slightly elevated at week 12 in all volunteers (range 0.82-1.77x10^9/L) and still elevated in two volunteers at week 52, one of which had already elevated eosinophils at baseline (baseline 0.74x10^9/L, week 52 1.18x10^9/L and baseline 0.34x10^9/L , week 52 0.72x10^9/L respectively).

Kato-Katz slides were all negative at week 5 and 6 and became positive in one volunteer at 7 weeks after infection. At week 9 all volunteers showed egg excretion by Kato-Katz. Median number of eggs per gram (epg) at the end of the first follow-up period at week 12 was 560 (range 160-1680). After the initial follow-up volunteers donated feces at irregular intervals, enabling data collection up to week 52.

Bayesian multi-level analysis showed that by week 10, egg counts had risen to about half the maximum level and reached their maximum level around week 13 (figure 2A). Thereafter, egg excretion remained relatively stable, with still considerable variation in daily averages within individuals. The estimated population-level plateau was 25.8 eggs per slide, corresponding to around 1000 epg. Assuming negative binomial variation instead of Poisson distribution in repeated egg counts on the same day did not improve the model fit (figure 2B).



Parameter	Description	Negative binomial variation (log eggs per slide)	Poisson variation (log eggs per slide)
β_{pop}	Population average at plateau level	25.0 (7.2-57.2)	25.8 (10.2 - 57.2)
σ_{β}	Inter-individual variation in stable level on logarithmic scale	0.7 (0.1 - 2.3)	0.7 (0.1 - 2.2)
μ_t	Time when faecal egg density has reached half of its stable level (weeks)	10.2 (8.9 - 11.8)	10.2 (9.0 - 11.7)
σ_t	Parameter determining slope of climb to plateau (97.5% of plateau level is reached at weeks)	1.5 (0.8 - 2.4)	1.6 (0.9 - 2.3)
k_{day}	Shape parameter for deviations of daily average egg counts from expected levels within individuals (lower values indicate higher variation)	1.5 (0.9 - 2.4)	1.5 (0.9 - 2.3)
k_{test}	Shape parameter for variation in repeated egg counts on same day within individuals (lower values indicate higher variation)	38.0 (10.3-99.5)	-

Figure 2. Individual fecal egg counts over time

A. Observed egg counts vs. estimated individual fecal egg density kinetics (solid line with grey band for 95%-Bayesian credible interval), assuming Poisson variation in repeated egg counts.
B. Parameters when assuming negative binomial or Poisson variation, posterior mean with 95%-Bayesian credible interval.

Discussion

This study shows that experimental infection with 50 L3 *Necator americanus* larvae divided over four application sites is well tolerated and leads to patent infection with eosinophilia and fecal egg excretion in all four volunteers. Long-term follow-up showed that egg counts increased from week 7 to week 13 post infection and then reached a stable level. The peak of adverse events, primarily abdominal complaints, occurred around six weeks after the infection, coinciding with peak eosinophilia. This time point is thought to mark the establishment of the larvae in the intestine.

To alleviate dermal symptoms, we divided the infectious larval dose over four extremities. Although median duration of rash was similar, the intensity of local events was decreased as compared to Diemert et al. [10] The levels of egg excretion, however, were much higher than any previous report [9 10] and were not accompanied by more severe abdominal adverse events. [10] This enhanced infectivity could be related to the viability of larvae, which had viability rates of >88% and were all used within ten days of harvesting. Alternatively, the application of larvae over several sites may have enhanced infectivity. The plateaus of egg counts observed in this study are below the WHO defined cut-off for a light infection of 2000 epg. [6] Comparable average egg excretions are widely observed in endemic areas. [14]

Although experimentally infected hookworm volunteers have been subjected to long follow-up before, [15] this is the first study to describe the kinetics of *Na* egg excretion over a prolonged period of time in multiple volunteers. We found low variability between egg counts on the same day within the same individuals, allowing for a Poisson distribution. This is remarkable as variation in egg counts in field studies is generally much higher and can only be described by a negative binomial distribution. [7] Possibly, the highly standardised method of slide preparation and feces homogenization in this trial and the lack of reinfection reduces variability in egg output. However, more data are needed to further quantify the day-to-day variation within and between individuals. Although PCR analysis may potentially show less variability, microscopic techniques are still the preferred primary endpoint in phase 2 vaccine or drug trials. Bayesian multi-level analysis is helpful to accurately assess the stabilizing levels of egg excretion from 13 weeks onwards despite several levels of variability and as such will provide key information for the future design of controlled human hookworm trials testing novel medicines or vaccines. Vaccine efficacy can be detected through a lower plateau level, later or slower rise in egg counts or a combination. The timing and level of the plateau in egg counts could be a reliable endpoint for clinical trials, assuming follow-up is extended beyond 13 weeks. Despite the small sample size, these promising findings add to the existing arguments for using CHHI-models for early vaccine efficacy studies.

In conclusion, we found that a controlled human infection with 50 L3 *Na* larvae was well tolerated, resulted in patent infection in all volunteers with unparalleled high egg counts.

Despite the fact that the number of subjects in this study was limited, our results show great promise in developing a sustainable human hookworm infection model that, aided by Bayesian statistical analysis of egg kinetics, could be of great value in accelerating clinical testing of novel vaccines and medicines.

Notes

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Supplementary material A

Description of procedure for larval production

Fecal samples were collected from a healthy donor confirmed negative for HIV, Hepatitis B and Hepatitis C carrying a chronic hookworm infection. Donor feces were confirmed negative for gastro-intestinal pathogens by real-time in-house multiplex PCR for Norovirus, Sapovirus, Astrovirus, Adenovirus, Rotavirus, Salmonella, Shigella, Campylobacter, Yersinia, *Entamoeba histolytica*, *Giardia lamblia* and Cryptosporidium. In addition, fecal samples were cultured for *Staphylococcus aureus* and highly-resistant Gramnegative rods using enrichment broth and subsequent subculture on non-selective and selective culture media for MRSA, ESBL-positive Enterobacteriaciae and ciprofloxacin- and tobramycin-resistant gram negative rods. Eggs were counted by Kato-Katz and hookworm egg morphology was confirmed by microscopy.

For culture of infectious L3 larvae, feces were mixed with gentamicin and amphotericin B and subsequently charcoal which was subsequently cultured in water for 7-10 days. At this time, larvae were collected and washed with betadine solution (100 mg/ml) to reduce bioburden. Larvae were counted, checked for viability and larval species morphology was confirmed by microscopy. A multiplex PCR was performed to distinguish *Necator americanus* from similar larvae (*Strongyloides stercoralis* and *Anylostoma duodenale*). Bacterial culture was performed to confirm absence of potentially pathogenic bacteria including *Staphylococcus aureus*, beta-hemolytic streptococci *Aeromonas* and *Pseudomonas aeruginosa* using general culture media. Upon preparing the correct dose on gauze, larvae were counted and checked for viability again.

Final batch release is performed by the QC and QP of the LUMC pharmacy according to pre-specified criteria.

Supplementary material B

Overview of all related adverse events present per week after infection.

Week	Rash	Itching	Abdominal pain	Nausea	Flatulence	Sore throat	Coughing	Headache
0	3	3	0	0	0	0	0	0
1	4	4	0	0	0	0	0	1
2	4	1	0	0	0	0	0	0
3	3	0	2	1	0	1	0	2
4	3	0	0	0	0	0	1	0
5	1	0	2	2	1	0	0	1
6	0	0	2	0	1	0	1	2
7	0	0	0	0	0	2	0	1
8	0	0	2	0	0	0	0	0
9	0	0	1	0	0	0	0	0
10	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0

All adverse events were grade 1 (mild), except two severe adverse events in week 6 (abdominal pain and headache, both lasting as severe for less than one day) and three moderate adverse events in week 6 (sore throat, coughing and headache).

Chapter 4

A randomized controlled trial to investigate safety and variability of egg excretion after repeated controlled human hookworm infection

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Abstract

Background

Controlled human hookworm infections could significantly contribute to the development of a hookworm vaccine. However, current models are hampered by low and unstable egg output, reducing generalizability and increasing sample sizes. This study aims to investigate the safety, tolerability and egg output of repeated exposure to hookworm larvae.

Methods

Twenty-four healthy volunteers were randomized double blind to one, two or three doses of 50 *Necator americanus* L3 larvae at 2-week intervals. Volunteers were monitored weekly and were treated with albendazole at week 20.

Results

There was no association between larval dose and number or severity of adverse events. Geomean egg loads stabilized at 697, 1668 and 1914 eggs per gram feces for the 1x50L3, 2x50L3 and 3x50L3 group respectively. Bayesian statistical modelling showed that egg count variability relative to the mean was reduced with a second infectious dose, however the third dose did not increase egg load or decrease variability. We therefore suggest 2x50L3 as an improved challenge dose. Model-based simulations indicates increased frequency of stool sampling optimizes power of hypothetical vaccine trials.

Discussion

Repeated infection with hookworm larvae increased egg counts to levels comparable to the field and reduced relative variability in egg output without aggravating adverse events.

Background

Hookworm infects around 230 million people worldwide. [1] Chronic infection causes iron-deficiency anaemia and protein loss among the world's poorest, [2] may impede children's cognitive and physical development[3] and adversely affect pregnancy outcomes. [4] This results in high losses in annual productivity (estimated between $7.5 and $138.9 billion), [5] perpetuating the poverty cycle. [6] The impact of periodical deworming, the cornerstone of current hookworm control programs, is impeded by high reinfection rates [7 8] and the looming threat of anthelmintic resistance. [9] A safe and efficacious hookworm vaccine is therefore needed to aid hookworm control,[10] however development is hampered by a lack of preclinical models since animal models do not reflect natural infection in humans.

Experimental infection of volunteers, so-called controlled human hookworm infection (CHHI) trials could be a method to screen novel products instead. [11] In other diseases, controlled human infection models have shown their usefulness in testing vaccine efficacy. [12 13] However, their pertinence hinges on safety of the model, generalizability of results to natural infection and adequately powered sample sizes.

Previous CHHI trials have shown that infections with 50 *Necator americanus (Na)* infectious larvae (L3) are well tolerated with mild abdominal adverse events and that skin eruptions can be alleviated by spreading the infectious dose over four sites. [14 15] Resulting egg counts however have varied, with some trials reaching 100% infectivity and egg counts comparable to field settings, [15] whereas other studies reached lower egg output. [14 16] In addition, microscopic egg counts, the gold standard endpoint of CHHI trials, are highly variable, [17] decreasing the power of hookworm infection studies to detect vaccine efficacy. In a previous trial we used Bayesian statistical modelling to describe the long-term kinetics of hookworm egg excretion investigated[15] and found that one dose of 50L3 larvae resulted in egg counts plateauing around 13 weeks after infection at a level comparable to low-endemic field settings. Although homogenizing the feces decreased the variability of egg counts in the same individual on the same day, considerable inter- and intra-individual variation remained. [15] We hypothesized that repeated infection could increase egg outputs and potentially reduce variability relative to the mean without increasing adverse events, enhancing the power of CHHI trials.

Here we report the outcome of a CHHI trial in which volunteers were exposed to multiple doses of *Na* L3. Based on these data we developed a Bayesian model for the in-depth analysis of the variation in egg counts by dose group and performed hypothetical sample size calculations to determine the power of CHHI models in vaccine trials.

Methods

This study was a randomized, double-blind, placebo-controlled trial investigating the safety and tolerability of cumulative doses of *Na* larvae up to 3x50 L3 and the variability in egg output after repeated infection with *Na* L3.
The trial was approved by the LUMC institutional review board (NL59186.058.17) and registered at clinicaltrials.gov (NCT03257072).

Study subjects
Healthy male and female volunteers aged 18-45 years were recruited from the Leiden area in January and February 2018. All volunteers provided written informed consent before inclusion. In- and exclusion criteria are described in supplement A. Participants were all confirmed hookworm-negative by PCR before inclusion.

Na L3 larvae were cultured from feces of chronically infected hookworm donors according to the principles of Good Manufacturing Practice following a procedure previously described. [15 18]

Study procedures
Volunteers were randomized 1:1:1 to receive one, two or three doses of 50 *Na* L3 at two-week intervals, totaling a cumulative dose of 50, 100 or 150 L3 (Figure 1). Volunteers in the 50 or 2x50L3 group first received respectively two or one dose of placebo. Randomization was performed through a randomization list made by an independent researcher. Larval doses were suspended in sterile water and divided over four gauzes which were applied to both upper arms (doses of 10 L3) and calves (doses of 15 L3) and left for one hour. As placebo, gauzes with sterile water were used. Investigators and participants were blinded to dose allocation.

Volunteers were followed weekly for twenty weeks. At each visit, adverse events (AEs) were collected and blood drawn for eosinophils and hemoglobin. Hemoglobin and eosinophils were measured on the Sysmex DI-60 automated cell morphology analyzer. Skin-related AEs, cough, sore throat, fever and abdominal AEs were solicited at each visit. For every AE timing, severity and causality were recorded. AEs were scored as unrelated, unlikely, possibly, probably or definitely related to hookworm infection, and mild (no interference with daily life), moderate (discomfort interfering with daily life) or severe (causing inability to perform usual daily activity). Two independent, blinded physicians divided the volunteers into two equal groups based on the safety data, one with “high” abdominal AEs signifying the half of volunteers with most abdominal AEs and one with “low” abdominal AEs, the half with the least abdominal symptoms. Division in groups was based on severity, duration and number of abdominal AEs, consensus was reached for each volunteer.

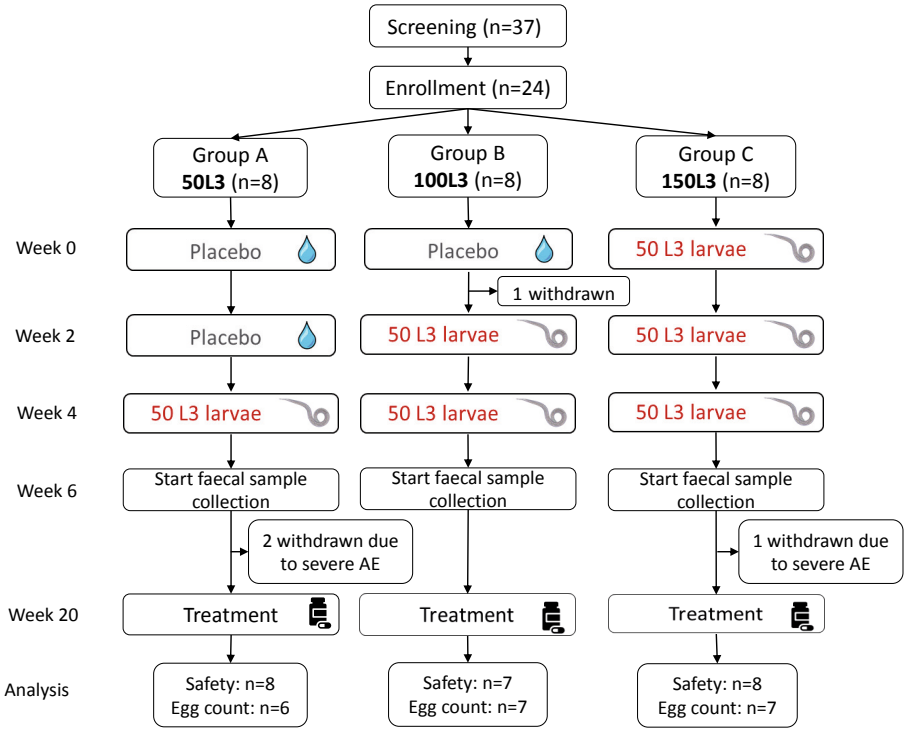


Figure 1. Trial set-up and flow chart. Abbreviation: AE, adverse event.

At week 20 of the trial all volunteers were treated with 400mg albendazole for three days. Two volunteers gave separate informed consent to postpone albendazole treatment and remain as donors for future studies. All other volunteers returned to the trial centre on weeks 1, 2, 3, and 8 after treatment to collect adverse events and ensure complete cure of the infection.

Immunological assays

Serum for analysis of antibodies was collected at weeks 0, 4, 8, 12, 16, 20, 23 and 28 of the trial. Hookworm-specific IgE, IgG, IgG1 and IgG4 were measured by ELISA. Procedures are described in supplement B. Specific antibody levels are expressed in AU/ml. Data was normalised with baseline set at 0 and other measurements presented as AU/ml above baseline, with peak value defined as the highest value measured from baseline.

Seroconversion was defined as any peak AU/ml value of 4x above standard deviation (SD). SD was determined in the week 4 samples of the 50L3 group, as baseline samples were normalised to 0 and at week 4 the 50L3 group had not yet been exposed therefore enabling its use as a control group.

Parasitological assays

Fecal samples were collected weekly from week 6 until week 20 of the trial and at one and three weeks after treatment. Kato-Katz slides were prepared fresh with 25 milligrams of stool, homogenized before preparation. [18] Two Kato-Katz slides per fecal sample were prepared, each read by a separate microscopist. The total number of eggs counted was multiplied by 20 and expressed as eggs per gram feces (epg) per collected stool. The method for detection of *Na* DNA in stool by PCR is described in supplement C.

Statistical analysis

Similar to other proof-of-concept vaccine efficacy studies, groups of 8 subjects were chosen. This sample size would give 80% power to detect a 50% relative reduction in egg counts.

Primary endpoint was the frequency and severity of adverse events per group, assessed in the intention to treat population. Differences between groups were analyzed using a Kruskall-Wallis or Mann-Whitney U test.

Secondary endpoint was the difference in egg load between groups. The egg load was defined as the excretion of eggs in faeces between week 16 and 20 of the trial by Kato-Katz. Mean egg counts per individual were calculated, log-transformed values were compared using a one-way ANOVA. Differences between groups for eosinophilic response and antibodies were evaluated using a Kruskall-Wallis test. The correlation between Kato-Katz and PCR was assessed by Spearman's rho.

To compare level and variability of egg counts between groups in more detail and perform power calculations for hypothetical vaccine trials, we developed a Bayesian non-linear regression model describing egg count time series of each individual. The development of this model is described in supplement D.

After establishment of the model and generation of the model parameters, power calculations were performed, primarily assuming a reduction in egg loads of 50% in a vaccine group as compared to the control group with additional calculations for a 30-70% effect size. Power was calculated with the aim to detect a significant difference defined as a p-value < 0.05 based on Wilcoxon signed rank test for the difference in individual average egg counts (averaged over days and Kato-Katz slides) between trials arms.

Results

Study flow

In February 2018, 24 volunteers were included in the trial. Trial flow is shown in figure 1, participant characteristics in table 1. Despite randomization more females than males were included in the 50L3 group. One volunteer withdrew informed consent after one week for reasons unrelated to the trial. Two volunteers (randomized to the 50L3 group) withdrew 6

weeks after first exposure, the third volunteer (from the 3x50L3 group) withdrew at 9 weeks due to severe abdominal AEs. All symptoms resolved with albendazole treatment. Safety data, eosinophils and Ig-results available until withdrawal for these volunteers has been included in the intention to treat analysis, however volunteers withdrew before detection of eggs and therefore are not included in the per protocol analysis of egg excretion.

Table 1. Participant characteristics and number and duration of adverse events for each study group with p-values (Kruskall-Wallis test)

	Total (n=23)	50 L3 (n=8)	2x50 L3 (n=7)	3x50 L3 (n=8)	P-value
Sex **Female** **Male**	15 8	7 1	4 3	4 4	
Age in years, median (range)	22 (19-41)	27.5 (19-38)	21 (19-33)	23 (19-41)	
History of travel to hookworm-endemic area	11	4	4	3	
History of gastro-intestinal disease	1	1*	0	0	
Median number of AEs per volunteer (min-max)	16 (8-31)	17 (11-24)	17 (9-31)	16 (8-27)	P=0.902
Median duration of rash	34 (0-77)	18.5 (0-49)	37 (15-50)	41.5 (21-77)	P=0.091
Total number of related abdominal AEs (% of total)	99 (100%)	32 (32)	39 (39)	28 (28)	P=0.681
Median number of related abdominal AEs per volunteer (min-max)	4 (0-10)	4 (0-6)	4 (1-10)	4 (0-7)	
Maximum severity of abdominal AEs, N= None **Mild** **Moderate** **Severe**		1 2 1 4	0 2 1 4	1 3 1 3	
Median duration of abdominal AEs in days, (range)	22 (0-125)	21.5 (0-38)	29 (4-125)	23.5 (0-110)	p=0.638
Number of volunteers with "high" abdominal AEs	12	4	4	4	

AE: Adverse events
*: gastric reflux

Clinical data

No serious adverse events occurred. Most common related AEs were pruritus and rash during the first weeks after exposure (n=23) and abdominal AEs from week 2 after first infection (n=21). Two volunteers reported no abdominal symptoms, five reported only mild

abdominal bloating or flatulence for less than 5 days. Other gastro-intestinal symptoms started from week 2 after exposure, peaked at week 4 and 5 and resolved 8 weeks after first infection in the majority of volunteers (13/16). Symptoms in the remaining three volunteers resolved with albendazole treatment. Based on the combination of severity and duration of AEs, 12 volunteers were classified as “high” abdominal AEs and 11 volunteers as “low”.

There were no significant differences in number and severity of AEs between study groups (table 1), the number of volunteers experiencing grade 3 AEs or any abdominal AE were similar across study groups.

Hemoglobin stayed stable throughout the trial (figure 2A). Eosinophil counts increased in all groups, starting at three weeks after first exposure, with a peak at week six after initial infection (figure 2B). There was no difference in mean peak eosinophil count between groups (p=0.4).

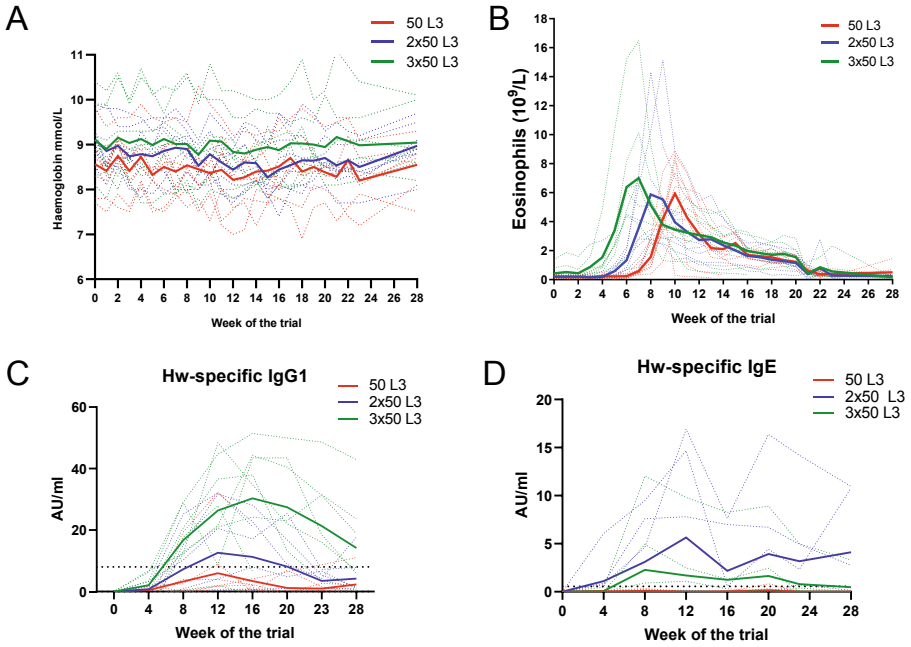


Figure 2. A, B, Hemoglobin levels (A) and eosinophil counts (B) for individual volunteers (dotted lines) and mean for each study group (continuous lines). C, D, Immunoglobulin (Ig) G1 antibody (C) and IgE (D) responses for individual volunteers (dotted lines) and mean for each study group (continuous lines); horizontal dotted line represents threshold for seroconversion (increase from baseline to 4 times the standard deviation). Abbreviation: AU, arbitrary units.

Hookworm-specific antibodies increased with additional doses and peaked at week 12 or 16 of the trial, although there was a large variation between individuals. Rise in IgG1 was significantly different between dose groups (p=0.013 for peak IgG1 value between groups)

(figure 2C). IgG1 seroconversion was reached in respectively 3, 4 and 7 volunteers for the 50 L3, 2x50 L3 and 3x50 L3 groups which showed a weak statistical significance (Chi-square test p=0.12). For IgE, seroconversion was observed in three volunteers in the 2x50 L3 group and two in the 3x50 L3 group. (figure 2D). IgG4 responses were less pronounced than for IgG1, did not show clear differences between dose groups and reached seroconversion in 2, 3 and 2 volunteers respectively (supplementary figure 1A). Total IgG is displayed in supplementary figure 1B.

Parasitological analysis

Volunteers in the 50 L3 group secreted hookworm eggs by Kato-Katz at a median of 7 weeks after first infection (range 5-8), the 2x50 L3 and 3x50 L3 group at 8 weeks (range 7-9) (p=0.676).

Egg loads differed significantly between groups: geomean of the mean counts per individual were 697 epg for the 50 L3 group (95%-CI 228-2131), 1668 (95%-CI 979-2840) for 2x50L3 and 1914 (95%-CI 1455-2517) for 3x50 L3 (one-way ANOVA: p=0.04) (table 2). Post-hoc testing for between group difference showed weak differences between the 50 L3 group and the other two groups (p=0.1 for 50 L3 vs 2x50L3, p=0.05 for 50 L3 vs 3x50 L3) and no difference between the 2x50 and 3x50 L3 groups (p=0.6) (figure 3).

Infection intensity as determined by PCR mirrored these findings (table 2, p=0.02) and were strongly correlated with Kato-Katz counts (p=<0.0001, rho=-0.79) (supplementary fig 2).

PCR and Kato-Katz were all negative three weeks after treatment.

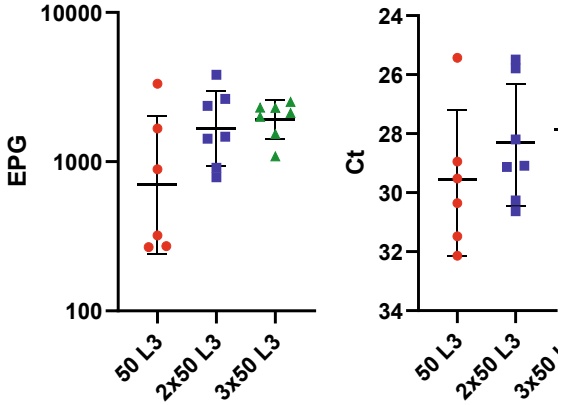


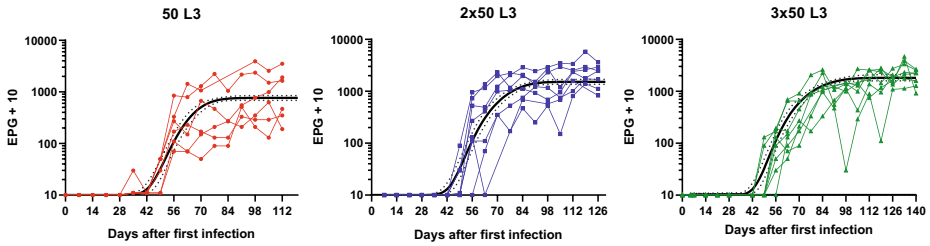
Figure 3. Individual mean Kato-Katz slide measurements (left) and cycle threshold values (right) at weeks 16–20 of the trial. Red circles represent the 50L3 group; blue squares, 2 × 50L3 group; green triangles, 3 × 50L3 group; lines, geometric means; and error bars geometric standard deviations. Abbreviation: epg, eggs per gram feces.

Bayesian statistical modelling

Using Bayesian statistical modelling we first assessed the relative contributions of each L3 dose to the overall egg load. Allowing dose contributions to vary freely between all groups confirmed an equal contribution of the first and second dose of L3 and a lesser contribution of the third dose (estimated at factor 0.4 relative to a single dose, 95%-Bayesian credible interval (BCI): 0.01–1.0). Next, when we allowed the level of temporal overdispersion (i.e. daily variation relative to the mean) to vary freely between all three groups, the level of overdispersion was a factor 1.9 higher (95%-BCI: 1.0–3.2) in the single-dose group compared to the two-dose group; there was no significant difference in temporal overdispersion (and thus relative variation) between the two- and three-dose groups(difference of factor 1.1, 95%-BCI: 0.5–1.9). We therefore further simplified the model assuming that temporal overdispersion differed only between the single dose group (k = 3.2, 95%-BCI: 2.0–4.9) compared to the other two dose groups (k = 5.6, 95%-BCI: 4.1–7.5; i.e. a difference of factor 1.9, 95%-BCI: 1.01–3.05). We verified that random effects for the plateau level and timing of rise in egg counts within each individual were uncorrelated (supplementary figure 3).

We estimated that one, two, or three doses of 50 L3 resulted in a posterior mean egg load at plateau level of 760 (95%-BCI: 640-840) to 1520 (95%-BCI: 1360-1680) and 1800 (95%-BCI: 1560-2080) epg, respectively, corresponding with the descriptive statistics of egg loads previously described (figure 4). The rise in egg counts due to each single dose reached 50% of its maximum level at 67 days (~10 weeks) after exposure (95%-BCI: 63–70) and 97.5% of its maximum after another 18 days (95%-BCI: 15–22). Inter-individual variation in the timing of the initial rising phase was minimal with a standard deviation on the logarithmic scale of 0.08 (95%-BCI: 0.05–0.11).

A summary of the final model estimated parameters is provided in supplementary table 1, and estimated and measured egg counts per individual in supplementary figure 4.



	50 L3	2x50 L3	3x50 L3
Geomean eggs per gram of mean egg count per individual week 16-20 of the trial (95%-CI)	697 (228-2131)	1668 (979-2840)	1914 (1455-2517)
Geomean of mean Ct-value per individual wk 16-20 of the trial (95%-CI)	29.6 (27.1-32.3)	28.3 (26.5-30.3)	27.8 (27.0-28.7)
Posterior mean in Bayesian model (95% BCI)	760 (640-840)	1520 (1360-1680)	1800 (1560-2080)
Temporal overdispersion (shape parameter k, higher values indicate less variability relative to mean) (95% BCI)	3.2 (2.0–4.9)	5.6 (4.1-7.5)	5.6 (4.1-7.5)

Figure 4. Egg counts as observed and predicted by a bayesian nonlinear regression model, for 50L3 (left), 2 × 50L3 (middle), and 3 × 50L3 (right) groups. Solid line in each panel represents the group average; dashed lines, upper and lower limits for the 95% bayesian credible interval (BCI) of the point estimate. Table below graphs shows mean values for Kato-Katz slides and polymerase chain reaction, bayesian posterior means, and shape parameter k for temporal overdispersion; for the latter, higher values indicate less variability relative to the mean. Abbreviations: BCI, bayesian credible interval; CI, confidence interval; Ct, cycle threshold; epg, eggs per gram feces.

Correlation between adverse events, egg counts and antibody response

Volunteers with a higher egg output had higher mean hookworm-specific IgG1 (R=0.537, p=0.015 for egg load, R=-0.586, p=0.007 for Ct value) but not more IgE, IgG4 or eosinophils. Volunteers with more abdominal adverse events had higher levels of eosinophils (mean 9.1x10^9 vs 4.4x10^9 in “high” versus “low” counts, p=0.003) but an equal number of eggs (geomean 914 epg vs 1086 epg, p=0.6) (figure 5). Antibody levels did not correlate with adverse events or eosinophils.

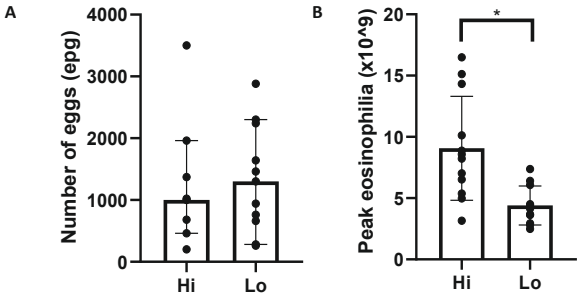


Figure 5. Comparison of volunteers in the upper ("high") and lower ("low") groupings according to the severity, duration, and number of abdominal adverse events, for both egg load (A; shown as median with 95% CI) and eosinophilia (B; shown as mean with standard deviation). *P = .003. Abbreviations: CI, confidence interval; epg, eggs per gram feces.

Power calculations

Using the Bayesian non-linear regression model, power calculations were performed by repeatedly simulating synthetic data for a two-armed trial assuming the use of a vaccine that reduces egg counts by 50% compared to placebo. Sampling of stools was assumed to start during the stable phase in week 15 after first infection. Although multiple doses of L3 increase study power somewhat (due to higher egg counts in the control group and lower random daily variation within individuals), power is mostly driven by the number of repeated samples that are collected at weekly intervals (figure 6). A power of >80% can be reached in groups as small as six volunteers if samples are taken for five weeks with two doses of 50 L3. In contrast, a power of only 60% is reached with a single sample in groups of 15 volunteers with three infectious doses. Assessing multiple Kato-Katz slides per sample does not improve power compared to a single slide. The power calculation was re-run with an expected vaccine efficacy of 30-70%. This showed that with sufficiently frequent sampling even at only 30% efficacy groups of 15 participants can reach 80% power (supplementary figure 5).

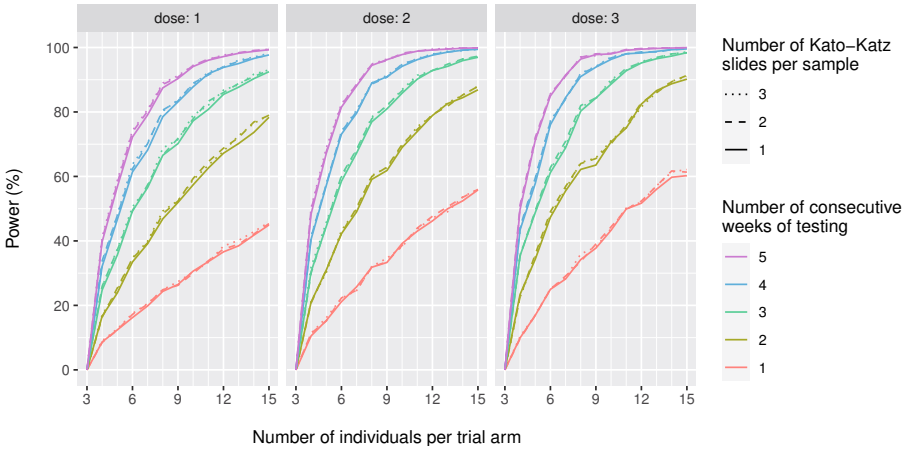


Figure 6. Power calculation using bayesian modeling of egg counts showing the number of individuals per trial arm in a vaccine trial, assuming 50% reduction in egg counts in the vaccine versus the placebo group, after 1 (left), 2 (middle), or 3 (right) doses of 50L3. Colors represent the numbers of weekly samples obtained with the top line in each graph representing 5 samples and each subsequent lower line one sampling less, the lowest line represents 1 sample.

Discussion

This dose escalation study showed that higher cumulative doses of hookworm L3 did not enhance adverse events, which displayed high inter-individual variability and a reasonable tolerability profile. Repeated infectious doses resulted in an increase in total egg counts and decreased relative variability in egg excretion. Power calculations based on a Bayesian non-linear regression model showed that repeated sampling is the most important parameter determining the power of the controlled human hookworm infection model to detect potential vaccine efficacy.

Volunteers reported a considerable number of abdominal adverse events, with three volunteers receiving rescue treatment early due to severe abdominal AEs. However, these events do not seem to be related to L3 dose, as two of the rescued volunteers were in the lowest dose group. Within each dose group we found considerable inter-individual variation in number and severity of adverse events. Severity of adverse events correlated with eosinophilic response, which both peak around week 6 after infection. We hypothesize that this may reflect symptomatic eosinophilic enteritis, which has previously been associated with hookworm attrition. [19] We however could not establish a relationship between egg load and eosinophil counts.

Larval dose and eosinophilic response were not related, however there was a clear relationship between dose and humoral responses to the larvae. Remarkably, we found

IgG1 to be the most pronounced responding Ig subtype in this acute infection, contrasting epidemiological studies where IgE and IgG4 are hallmarks of active chronic infection.[20] IgE has previously been observed to develop only after repeated exposure[20] and may confer protection.[21] We did observe an IgE response in some volunteers after repeated exposure however given the limited response in only a few volunteers, we were not able to confirm any protective effect.

PCR results showed a similar pattern of increased infectious loads with higher cumulative infectious doses and showed good correlation with Kato-Katz outcomes. However, the high SD of between 2.0 and 0.9 Ct, corresponding to a variation of double or quadruple the amount of DNA indicate high variability in PCR outcomes. This may be caused by both variability in the egg excretion itself and by differences in the number of DNA-copies per egg, as a Na egg may be in a 2-16 cell stage, increasing possible sources for variation.[22] In addition to the more difficult field applicability of PCR-techniques and supplementary variability of Ct-values between labs,[22] this underscores that as yet microscopic techniques remain the cornerstone of hookworm diagnosis and highlights the necessity to improve power and accuracy of diagnostic outcome measures.

Repeated dosing resulted in egg counts that are more representative of infection levels in endemic areas, with mean epg only slightly below the WHO threshold for moderate infection (2000 epg).[23] These egg counts are higher than previously reported using a single inoculation,[14 15] enabling a better comparison with natural infection. However, the third dose does not seem to add significantly to the total egg output compared to two doses. Possibly, repeated exposure induces some immunity against the larvae of the subsequent infection resulting in less surviving worms, or competition for nutrients and feeding sites occurs as the number of larvae establishing in the intestine simultaneously is higher than in a natural, trickled infection.

The high variability of egg secretion complicates the use of the CHHI model for vaccine testing. We therefore used Bayesian statistical modelling to more accurately estimate individual and population egg outputs and to better describe the variability in egg counts. Use of Bayesian modelling carries the advantage of a robust inference and the possibility to carry uncertainties of parameter estimates into power calculations, providing more accurate power estimates. This confirmed that the third infectious dose has a very limited contribution to the egg count plateau and to reduction of relative variability compared to two doses. Consequently, the use of the 2x50L3 dose seems the most rational option for future use in CHHI, as it leads to egg counts comparable to endemic areas with reduced relative variability compared to a single dose without aggravating adverse events. The power calculation based on the Bayesian statistical model underscores the importance of repeated sampling over time, which has higher impact on study power than the number of infections. More importantly, if we assume that variation between sampling on consecutive days is similar as variation within weekly samples and therefore take five consecutive samples

during the first weeks of the plateau phase (from week 13 after infection), CHHI trial duration may be substantially shortened from our current 20 weeks follow-up to for example 15 weeks. Naturally, sample sizes in future vaccine studies may be further increased to also detect differences in AEs or SAEs.

Due to the staggered design timing of first infection varied between different dose groups. However, egg load was analyzed at plateau level for all groups, ensuring comparability of outcomes. The study was underpowered to detect a difference in adverse events. Given the remarkably equal distribution of adverse events in the groups, significant differences seem unlikely. Although study set-up cannot mimic natural repeated infection with very frequent exposure to small inoculae and group sizes were diminished due to withdrawal of four volunteers, this remains the largest study to date to investigate any kind of repeated exposure to hookworm infection.

In conclusion, this trial has further advanced the controlled human hookworm infection model by introducing repeated challenge in the model and underscoring the importance of repeated sampling after development of the plateau phase. These findings are an important step in the applicability of the CHHI model for future vaccine- and drug research.

Notes

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The authors wish to thank Chelsea Gootjes and Mark Dekker for their contribution to the hookworm culture and preparing and reading Kato-Katz slides, Beckley Nosoh for running the PCR and dr. Martijn Bauer for his advice as safety monitor of this study.

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Supplementary material

Supplement A: In- and exclusion criteria

Inclusion-criteria:

1. Subject is aged ≥ 18 and ≤ 45 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. Subject agrees to refrain from blood donation to Sanquin or for other purposes throughout the study period.
5. For female subjects: subject agrees to use adequate contraception and not to breastfeed for the duration of study.
6. Subject has signed informed consent.

Exclusion-criteria:

A potential subject who meets any of the following criteria will be excluded from participation in this study:

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, 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 Mass Index (BMI) <18.0 or >30.0 kg/m2 at screening;
- positive HIV, HBV or HCV screening tests;
- the use of 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;
- having one of the following laboratory abnormalities: ferritine <10 ug/L, transferrine <2.04 g/L or Hb <7.0 mmol/L for females or <8.0 mmol/L for males;
- 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 one year prior to study onset.

2. Known hypersensitivity to or contra-indications for use of albendazole, including co-medication known to interact with albendazole metabolism (e.g. carbamazepine, phenobarbital, phenytoin, cimetidine, theophylline, dexamethasone)
3. Known allergy to amphotericin B or gentamicin
4. For female subjects: positive urine pregnancy test at screening
5. Positive fecal qPCR or Kato-Katz for hookworm at screening, any known history of hookworm infection or treatment for hookworm infection or possible exposure to hookworm in the past
6. Being an employee or student of the department of Parasitology of the LUMC
7. Current or past scars, tattoos, or other disruptions of skin integrity at the intended site of larval application
8. Subjects with planned travel to hookworm endemic areas during this trial
9. Receipt of a vaccine within 4 weeks prior to the study initiation
10. Known food allergy

Supplement B: ELISA Procedure

Hookworm antigen: *Necator americanus* L3 extract.

Cultured Hookworm L3 larvae were collected in sterile water and stored in 50ml tubes at -80°C after which they were freeze dried and stored at -80°C again. After thawing, the larvae were suspended in PBS, transferred to a glass homogenizer and kept on ice whilst washing 4 times. The solution in the homogenizer was crushed for 15 minutes and left for one hour on ice. Crushing and resting was repeated three times. Crushed hookworm larvae were transferred to a glass tube, and the homogenizer was washed once with PBS. The crushed hookworm larvae were sonified (Branson Sonifier) 6 times for 30 seconds with an interval of 20 seconds. The samples were kept overnight at -80°C. The frozen samples were thawed and centrifuged for 25 minutes, 13.000 rpm at 4°C. The supernatant as well as the resuspended pellet were centrifuged again, after which supernatants from both tubes were collected and pooled. Antigen concentration was determined using a BCA kit (ThermoFisher 23225) following manufacturers instruction.

<u>IgG, IgG1 and IgG4</u> ELISA: High-binding C96-wells maxisorp plates (Nunc-Immuno™ 430341) were coated with 5ug/ml hookworm antigen in 0.1M Na-carbonate pH 9.6. After overnight incubation at 4°C, plates were washed 4 times with 0.05% Tween 20 (Sigma-Aldrich, 27, 434-8) in PBS wash buffer. Plates were blocked with 5% BSA/PBS for one hour at 37°C. After washing, a mix of positive serum was used in a serial dilution of 1:2 as positive controls, samples were at least 1:2 diluted in PBS 0.05% Tween 20 assay buffer. Plates were incubated overnight at 4°C for IgG1 and IgG4, and 1 hour at 37°C for total IgG. For IgG1 detection, conjugated monoclonal antibody of HRP-labelled anti human IgG1 (Fc) (clone MH161-1, HP6188, Sanquin; cat no: M1328) was added and for IgG4 detection HRP-labelled anti human IgG4 (CH3) (clone MH164-1, HP6196, Sanquin; cat no: M1331) was added, both at a

concentration of 1.8uL diluted in 5.5 ml PBS 0.05% tween and both 50 uL/well. For total IgG detection, 50 uL/well 1.8 uL alkaline phosphate conjugated anti-human IgG (Sigma A9544, 4°C) in 5.5 ml PBS 0.05%Tween-4% BSA was added. Plates were incubated at 37°C (one hour for IgG, four hours for IgG1 and IgG4). After washing, TMB substrate was added (TMB Microwel substrate system(KPL, 50-76-00) for IgG1 and IgG4, 6mg p p-nitrophenylphosphate (p-NPP) in 6 ml diethylanolamine (DEA) buffer for IgG)) and development was stopped with 18M H_2SO_4 in water, after which plates were read at by 450 nm at the ELISA plate reader. Total IgG plates were incubated in the dark for one hour at room temperature and then read at wavelength 405 nm.

IgE ELISA: Polysorp F96-wells plates (Nunc-Immuno™ 475094) were coated with 5ug/ml hookworm antigen in 0.1 M Na-carbonate buffer, pH 9.6. After overnight incubation at 4°C wells plates were washed 4 times with 0.05% Tween 20 (Sigma-Aldrich, 27, 434-8) in PBS wash buffer. Blocking with 5% BSA/PBS was performed for one hour at room temperature. After washing four times, a mix of positive sera were added in a serial dilution of 1:2 in PBS 0.05% Tween 20 assay buffer and serum samples were diluted at least 1:2 in assay buffer. Plates were incubated overnight at 4°C. After five washings 100uL/well of 11uL biotinylated goat anti human IgE (Vector BA3040, 0.5 mg/ml) diluted in 11 ml PBS 0.05%Tween was added and plates were incubated for three hours at 37°C. Samples were flicked off and washed five times after which 100uL/well of 3,6 uL of Streptavidin alkaline phosphatase (strep-AP-conjugate, Roche 11.089.161.001, 4ºC) diluted in 11 ml PBS 0.05%Tween was added and incubated for three hours at 37°C. After flicking the conjugate and washing 5 times 100uL/well p p-nitrophenylphosphate was added at a concentration of 1 mg/ml in diethylanolamine buffer. Plates were incubated for two hours in the dark and then read at 405nm at the Elisa plate reader.

All Ig analyses:
OD values are converted to AU/ml using a standard curve with unknown concentration, enabling comparison of a rise or fall in serum concentration within the same subject.

Supplement C: PCR procedure
For PCR, DNA was extracted following a previously described method[1], including the addition of Phocin Herpes Virus (PhHV-1) to the lysis buffer as an internal control. Amplification reactions were performed in white PCR plates in a volume of 25 µl with PCR buffer (HotstarTaq master mix, QIAgen, Germany), 5 mM MgCl2, 2.5 µg Bovine Serum Albumin (Roche Diagnostics Nederland B.V., Almere, the Netherlands), 5 pmol of each Na-specific primer, 1.25 pmol of Na-specific XS-probe and 5ul of the DNA sample[2] 3. Primers and probe for the detection of Na are described in supplementary table 1. Amplification, detection and analysis were performed using the Bio-Rad CFX96™ realtime detection system, resulting in Cycle threshold (Ct)-value as the output, reflecting the parasite-specific DNA load in the tested sample. All samples were analysed in the same PCR-run.

Primers and Probes for Na PCR[2 3]

Oligonucleotide name	Oligonucleotide sequence	Genbank accession no of target sequence
Necator americanus		
Na58F	5'-CTGTTTGTCGAACGGTACTTGC-3'	AJ001599
Na158R	5'-ATAACAGCGTGCACATGTTGC-3'	AJ001599
Na81t_XS_FAM	FAM-5'-CTGTACTACGCATTGTATAC-3'-BHQ1	AJ001599

Supplement D: Development of Bayesian model

The initial rise in egg counts in each individual was described with an S-shaped function, using a cumulative normal distribution function, which was scaled to the plateau level within each individual. The model captures differences between groups in terms of the level of stabilization (the plateau level) and daily variation in egg counts (fixed effects) and inter-individual variation in the timing of the initial rise and the level of stabilization (random effects). The duration of the initial rise in egg counts after a single dose was assumed to be the same for all individuals. Multiple doses of L3 over time were assumed to result in a plateau which reflects the sum of multiple (shifted) S-curves (figure 1). Repeated doses of L3 larvae were allowed to result in diminishing, equal or increased effect contribution to the final plateau egg level. Random effects for timing of the rise and the level at which egg counts stabilized within individuals were assumed to each follow an independent log-normal distribution. To make the model identifiable, the random effect for variation in stabilization level was constrained to sum to zero (on the logarithmic scale) within each dose group. Average fecal egg densities within individuals were assumed to vary randomly between days following a gamma distribution, where the level of overdispersion (i.e. daily variation relative to the mean egg count, determined by the shape parameter k of the distribution with lower values for k signifying higher variation) was allowed to differ between the three dose groups. Repeated egg counts on the same day (i.e. performed on a single homogenized stool sample) were assumed to follow a Poisson distribution; a negative binomial distribution did not provide a better fit. This corresponded to the findings in the previously developed model[4], underscoring the importance of feces homogenization.

The joint posterior distribution of model parameters was estimated with dynamic Hamiltonian Monte Carlo, using *rstan* version 2.18.2 in *R* version 3.6.0 and *RStudio* version 1.2.1335[5]. Parameter estimates were summarized as posterior means and 95%-Bayesian credible intervals (BCI). The parsimonious structure of the Bayesian model (i.e. the model with the fewest parameters that adequately describes the data) with regard to relative differences between groups (contribution of repeated L3 doses and level of random daily variation in fecal egg density) was identified based on whether 95%-BCIs included the value 1.0 (i.e. no multiplicative effect). To estimate model parameters more accurately, additional data on egg counts from four additional volunteers from a previous pilot study was included in the analysis[4].

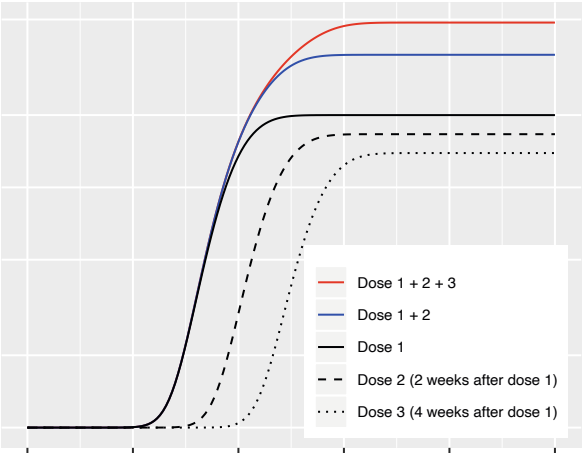
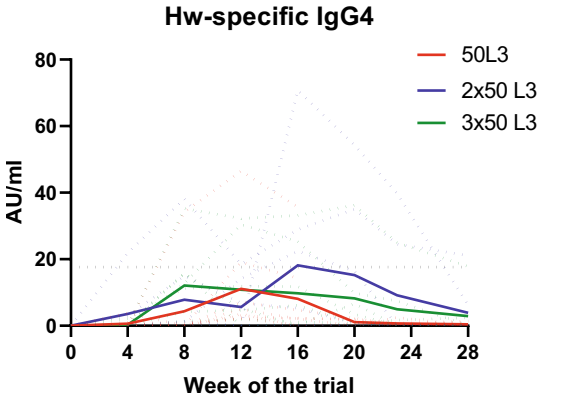


Figure 1. Hypothetical S-shaped curves of repeated doses, assuming that dose 1 leads to on average 19 eggs, dose 2 to 15, and dose 3 to 12.

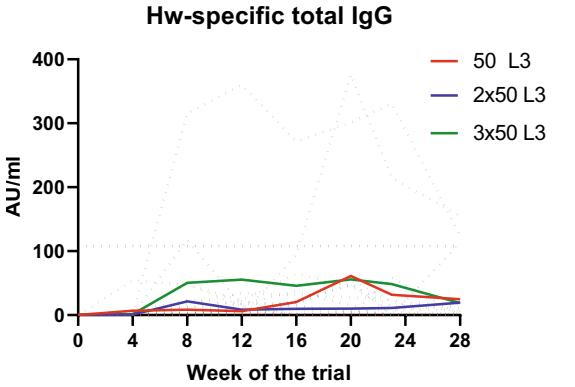
References for supplementary material

1. Verweij JJ, Pit DS, van Lieshout L, et al. Determining the prevalence of Oesophagostomum bifurcum and Necator americanus infections using specific PCR amplification of DNA from faecal samples. Tropical medicine & international health : TM & IH 2001; 6:726-31.
2. Kaisar MMM, Brienen EAT, Djuardi Y, et al. Improved diagnosis of Trichuris trichiura by using a bead-beating procedure on ethanol preserved stool samples prior to DNA isolation and the performance of multiplex real-time PCR for intestinal parasites. Parasitology 2017; 144:965-74.
3. Verweij JJ, Brienen EA, Ziem J, Yelifari L, Polderman AM, Van Lieshout L. Simultaneous detection and quantification of Ancylostoma duodenale, Necator americanus, and Oesophagostomum bifurcum in fecal samples using multiplex real-time PCR. The American journal of tropical medicine and hygiene 2007; 77:685-90.
4. Hoogerwerf MA, Coffeng LE, Brienen EAT, et al. New Insights Into the Kinetics and Variability of Egg Excretion in Controlled Human Hookworm Infections. The Journal of infectious diseases 2019; 220:1044-8.
5. Stan Development Team (2019). RStan: the R interface to Stan. R package version 2.18.2, http://mc-stan.org/.

Supplementary figures

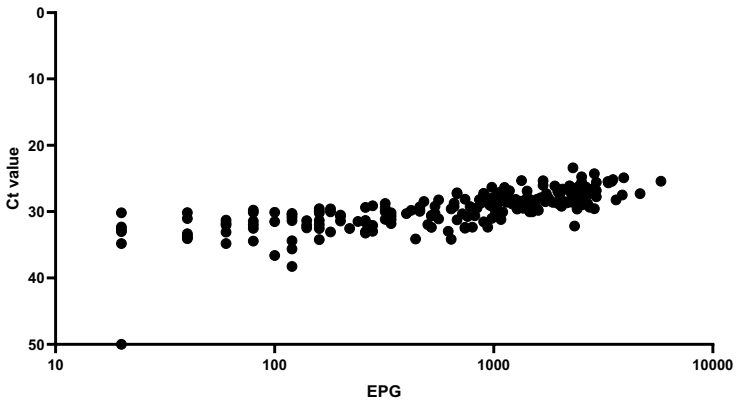


A

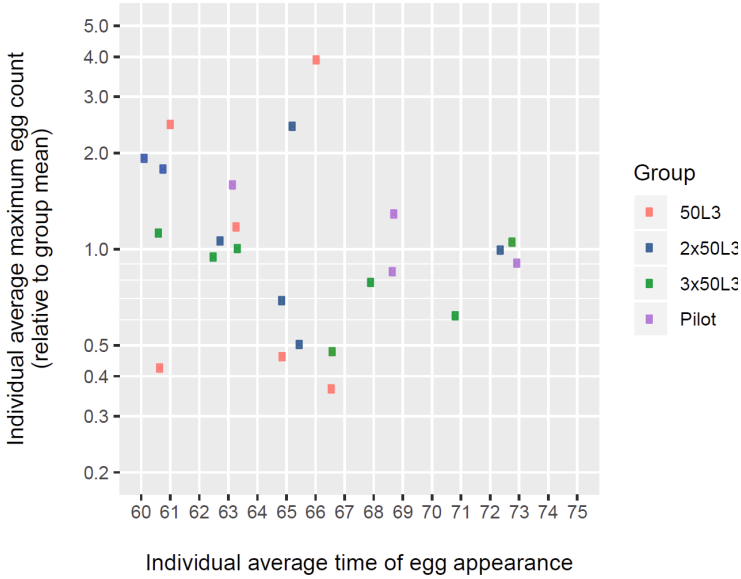


B

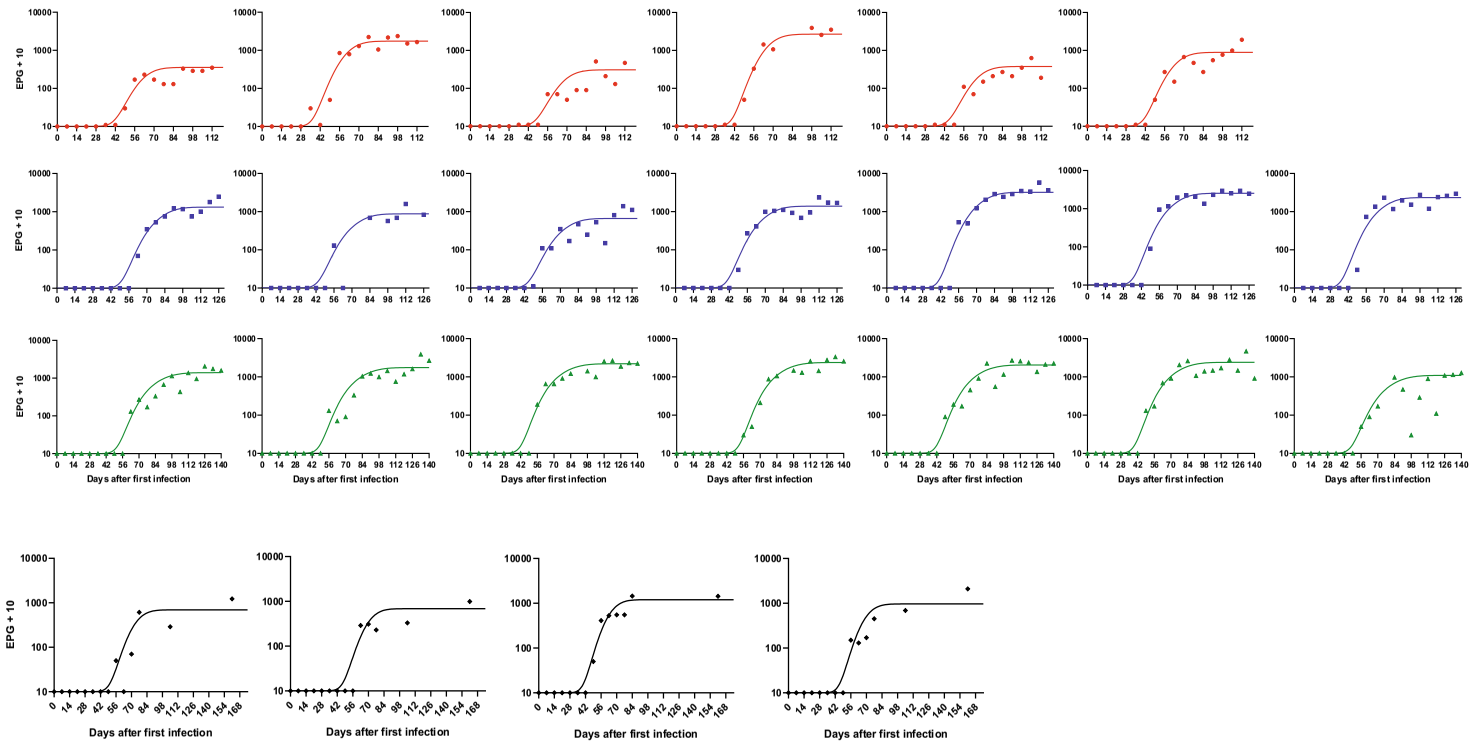
Supplementary figure 1. IgG4 antibody responses (panel A) and total IgG responses (Panel B) during the trial for individual volunteers (dotted lines) and mean per study group (continuous lines) Horizontal dotted line indicates threshold for seroconversion (rise from baseline 4x above SD). (50L3: red lines, 2x50L3: blue lines, 3x50L3: green lines)



Supplementary figure 2. Correlation between Kato-Katz measurements and PCR Ct-values. Spearman's rho: r=-0.79, p<0.001

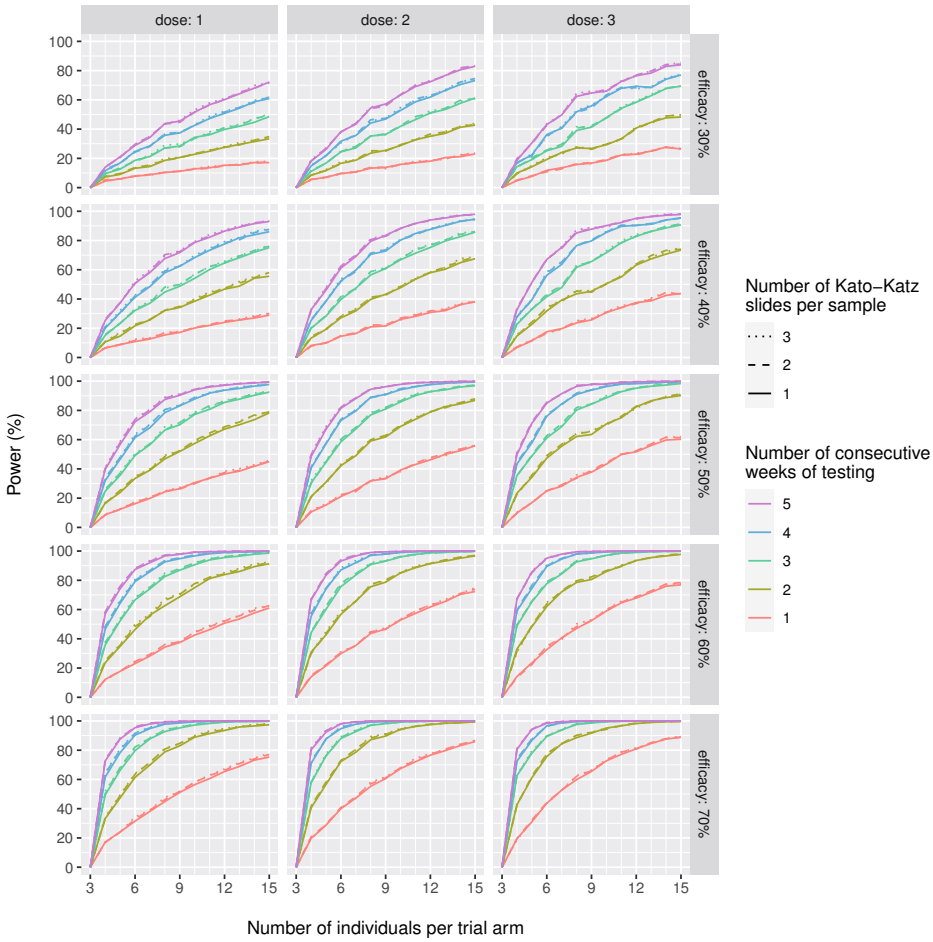


Supplementary figure 3. Relation between appearance of eggs and plateau level. X-axis: individual time of egg appearance in days, plotted against the dispersion from group mean of individual maximum egg count. Pilot study: 4 volunteers included in previous study with infectious dose of 50 L3.



Supplementary figure 4. Individual measured (dots) and estimated (S-shaped curve) egg counts

Red circles: 50L3, blue squares 2x50 L3, green triangles 3x50 L3, bottom panel in black diamonds displays data from pilot study (infectious dose 50L3)



Supplementary figure 5. Power calculation using Bayesian modelling of egg counts showing the number of individuals per trial arm in a vaccine trial assuming 50% reduction in egg counts of the vaccine versus the placebo group, following 1, 2 or 3 doses of 50L3 and assuming vaccine efficacy between 30-70%. The number of slides per KK samples are shown as dotted (3 slides), dashed (2 slides) and solid (1 slide) lines. Different colors represent the number of weekly samples taken.

Chapter 5

Dynamics of the bacterial gut microbiota during controlled human infection with *Necator americanus* larvae

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Abstract

Hookworms are soil-transmitted helminths that use immune-evasive strategies to persist in the human duodenum where they are responsible for anemia and protein loss. Given their location and immune regulatory effects, hookworms likely impact the bacterial microbiota. However, microbiota studies struggle to deconvolute the effect of hookworms from confounders such as coinfections and malnutrition. We thus used an experimental human hookworm infection model to explore temporal changes in the gut microbiota before and during hookworm infection. Volunteers were dermally exposed to cumulative dosages of 50, 100 or 150 L3 *Necator americanus* larvae. Fecal samples were collected for microbiota profiling through 16S rRNA gene amplicon sequencing at weeks zero, four, eight, fourteen and twenty. During the acute infection phase (trial week zero to eight) no changes in bacterial diversity were detected. During the established infection phase (trial week eight to twenty), bacterial richness (Chao1, $p=0.0174$) increased significantly over all volunteers. No relation was found between larval dosage and diversity, stability or relative abundance of individual bacterial taxa. GI symptoms were associated with an unstable microbiota during the first eight weeks and rapid recovery at week twenty. *Barnesiella*, amongst other taxa, was more abundant in volunteers with more GI symptoms throughout the study. In conclusion, this study showed that clinical GI symptoms following *N. americanus* infection are associated with temporary microbiota instability and relative abundance of specific bacterial taxa. These results suggest a possible role of hookworm-induced enteritis on microbiota stability.

Introduction

Helminths such as hookworms can have beneficial effects on auto-immune diseases[1, 2] such as celiac disease,[3, 4] but also cause eosinophilic gastroenteritis, anemia and protein loss and are therefore responsible for a high burden of disease in low- and middle-income countries.[5] As a part of the human gut microbiome in developing countries with a high rate of hookworm infections, hookworms can exert evolutionary pressure on the bacterial gut ecosystem through intestinal motility, mucin glycosylation, mucus secretion, epithelial damage and worm products.[6] For example, several helminths and their products have been shown to increase permeability of monolayers in cell culture.[7, 8] In addition, worm products can have direct antibacterial activity, thereby having the potential to directly alter the bacterial gut microbiota.[9, 10] However, the complex interplay between hookworms such as *Necator americanus* and the bacterial microbiota is largely unknown.

In real-world settings, most studies have focused on characterizing the gut microbiota of infected individuals in highly endemic regions with limited follow-up on individuals.[11, 12] However, effects of confounding factors cannot always be uncoupled from the bacteria-helminth relationship, as mixed helminth infections, other intestinal diseases and malnutrition are also common in endemic regions.[13] These factors may explain a large part of inconsistent findings between studies.[13] In addition, due to the high inter-individual variability of the microbiome, cross-sectional studies only yield limited information.

In the current study, we studied the effect of hookworm infection on the gut microbiota using a longitudinal model for human hookworm infection in healthy volunteers (controlled human hookworm infection model, CHHIM). Here, samples can be obtained at baseline, where the gut microbiome is unperturbed, and longitudinally in order to model the ecosystem's dynamics and perturbation after exposure to *N. americanus*. This model allows for studying the changes in the bacterial microbiota in the different stages of infection; skin penetration, (pulmonary) migration and gut establishment, a process which takes roughly four weeks.[14] In addition, potential confounding factors which could affect the outcome of studies investigating bacterial-helminth interactions are minimized.[13] The power of CHHIM to investigate changes in the human microbiota has been demonstrated in a small study where patients with celiac disease were experimentally infected.[15-17] Although a very small study (n=8), a minor increase in richness was seen after infection, while no changes in community, diversity or abundance of individual taxa occurred.[15] This study was however limited by the use of a low infectious inoculum of twenty larvae which resulted in egg output much lower than commonly found in endemic areas and by only including patients with celiac disease.[18] In this study we infected individuals with 50-150 L3 larvae, after which we found mean egg counts of around 1500 eggs per gram feces at plateau level, which is more in line with the endemic situation where mild infection is defined by WHO as <2000 eggs per gram feces.[18, 19] Still, infection levels in CHHIMs are not fully comparable to areas with a high infectious burden, defined as >4000 eggs per gram by WHO. The current study had

two main aims. First, to investigate temporal changes in the gut microbiota in response to different dosages (ranging from 50 to 150L3) *N. americanus* larvae in healthy young volunteers. Second, to investigate temporal differences in the gut microbiota between healthy volunteers experiencing different amounts of clinical symptoms.

Results

Results of the clinical trial have been published elsewhere.[19] Briefly, of the 24 randomized volunteers, twenty completed follow-up and were included in the microbiota analysis, providing a total of 100 fecal samples. The primary aim of the clinical trial was to investigate the effect of repeated infectious dosages on hookworm egg excretion and variability. From our 20 volunteers, eight (40%) were male and twelve (60%) were female and the mean age was 25.7 years (standard deviation 6.1 years). No volunteers had used antibiotics in the six weeks prior to enrolment. All volunteers developed patent hookworm infection as shown by positive microscopy for hookworm eggs at a median of eight weeks (range five-nine) after first skin infection with L3 larvae.[19] Abdominal adverse events in many volunteers starting at three to four weeks after infection were paralleled by eosinophil increases which likely marked the timepoint of arrival and establishment of the hookworm in the duodenum. Abdominal adverse events consisted of bloating, nausea, vomiting, diarrhea or abdominal cramping. Volunteers exposed to higher larval dosages (n=6 volunteers with 50L3 larvae, n=7 with 100L3 larvae and n=7 with 150L3 larvae) generally had higher egg loads in feces, but there was no relation between cumulative larval dosage and number and severity of adverse events.[19] Based on the severity, number and duration of adverse events, nine volunteers were classified into the “hi” GI symptoms group, whereas eleven were categorized into the “lo” GI symptoms group by two independent physicians. All volunteers with severe adverse events were placed in the “hi” category, together with two volunteers who did not have severe adverse events but moderate adverse events of long duration (Table S1). Median number of related abdominal adverse events was 4 in the whole cohort (range 0-10), split per dosage group this was 4.5 in the 50L3 group, 4 in the 100L3 group and 3 in the 150L3 group. This difference was not statistically significant. Originally, twelve volunteers were classified in the “hi” category, however, due to severe abdominal adverse events three participants from the “hi” group were treated early and could not be included in the microbiota analysis.

On average 28,600 reads (range=6,524-49,476 reads, median 29,244 reads) were generated per volunteer sample (total n=100), resulting in a total of 1,258 unique OTUs (after filtering on 0.005% abundance). Both positive controls were highly similar to theoretical expectations, with the DNA standard (n=2) being more similar to theoretical expectation than DNA extraction controls (n=3) based on Bray-Curtis distances (Figure S1A + B). Two out of three negative extraction controls did not contain any reads post-filtering and one negative control contained only five reads in total.

High individual-specific clustering despite N. americanus infection

To explore data and understand potential shifts in microbiota composition, we performed t-Distributed Stochastic Neighbor Embedding (t-SNE), using Bray-Curtis dissimilarity of all samples, which revealed individual-specific clustering (Figure 1A), but no clear clustering according to GI symptoms group (Figure 1B) or larval dosage group (Figure 1C). Two individuals clustered separately, one of which had taken a course of amoxicillin (volunteer 18), while the other was strictly vegetarian (volunteer 11). It needs to be taken into account that t-SNE preserves the local structure rather than the global structure of the data (like in PCA), so large distances in the 2D plot do not necessarily reflect large distances in the high-dimensional space. Other people taking antibiotics during the study course, all for reasons unrelated to the study, did not show large compositional changes (Figure 1 + Figure S2 + Table 1).

Table 1: Volunteer characteristics. Included information is larval dosage group, GI symptom group and whether individuals took antibiotics during the study.

Volunteer ID	Dosage group	GI-symptoms	Gender	Antibiotic use
1	C	Light	Male	Amoxicilin, 3 times per day 500mg, for 7 days, between week 0 and 4.
2	C	Heavy	Female	
3	C	Light	Male	
4	B	Heavy	Female	
5	A	Light	Female	
6	C	Heavy	Female	
7	B	Heavy	Female	
8	A	Light	Female	
9	B	Heavy	Male	Single iv Kefzol administration, between week 14 and 20.
10	B	Light	Male	
11	C	Light	Male	
12	A	Heavy	Male	
13	C	Light	Male	
14	A	Heavy	Female	Single Azitromycin (1000mg), between week 4 and 8.
15	B	Light	Female	
16	A	Heavy	Female	Amoxicilin, 3 times per day 500mg, for 5 days, between week 0 and 4.
17	B	Light	Male	
18	B	Heavy	Female	Amoxicilin, 3 times per day 500mg, for 6 days, between week 0 and 4.
19	C	Heavy	Female	
20	A	Light	Female	

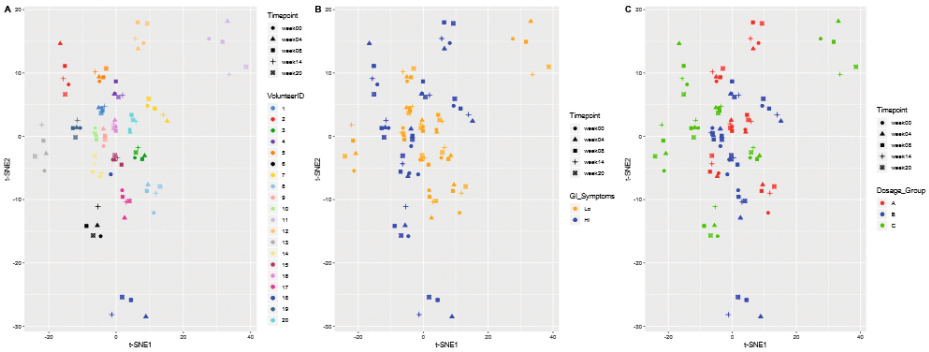

Figure 1: t-Distributed Stochastic Neighbor Embedding (t-SNE), using Bray-Curtis dissimilarity. Volunteer-specific clustering is observed, with no obvious shift according to timepoint. Volunteers (n=20) are colored by their volunteer number (A), the GI symptoms group (B) or the larval dosage group (C), while each shape corresponds to a timepoint.

Larval dosage does not differentially impact alpha diversity or stability in the acute phase of infection

To investigate whether larval dosages induce differential effects on the gut microbiota, we compared alpha diversity and stability measures between dosage groups. Group A (n=6 volunteers) received 50L3 larvae, group B (n=7 volunteers) 100L3 larvae and group C (n=7 volunteers) 150L3 larvae (Figure 2). First, we investigated potential changes in alpha diversity and stability during the acute phase of infection (which includes trial week zero, four and eight). To test for differences in these parameters, normality was tested using Shapiro-Wilk test and equal variance using an F-test. Subsequently, depending on outcome of these tests, appropriate tests were performed.

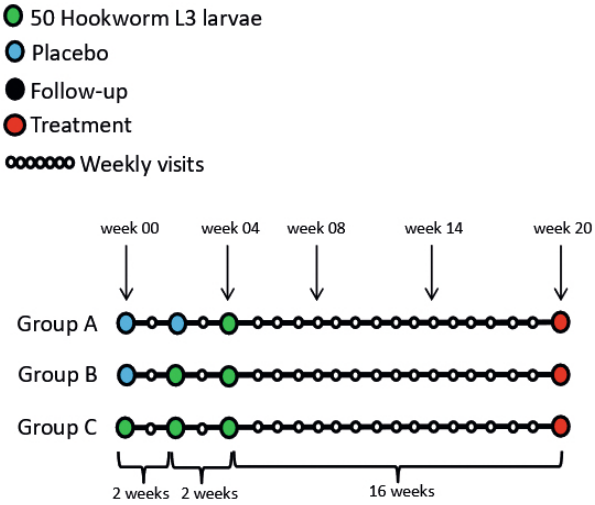

Figure 2: Study design. At indicated trial weeks (week zero, four, eight, fourteen and twenty) feces were collected for microbiota analysis.

We started to compare the effect of acute infection compared to an uninfected state. As at trial week four group A was not yet exposed, and group C twice (Figure 2), we compared their deltas at trial week four (Chao1/Shannon at week four minus Chao1/Shannon at week zero). Group B was not included in this analysis, since this group was infected at week two of the study and thus at the time samples were taken (week four), patent infection was not yet established in the gut. No differences in deltas were found at OTU level (independent t-test, p=.76) or genus level (Mann-Whitney test, p=.61) between A and C. No difference within group C between trial week zero and four at OTU level (paired t-test, p=.49) or genus level (paired t-test, p=.41) was observed either (Figure 3A + B). The same tests were performed for Shannon diversity, stability measures (1-Bray-Curtis and 1-Jaccard, Welch t-test p=.742 and independent t-test p=.219 respectively), but no differences were observed (Figure 3C-F).

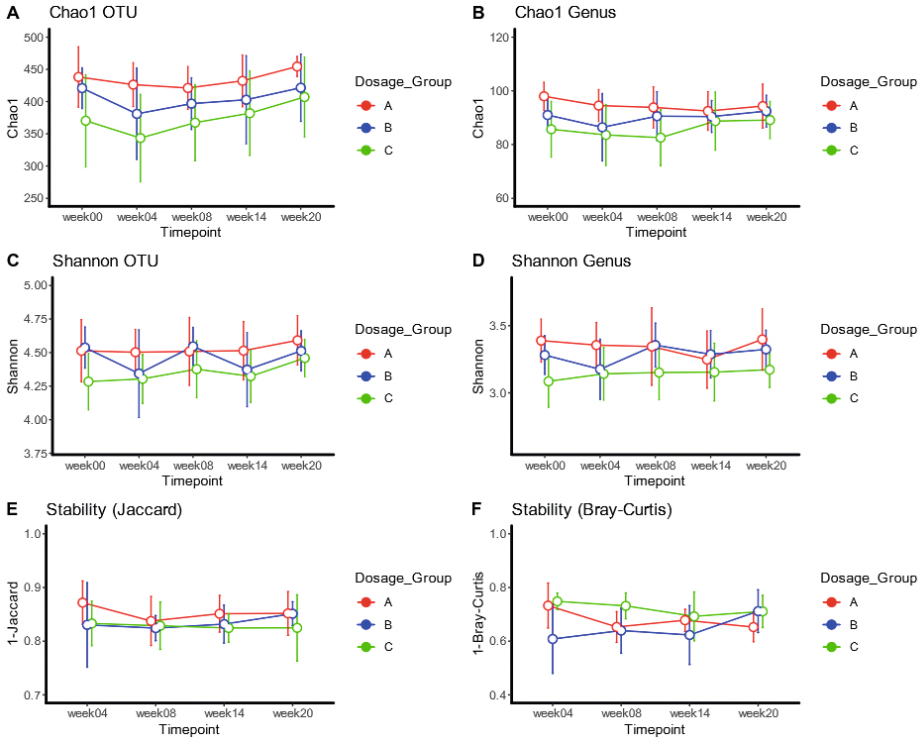

Figure 3: Richness (Chao1) and diversity plots at OTU and genus level (A-D) and stability measures (Jaccard and Bray-Curtis) for larval dosage groups (E-F). Total infectious dosages for group A (red): 50L3 larvae, group B (blue): 100L3 larvae and C (green): 150L3 larvae. Means and the 95% CI of the standard error of the mean are displayed. *p<.05, **p<.01, ***p<.001

At trial week eight all volunteers likely had established intestinal hookworm infection. Therefore, trial week zero and eight were compared to all individuals. No differences were observed in Chao1 at OTU level (Wilcoxon signed rank test, p=.391) nor at genus level

(Wilcoxon signed rank test, p=.152) (Figure 3A + B). No differences were observed in Shannon diversity either (Figure 3C + D).

In conclusion, we did not observe any changes in diversity or stability of the microbiota during the acute phase of infection between or within dosage groups.

Microbiota richness increases in all volunteers during the established infection phase
Subsequently, we investigated the effect of established infection (trial week eight to twenty) on the gut microbiota using a linear mixed model (LMM). Chao1 at OTU level increased from trial week eight to twenty (p=.0174), and less clearly so at genus level (p=.0905) over all volunteers, but no differential effect between larval dosage groups was observed (Figure 3). No differences in Shannon diversity or stability were seen between or within larval dosage groups or over time across all individuals. In conclusion, we found an increased richness over all volunteers during established infection, but Shannon diversity and stability remained unchanged. It is however unclear whether this increased richness is a direct result of the infection, as no non-infected group was available at this time point.

Individual bacterial taxa do not display major differential changes between larval dosage groups
Lastly, we performed differential abundance analysis between larval dosage groups A and C over time using the MetaLonDA package and investigated an overall hookworm effect over all volunteers using DESeq2. Group B was not included in this analysis, as data was only available at timepoint two weeks after infection. At this timepoint no patent infection is established in the gut, but systemic effects or effects of early larval migration cannot be excluded. In addition, the antibiotic-induced effect on the gut microbiota of volunteer 18 (who was in group B) could affect the analysis, especially considering the small number of volunteers in each dosage group. MetaLonDA at genus revealed that *Dorea* was significantly increased between trial week four and week eight in group A (p=0.04) (Figure S3A). However, as this is the only differentially abundant taxa at a single time interval, this is unlikely to represent biological relevance. This analysis was also performed at OTU level (Table S2 and Figure S4A). No differences in relative abundance were observed across all volunteers from trial week zero to twenty either at both genus and OTU level (adj. p-value > 0.05). We subsequently continued analyzing the relationship between the gut microbiota and severity of GI symptoms.

Hi GI symptoms were associated with an unstable microbiota at trial week eight

Our next goal was to investigate whether baseline differences in microbiota composition could be associated with severity of GI symptoms, so we compared the “lo” (n=11 volunteers) and “hi” (n=9 volunteers) GI symptoms groups. No difference in Chao1 was observed at OTU level (independent t-test, p=0.244) or genus level (Mann-Whitney test, p=0.446) (Figure 4A

+ B) at week zero. Comparing week zero with week eight did not show differences at OTU level (Wilcoxon signed rank test for the “lo” group, p=0.391 and paired t-test for “hi” group, p=0.382, Figure 4A) or genus level (Wilcoxon-signed rank test for “lo”, p=0.152 and paired t-test for “hi”, p=0.132) (Figure 4B). No differences were seen at trial week eight between symptom groups at OTU level (Mann-Whitney test, p=0.412) or genus level (independent t-test, p=0.674) (Figure 4A + B). The same tests were performed for Shannon diversity, but no differences were observed either (Figure 4C + D).

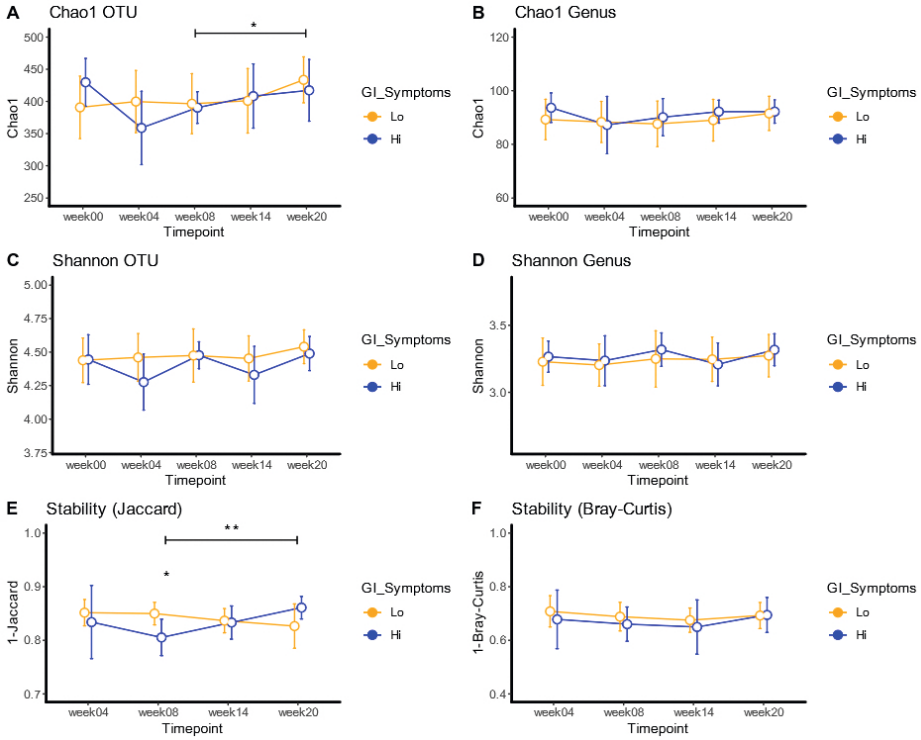

Figure 4: Richness (Chao1) and diversity plots at OTU and genus level (A-D) and stability measures (Jaccard and Bray-Curtis) for GI symptoms groups (E-F). For Figure A, significance between trial week eight and week twenty is for the “lo” GI symptoms group (orange). For Figure E, significance between trial week eight and week twenty is for the “hi” GI symptoms group (blue). Means and the 95% CI of the standard error of the mean are displayed. *p<.05, **p<.01, ***p<.001.

Microbiota stability of the ”hi” GI symptoms group was significantly decreased at trial week eight as compared to the “lo” GI symptoms group (Jaccard, independent t-test, p=.036) (Figure 4E). No difference was found using Bray-Curtis dissimilarity (Figure 4F).
In conclusion, we did not find any changes in alpha diversity between GI symptoms groups, but microbiota stability (here Jaccard) was significantly reduced in the “hi” GI symptoms group at trial week eight compared to the “lo” GI symptoms group.

Microbiota stability recovers over time

LMM was applied to investigate changes in diversity during the established phase in the GI symptoms groups. We found a significant increase in Chao1 from trial week eight to twenty in the "lo" GI complaints group at OTU level (LMM, p=.045) (Figure 4A), but not at genus level (LMM, p=.120) (Figure 4B). No differences were found for Shannon diversity.

As previously mentioned, stability in the "hi" GI symptom group was reduced at trial week eight. This instability quickly recovered from trial week eight to twenty (Jaccard, paired t-test, p=.004) (Figure 4E). In addition, the slopes between the symptom groups were significantly different from trial week eight to twenty, confirming the recovery within the "hi" symptoms group (LMM interaction, p=.002). This also means that there was increased dissimilarity in the "lo" symptoms group in this time period. We further hypothesized that this stability may perhaps be related to eosinophil count, but we did not find a significant correlation between eosinophil count and microbiota stability when stratifying by trial week (Figure S5A). No differences in Bray-Curtis dissimilarity were observed during established infection, nor was a correlation found with eosinophil counts at any trial week (Figure S5B). Eosinophil counts are visualized per time point in Figure S6.

In summary, the "hi" GI symptom group was characterized by transient microbiota instability and subsequent recovery.

Specific bacterial taxa differ between symptoms groups during the entire study course

To investigate whether changes in individual bacterial taxa over the entire study course could be linked to symptom groups ('hi' n=9, 'lo' n=11), we employed the MetaLonDa package (Figure 5, Figure S3B-F and Table S2) and found several bacterial taxa in the "hi" GI symptoms group significantly increased at genus level. *Barnesiella* was found to be significantly increased in this group at all intervals from trial week zero to week twenty (p<.05), *Lachnospiraceae_ND_3007* was significantly higher from trial week zero to fourteen (p<.05), *Bilophila* was more abundant between trial week zero and four, and between trial week eight and twenty (p<.05) and *Escherichia-Shigella* was significantly more abundant between trial week zero and four (p<.05). In the "lo" GI symptoms group, *Allisonella* was more abundant between trial week fourteen and twenty, at which time a chronic infection had been established (p<.05). Lastly, relative abundance over time of these significantly different genera was visualized, to investigate whether significance was driven at the group level or by a single individual (Figure S3B-F). This showed that the difference in *Escherichia-Shigella* was driven by a single person, namely volunteer 18, while all other differences were largely group-driven. When analysis was repeated without volunteer 18, *Escherichia-Shigella* was indeed non-significant (p=.292). In addition, the association with *Barnesiella* and *Bilophila* persisted throughout the study (p=.074 and p=.072), *Allisonella* became more abundant in the "lo" group throughout the entire study (p=.017) and *Oscillibacter* was more abundant in the "lo" group (p=.012) from week zero to fourteen (Table S2). All analyses were also performed at OTU level (Figure S4B+C and Table S2). These results confirm that

differences in relative abundance of taxa between the symptom groups were largely group-driven, apart from *Escherichia-Shigella*.

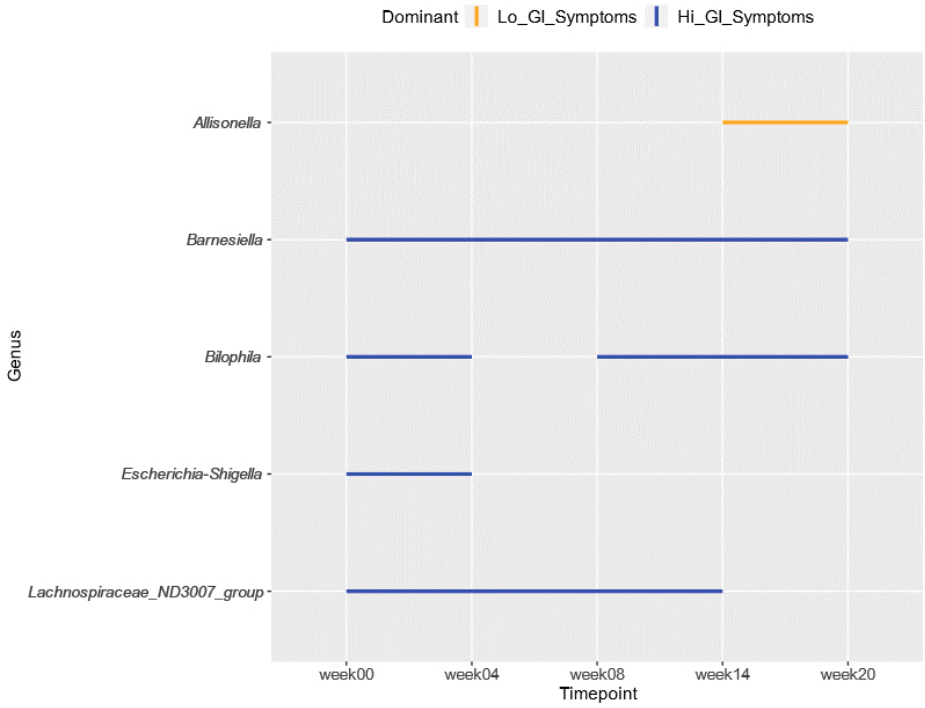

Figure 5: Time intervals of significantly different bacterial genera between GI symptoms groups. Each line interval represents a significant time interval, with significance being considered p<.05.

Orange lines indicate higher abundance in the "lo" GI symptoms group, while blue indicates higher abundance in the "hi" GI symptoms group.

Discussion

Herewith we present the first longitudinal assessment of microbiota changes over the course of an experimental *N. americanus* infection in healthy individuals. Although no convincing relationship between microbiota and larval dosage was observed, stability of the bacterial microbiota was linked to severity of clinical symptoms. In addition, we found several statistically significant changes in relative abundance of individual bacterial taxa over time between symptom groups.

We found a very strong volunteer-specific clustering, despite a patent hookworm infection. This corroborates previous findings showing that the gut microbiota is stable over time in healthy adults at a compositional level[20-22] and the previous assessment of experimental

hookworm infections in patients with celiac disease where minor changes were detected over time. [16]

We detected an interesting link between microbiota stability from trial week eight to twenty and clinical GI symptoms. Recovery of stability in the "hi" symptoms groups leads us to believe that either volunteers with a more unstable microbiota in early weeks post-infection are more likely to experience GI symptoms during the infection, or the GI symptoms are caused by a more severe enteritis that also affects microbiota stability. The latter hypothesis seems most likely whereby symptoms are caused by an eosinophilic enteritis, with eosinophils having been described to correlate with severity of enteritis, [19, 23] and the enteritis may in turn affect the gut microbiota. Although cause and effect cannot be determined, it does suggest an important bacterial-helminth-host interplay, which deserves further investigation.

We observed increased richness over all volunteers during the established infection phase. This is in line with previous studies which have mostly shown that individuals with a parasitic infection show either equal or increased microbial richness and diversity. [11, 15, 24] However, we cannot be fully certain this is an infection-induced effect, as no "no-infection" control group was included in our study. It is unclear why alpha diversity may increase during hookworm infection, although several hypotheses can be formulated. Potentially, the immune regulatory effects of helminths might facilitate increased bacterial microbiota richness and diversity. [25, 26] On the other hand, expansion of the current bacterial community could also be promoted. Another hypothesis is that *N. americanus* affects the gut metabolome. While the effect of *N. americanus* infection on the full gut metabolome has not yet been investigated in humans to our knowledge, short chain fatty acid (SCFA) levels were measured in eight volunteers undergoing *N. americanus* infection. [27] Out of these eight volunteers, six showed an increase in total fecal SCFA, while two showed a reduction. [27] Even though this suggests an effect of *N. americanus* on the gut metabolome, this should be confirmed with a larger sample size.

We observed several changes in individual bacteria taxa between the GI symptoms groups, although the biological relevance of these changes remains unclear. The increased abundance of *Barnesiella* and the decreased abundance of *Allisonella*, a histidine-consuming and histamine-producing taxon, in the "hi" symptoms group are puzzling. While *Barnesiella* is associated with a healthy microbiota and beneficial intestinal effects, [28-31] *Allisonella* and its metabolic product histamine are associated with increased GI symptoms. [32-34] This would counterintuitively suggest that individuals with a microbiota generally regarded as more beneficial respond more heavily to hookworm invasion. The opposite holds true however for *Bilophila*, a taxon which thrives under high-fat and animal-based diets. [35, 36] It is associated with increased inflammation, impaired intestinal barrier function and production of hydrogen sulfide. [35-37] Being more abundant in the "hi" symptom group, this contradicts with the hypothesis that a more beneficial microbiota responds more vigorously

to hookworm invasion. All in all, the relevance of these findings should be tested in larger groups and with more functional techniques than 16S rRNA gene amplicon sequencing. In addition, answering this hypothesis would require a clear definition of a 'healthy or beneficial' microbiota, a phenomenon which is currently incompletely understood.

The current study had several strengths and limitations. During the study period, five volunteers were prescribed antibiotics. While a clear effect of antibiotic usage was only observed in volunteer 18, we cannot exclude that an effect occurred in other volunteers and might have confounded our results. However, given the small sample size we were unable to partial out any confounders (e.g. antibiotic use and diet) which is a limitation of our study. It should be noted that investigating fecal material is probably not reflective for local microbiota changes in the duodenum and ideally duodenal biopsies would be taken, as was done previously by Giacomin *et al.* [17] This would however pose a sharply increased burden to volunteers. By using healthy volunteers, the effects of *N. americanus* infection on the bacterial gut microbiota could be studied without many external confounders. In addition, the longitudinal study setup allowed us to investigate the dynamics of the bacterial gut ecosystem. Implementation of well-controlled longitudinal studies were also recently described to be crucial for advancing the microbiome field. [38, 39] Future studies should include functional approaches (e.g. coupling metagenomics with metabolomics) to obtain insight into potential changes in microbial metabolism which could be a result of *N. americanus* infection. By using positive and negative controls, we confirmed that we should investigate richness and diversity both at OTU and genus level, and that our DNA extractions were well performed, although minimal contamination may have occurred. The original clinical trial reported the highest egg counts described in CHHI experiments in literature yet, reaching egg counts similar to those seen in mild infection in endemic areas, allowing for better comparison with natural infection. [18] Although the groups in this controlled study were small, the combination of infecting healthy volunteers with the highest infectious as of yet and describing their bacterial gut microbiota longitudinally is unique.

In conclusion, this is the first study to investigate longitudinal changes in gut microbiota during *N. americanus* infection in healthy individuals. We observed high stability of the gut microbiota despite this infection over the twenty-week study period, although transient instability was observed in individuals with "hi" GI symptoms. These data open new avenues for exploring helminth-bacterial interaction in the human intestine.

Materials and methods

Twenty-four healthy male and female volunteers aged 18-45 years were included in a randomized controlled clinical trial investigating the effect of repeated infectious dosages on hookworm egg excretion and variability as previously described. [19] Volunteers were dermally exposed with two week intervals to either one, two or three dosages of 50 infectious larvae, resulting in cumulative dosages of 50, 100 and 150 larvae respectively. Study set-up was

such that the 150 larvae group (C) received first infection at trial week zero, the 100 larvae group (B) at trial week two and the 50 larvae group (A) at trial week four. Volunteers were allocated equally to one of three groups at random according to an independently prepared randomization list. Group allocation was defined by the randomization number which was linked to the volunteer identification code at the first infection. Investigators and participants were blinded to group allocation. A schematic overview of study setup can be found in Figure 2.

Culture of larvae and procedure of infection was performed according to a previously described method. [40] In short, infective L3 larvae were cultured from feces from a chronic donor, were suspended in water and applied on upper arms and calves using gauzes. Volunteers were followed for twenty weeks after first exposure, after which treatment with albendazole was given to eradicate the infection. Volunteers visited the trial center weekly for collection of adverse events, safety laboratory evaluation and collection of fecal samples for egg count. Adverse events were collected at weekly visits. For every adverse event, time and date of onset, end, severity and causality was recorded. Adverse events were characterised using ICD-10 as unrelated, unlikely, possibly, probably or definitely related to hookworm infection, and mild (no interference with daily life), moderate (discomfort interfering with daily life) or severe (causing inability to perform usual daily activity). Adverse events were then assessed by two independent physicians who divided the participants in two groups with 'hi' and 'lo' adverse events. Originally, twelve volunteers were classified in the "hi" category. All volunteers with severe adverse events were placed in the "hi" category, together with two volunteers who did not have severe adverse events but moderate adverse events of long duration (Table S1). Consensus was reached for every participant. Unfortunately three participants in the "hi" group withdrew early from the trial due to severe abdominal adverse events and insufficient follow-up fecal samples were collected to include these participants in the microbiome analysis. For analysis, adverse events scored as possibly, probably and definitively related were considered related and were included in the symptom grouping. Duration of adverse events was recorded. Detailed information on adverse events for each volunteer can be found in Table S1. Samples for analysis of fecal microbiota were collected at baseline and trial weeks four, eight, fourteen and twenty of the trial. For analysis of the relation between clinical symptoms and fecal microbiota, gastrointestinal symptoms were categorized as either "hi" or "lo". [19]Fecal egg counts were measured by microscopy using Kato-Katz. Eosinophils were measured weekly and egg counts were measured weekly from trial week five using Kato Katz microscopy. The trial was approved by the LUMC IRB (P17.224) and was registered at clinicaltrials.gov under NCT03257072.

Microbiota analysis

Fecal samples were aliquoted and immediately stored at -80°C. DNA was extracted from 0.1 gram feces using the Quick-DNA™ Fecal/Soil Microbe Miniprep Kit (ZymoResearch, CA, USA) according to manufacturer instructions with minor adaptations, as described previously. [41]

Quality control, library preparation and sequencing were performed by GenomeScan B.V. (Leiden, The Netherlands) using the NEXTflex™ 16S V4 Amplicon-Seq Kit (BiooScientific, TX, USA) and the Illumina NovaSeq6000 platform (paired-end, 150bp). Raw read processing was performed using the NG-Tax 0.4 pipeline with following settings: forward read length of 120, reverse read length of 120, ratio OTU abundance of 2.0, classify ratio of 0.9, minimum threshold of $1*10^{-7}$, identity level of 100% and error correction of 98.5, using the Silva_132_SSU Ref database.[41-43] The obtained OTU table was filtered for OTUs with less than 0.005% relative abundance.[44] As quality controls for both DNA extraction and sequencing, we included ZymoBiomics Microbial Community Standard, (Zymo Research, Irvine, California, USA) ZymoBiomics Microbial Community DNA Standard (Zymo Research) and three negative DNA extraction controls. Raw sequencing data is available at ENA (https://www.ebi.ac.uk/ena) under accession number PRJEB36316. All analytical R code will be uploaded to GitHub upon acceptance of this manuscript.

Statistical analysis

All analyses were performed in R (v3.6.1) using the packages phyloseq (v1.28.0), microbiome (v1.6.0), Metalonda (v1.1.5), DESeq2 (v1.24.0), lme4 (1.1-21), lmerTest (v3.1-0).[45-50] Richness and diversity were computed at OTU and genus level, as richness was found to be overestimated based at OTU level in the positive controls. Genus level was obtained by agglomerating OTUs at genus level. Bray-Curtis and Jaccard indices were computed at genus level. Bray-Curtis and Jaccard indices were computed intra-individually, using trial week zero as the baseline measurement. As Bray-Curtis and Jaccard indices are dissimilarity indices, we computed 1- respective index to obtain similarity, where a value of 1 represents 100% similarity. For alpha diversity and stability measures, data was split into an “acute infection phase” (trial week zero to eight) when most symptoms occurred and an “established infection phase”(trial week eight to twenty) when symptoms subsided. To test for differences in these parameters, normality was tested using Shapiro-Wilk test and variance was tested using an F-test. Subsequently, depending on outcome of the normality and variance test, independent t-tests, Welch t-tests, paired t-tests, Mann-Whitney U tests and Wilcoxon signed-rank tests were performed.. Clustering using t-Distributed Stochastic Neighbor Embedding (t-SNE) method was performed using the tsne_phyloseq function with default parameters.[51] t-SNE aims to preserve the local structure of the original high-dimensional space while projecting the data points in a low dimensional (2D) space. All Figure were created in R and only minimally formatted in Adobe Illustrator when necessary.

Correlation analysis

We used Spearman’s Rank correlation to examine the relationship between eosinophil count and microbiota stability. Microbiota stability was defined in the same manner as previously, with Bray-Curtis and Jaccard indices computed intra-individually, using trial week zero as the baseline measurement. As both indices are dissimilarity indices, 1- respective index was computed to obtain similarity. Eosinophil count was measured weekly, and therefore each individual at each time point had a measured eosinophil count. Timepoints were stratified to

account for the repeated measurements design. In order to avoid skewing of the correlation by baseline data, at which point eosinophils were low and microbiota was 100% similar due to baseline to baseline comparison, this timepoint was excluded.

Linear Mixed Models

We performed linear mixed modelling (LMM) using the lmer function from the lme4 package[49] for alpha diversity and both stability indices from trial week eight until week twenty, as all groups had established infection in the gut from trial week eight onwards. Volunteer ID was included as a random intercept to control for inter-individual baseline differences and repeated measurements design. Included fixed effects were dosage group/ symptom group and timepoints. In case an interaction effect was suspected by visually inspecting plots, an additional interaction model was also performed with dosage group/ symptom group*timepoint. Models were checked by inspecting whether residuals were normally distributed using qq-plots. P-values were obtained using the lmerTest package and considered significant when < 0.05. [50]

Time series modelling of individual taxa

Differential abundance testing was performed at genus and OTU level. The metagenomic longitudinal differential abundance method (MetaLonDA) package was used to identify differentially abundant taxa between groups over time. [47] It is a flexible method capable of handling inconsistencies often observed in human microbiome studies and relies on two main modelling components, the negative binomial distribution for modelling read counts and smoothing spline ANOVA for modelling longitudinal profiles. The function metalondaAll was used with the following settings: n.perm=1000, fit.method=”nbinomial”, num. intervals=4, pvalue.treshold=0.05, adjust.method=”BH”, norm.method=”median_ratio”. These settings indicate that the function was run with 1000 permutations using the median ratio method to normalize count data and fitting a negative binomial distribution. Four intervals were included (between each included trial week) and p-values were corrected using the Benjamini-Hochberg procedure. DESeq2 was used to establish an overall time effect across all volunteers using the likelihood-ratio-test (full model included volunteer ID and timepoint, reduced model included volunteer ID) and for identifying differentially abundant taxa in pair-wise comparisons. [48] Prior to the use of both MetaLonDA and DESeq2, genera and OTUs were filtered for presence in at least 25% of all samples. Relevant tests performed are indicated in all Figures and in the text.

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Author contributions

QD and MH wrote drafts of the manuscript. QD performed microbiota data analysis and created Figures. MH, JJ and JK performed the clinical study. AG performed DNA extraction and lab-related procedures. RZ processed raw sequence data. JG provided statistical advice. MR and EK designed the study. MR was the study principal investigator. All authors interpreted data, reviewed manuscript versions and approved the final manuscript.

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Chapter 6

Protective efficacy of short-term infection with *Necator americanus* larvae in healthy volunteers in the Netherlands: a single-center, placebo-controlled, randomised controlled trial

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Abstract

Background

Vaccine development against hookworm is hampered by lack of natural immunity, limiting discovery of mechanisms of protective immunity and new vaccine targets. Immunisation with attenuated larvae has proven effective in dogs and partial immunity has been reached using an irradiated larvae model in healthy volunteers. This study investigates the protective efficacy of immunisation with short-term larval infection against hookworm challenge.

Methods

A double-blinded randomized controlled trial was conducted in the Netherlands at Leiden University Medical Center. Healthy volunteers were randomised to three short-term infections with 50 infectious *Necator americanus* L3 larvae or placebo. Infection was abrogated with a three-day course of 400mg albendazole two weeks after each exposure. Subsequently all volunteers were challenged with 2 doses of 50L3 at a two week interval. Primary endpoint was egg load at week 12-16 after challenge. The study is registered at clinicaltrials.gov under NCT03702530.

Findings

Participants were recruited between November 8, 2018 and December 14, 2018. The first immunisation was conducted December 18, 2018. Twenty-three volunteers were randomised, 15 to the intervention group and 8 to placebo. Immunised volunteers showed a trend towards lower eggs per gram (epg) faeces (geometric mean 571 vs 873, p=0.1). Five immunised volunteers developed a severe skin rash which was associated with 40% reduction in worm burden after challenge (geometric mean 441 epg vs 742 epg after challenge, p=0.003) and associated with higher peak IgG titers.

Interpretation

This is to our knowledge the first study to describe a protective effect of short-term exposure to hookworm larvae and show an association with skin response, eosinophilic response and IgG1.These findings open novel avenues for future vaccine discovery.

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Introduction

Worldwide around 300 million people are infected with hookworms, mostly in tropical or subtropical climates.[1] These nematode helminths of the species *Ancylostoma duodenale, Ancylostoma ceylanicum* or *Necator americanus* are transmitted from human-to-human through the faecal excretion of eggs. Of these, *Necator americanus* is the most prevalent species in humans.[2] The eggs from faeces hatch in warm, humid soil and develop into infectious filariform larvae (L3) which can penetrate the skin of the human host. After invasion of the skin, larvae migrate to the lungs, are coughed up, swallowed and enter the duodenum, where they attach to the duodenal wall and mature into adult worms.[2] Blood loss from the worm intestinal attachment site causes anaemia and malnutrition, especially in high-intensity infections and children and women of childbearing age with inadequate capacity to replenish their iron and protein stores.[2] Mass drug administration programs aim to control the hookworm burden in endemic areas, but due to high levels of re-infection have so far not been successful in eradicating human hookworm infections.[3]

Individuals in endemic areas are repeatedly exposed to hookworm infection, but do not develop protective immunity.[4] Active immune suppression by adult worms has been suggested to prevent the development of protective responses.[5] However, dogs can be immunised through repeated exposure to irradiated hookworm larvae, which cannot mature to adulthood. The irradiated *Ancylostoma* larvae induced a 55-90% reduction in egg output in faeces and a 60% reduction in intestinal worm burden,[6 7] which was replicated in mice.[8] The irradiated larvae are thought to develop until the lung stage where they die, inducing immunity in their lung sojourn.[5] Similarly, short-term infection of hamsters abrogated with antihelminthic treatment before the adult stage resulted in a reduction of intestinal worm burden of 97% upon subsequent exposure.[9] Animal models, however, cannot be directly translated into humans, as hookworm species and immune responses differ between hosts.[4]

Controlled human infection models are unique tools to obtain insight in human immune responses to different pathogens including hookworms, allowing for dissection of (antigen-specific) responses with little interference of coinfections, prior exposure, or simultaneous adult worm immune interference. The controlled human hookworm infection model has been developed with small numbers of larvae to study the possible beneficial effects of hookworm-induced immune regulation in auto-immune diseases.[10-12] Benefitting from this experience, the protective effects of exposure to radiation-attenuated larvae was recently explored in the human host.[13] In this study, exposure to UV-irradiated larvae did not significantly impact egg output after challenge with 30 wild-type L3 as measured by PCR, but a lower number of larvae were recovered after culture of eggs in faeces possibly indicating mildly protective immune responses.[13] However, the high level of variability in egg output in this study, measured on a single stool sample may have hampered the power to detect differences.[12 14] We have previously shown that higher levels of infection (cumulatively 100 larvae) and repeated sampling increases the power of such controlled infection models.[15] In

addition, we hypothesize that a form of chemo-attenuation, using an abrogated infection in which larvae are treated before maturing to the adult stages, in analogy to other parasitic diseases such as malaria, might result in a more homogenous attenuation phenotype. We thus designed a randomized controlled trial investigating the protective efficacy of repeated exposure to short-term larval infection with *Necator americanus*.

Methods

Study design and participants

This study was a randomized, double-blind, placebo-controlled clinical trial investigating the protective efficacy of repeated short-term exposure to hookworm infection. This trial was approved by the LUMC institutional review board (NL66725.058.18) and is registered at clinicaltrials.gov under NCT03702530. All participants provided written informed consent. Healthy male and female volunteers aged 18-45 years were recruited through advertisements on social media and in in publicly accessible areas at Leiden University Medical Center (Leiden, Netherlands). Before inclusion in the trial, potential participants were screened for concomitant illnesses, previous exposure to hookworm or other conditions that could interfere with the trial. Full in- and exclusion criteria can be found in supplement 1.

Randomisation and masking

Volunteers were randomized to the intervention or placebo group in a 2:1 ratio. Treatment was allocated according to a master randomisation list generated using excel with “random number generator” function, which was prepared by an independent data manager and used when preparing the treatment by the manufacturing team. All investigators and participants were blinded to treatment allocation. Individual deblinding envelopes were prepared to allow emergency deblinding for individual participants. Study procedures can be found in the study protocol.

Study procedures

Infective *Necator americanus* L3 larvae were cultured following the principles of Good Manufacturing Practice principles and adhere to the guidance stipulated in the published white paper.[16] Larvae were cultured from faeces provided by a chronically infected donor, according to a modified copro-culture method following procedures previously described.[18] Four chronic donors were part of an ongoing study approved by the institutional review board under P20.100. For infection of the chronic donors, larvae were originally provided by prof A. Loukas (James Cook University, Australia).[12]

The study consisted of an immunisation phase, in which the intervention group was exposed to 50L3 (infective larvae stage L3) on three occasions at three-week intervals and a challenge phase (starting at week 13 of the trial) in which all participants were challenged with 50L3 on two occasions (Figure 1). During the immunisation phase, volunteers were exposed to a dose of 50L3 divided over four sites (both upper arms 10L3, both calves 15L3) for the

intervention group or water for the placebo group. This was followed by treatment with 400mg albendazole for both intervention and placebo group, ingested with fatty food, during three days at a two weeks timepoint after each infection. For the challenge phase, all participants were exposed to 50L3 at week 13 and 15 of the study (7 and 9 weeks after the last immunisation). During the challenge phase participants were followed for 16 weeks and then treated with albendazole except for one volunteer who gave written informed consent to remain infected as a chronic donor in the ongoing study. Trial schedule was based on previous studies showing stabilizing egg excretion after 12 weeks of infection that can be used as primary endpoint, resulting in treatment at week 16.[14] The immunisation schedule was based on previous animal studies using triple immunisations. [6 7] Treatment schedule was determined following national guidelines for the treatment of hookworm infection.[17]

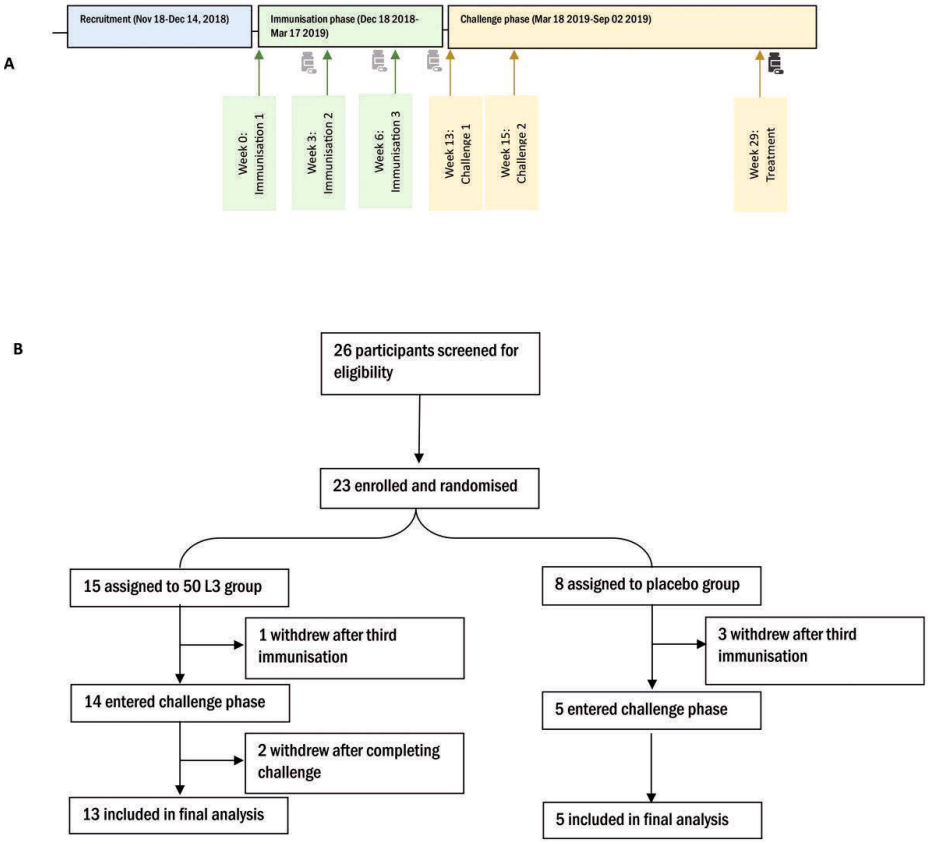


Figure 1. Trial flowchart

At each immunisation and treatment timepoint, volunteers visited the trial centre at Leiden University Medical Center. At these timepoints adverse events were collected, blood samples taken and stool samples collected by the participants. And blood and stool samples taken for safety analyses, all conducted at the study center. In between these timepoints

volunteers reported adverse events through e-mail or phone contact. During the challenge phase volunteers visited the study centre weekly for collection of adverse events, blood and stool samples. Adverse events were classified as unrelated, unlikely related (considered unrelated in dichotomous analyses), possibly related, probably related or definitely related (considered related in dichotomous analyses) and as mild (no impairment to daily life), moderate (some impairment) or severe (unable to carry on daily activities). Photos of skin rash were taken three weeks after each immunisation and weekly for six weeks after the challenge infections. Severity of rash was defined separately through assessment of photos of skin rash independently by two blinded physicians as mild (localised mild erythema) moderate (erythema at site of larval entry without further spread to surrounding skin) or severe (ardent red rash with serpentine lesions extending beyond site of entry or pustules). In case of dispute, photos were re-reviewed by the physicians and consensus was reached through debate.

During the immunisation phase, stool samples were analysed by Kato-Katz and *Necator americanus* real time PCR at week 8, 9 and 12 (2, 3 and 6 weeks after the final immunisation) according to previously described protocols.[16] During the challenge phase Kato-Katz and PCR were performed on weekly stool samples collected from week 18 onwards (5 weeks after challenge). For every sample, two Kato-Katz slides were prepared with 25 milligrams of homogenised stool, read by two separate technicians, egg counts were added and multiplied by 20 to calculate eggs per gram faeces. The quantitative real-time PCR results are expressed as cycle threshold (Ct)-values, which are inversely related with the parasite-specific DNA in the sample.

Hatching assays were performed on stool samples collected at week 25 and 29 (12 and 16 weeks after challenge). For the hatching assay, eggs from 5 gram of collected stool were cultured according to the method previously described.[14 18] Larvae were filtered after culture and washed, after which 250 µL of larval suspension was counted for the number of viable, motile larvae, in triplicate after stimulation with water at 50°C. Samples for antibody analysis were collected before each immunisation, two weeks after the last immunisation, before each challenge and at 4, 8, 12, 16 and 24 weeks after the first challenge (supplementary table 1). Hookworm-specific IgG1, IgG4 and IgE were measured by ELISA using *Necator americanus* L3 extract as hookworm antigen (Supplement 2). Data was expressed as AU/mL and expressed as fold change from baseline level at study start. Seroconversion was defined as at least a three-fold change from baseline.

Serum samples were tested for the presence of cytokines using a commercially available Bio-Rad Bio-Plex Pro Human Cytokine 27-plex assay according to the manufacturer instructions. The following cytokines were measured: FGF, Eotaxin, G-CSF, GM-CSF, IFN-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17A, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF, RANTES, TNF-α and VEGF using the Bio-plex 200 Luminex (Bio-Rad).

Outcomes and statistical analysis

Primary endpoint was defined as the difference in egg load between the intervention and placebo group, with egg load defined as the geometric mean (GM) epg (eggs per gram faeces) as measured by Kato-Katz between weeks 12-16 after first challenge in the per protocol population (all available data). Geometric mean of egg counts per individual was calculated and compared using a student's t-test. Differences in eosinophil counts, antibody response and circulating cytokines were compared with t-test or Kruskall-Wallis for non-parametric data and Chi-square or Fisher's exact test for categorical data. Safety data was assessed on the intention to treat population. A p-value of 0.05 was considered statistically significant. Statistical analyses were performed using IBM SPSS v23. [19]

Sample size was based on statistical modelling performed on our previous controlled infection studies[14 15] which showed that groups of 6 volunteers each with 5 stool samples taken from 12 weeks after challenge and analysed using Kato-Katz would result in 80% power at α=0.05 to detect an expected reduction in egg load of 50%. To anticipate loss to follow-up we increased sample size to 8 volunteers in the placebo group. For immunological dissection of potentially protective responses, we opted to increase the intervention group to 16 participants.

Data integration was performed through sparse Partial Least Squares (sPLS) regression using the 'mixOmics' package (v6.12.2) in R software (4.0.1).[20] PLS regression is suited for high-dimensional datasets and datasets with multicollinearity among the parameters. Sparsity is induced through a Lasso-like regularization, whereby most predictive features are selected. All datasets were included up to week 16 post challenge, antibodies and serum cytokines were normalized to baseline and $\log_2$-transformed. We filtered out features with a variance below 0.1 to reduce the change of spurious, but not necessarily meaningful, results. To determine the number of features to retain within the sPLS regression, we used leave-one-out validation from 1 to 50 features and selected the number of features giving the lowest mean average error, using the 'tune.spls' function, including 8 features in the final model. The leave one out average error was used to select the model with the best prediction after regularization. Plots were made with 'ggplot2' (v3.3.5) and 'pheatmap' (v1.0.12) packages. Heatmap clustering was performed using standard parameters: complete linkage based on Euclidean distance.

Role of the funding source

This trial was funded by a grant from Dioraphte foundation. The funders had no role in the design or conduct of the study, interpretation of data or manuscript preparation.

Results

Between November 8, 2018 and December 14, 2018, 26 volunteers were screened for eligibility, of whom 23 enrolled in the trial on December 18, 2018. The trial flow chart is depicted in figure 1. Six volunteers withdrew informed consent for reasons unrelated to the trial, four in the immunisation phase and two in the challenge phase. Three had been randomised to the placebo group and three to the intervention group. All safety data was included in the intention to treat analysis for adverse events. All available data from Kato-Katz and PCR analyses were taken forward in the per protocol analysis. One volunteer who withdrew after the challenge had enough faecal samples to be taken forward in the analysis of egg counts, the other did not. For immunological analysis only those volunteers who completed the trial were included. Baseline characteristics are included in table 1.

There were no serious adverse events. During the immunisation phase the most common adverse events (AEs) were itching and skin rash (table 1). Severity of itching increased significantly with each subsequent exposure to infective larvae, progressing to severe itching interfering with sleep in 4 of 15 volunteers, all in the immunisation group (figure 2A). Six volunteers were prescribed cortisone topical treatment due to severity of itching after the second and third immunisation. Abdominal AEs were rarely reported during the immunisation phase.

Table 1. Baseline characteristics and adverse events data. *: p<0.05

	Intervention group	**Placebo group**	**All**	
Immunisation phase n=	15	8	23	
Challenge phase n=	14	5	19	
Median age in years (IQR)	23 (20-26)	21 (18-28)	22 (20-26)	
Sex **Male** **Female**	7 (47%) 8 (53%)	3 (12.5%) 5 (62.5%)	10 (43%) 13 (57%)	
Mean Nº AEs per volunteer, related (SD) **Immunisation phase:** **Challenge phase:**	8.6 (1.7) 12.4 (4.8)	1.8 (2.4) 8.6 (5.0)	6.7 (3.6) 11.4 (5.0)	**p=<0.001*** p=0.15
Mean Nº skin AEs, related (itching and rash) (SD) **Immunisation phase:** **Challenge phase:**	7.1 (0.8) 4.8 (0.9)	0.8 (1.0) 3.6 (0.5)	5.3 (3.0) 4.5 (1.0)	**p=<0.001*** **p=0.01***
Nº of volunteers with grade 3 itching after challenge (%)	8 (57%)	1 (20%)	9 (47%)	**p=0.02***

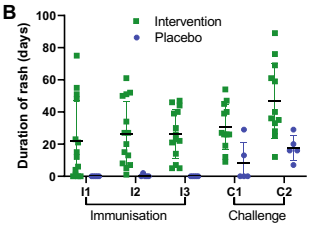
Table 1. Baseline characteristics and adverse events data. *: p<0.05 *(continued)*

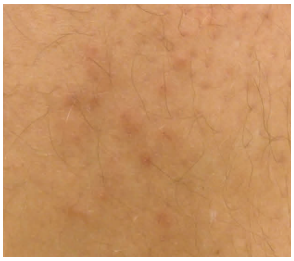
	Intervention group	Placebo group	All	
Mean Nº related abdominal AEs per volunteer in challenge phase (SD)	6.4 (4.0)	4.8 (4.3)	6.0 (4.0)	p=0.46
Nº of volunteers with related grade 3 abdominal AEs in challenge phase (%)	7 (50%)	2 (40%)	9 (47%)	p=0.55

During the challenge phase the most frequently reported AEs were itching and rash after the challenge and gastro-intestinal symptoms (table 1). Itching further increased particularly in the intervention group with a significantly higher number of volunteers reporting grade 3 itching after challenge compared to the placebo group (table 1, figure 2A) and significantly more skin-related AEs in the intervention group (table 1). Rash after challenge lasted significantly longer in the intervention group compared to the placebo group (mean number of days (SD): 30.8 (SD 14.2) vs 8.37 days (12.9), p=0.03 for the first challenge and 46.9 (SD 23.2) vs 17.6 days (8.1) p=0.009 for the second challenge) (figure 2B). Five immunised volunteers developed a grade 3 rash with erythema, blistering, fluid exudate or serpentine eruptions. Such severe rash was not observed in participants in the placebo group (Figure 2C + 2D). Seven volunteers with severe itching were prescribed antihistamines, next to the use of cortisone cream. Eight volunteers in the intervention group reported severe abdominal AEs ranging from three to eight weeks after challenge, either abdominal cramping or nausea and vomiting, all lasting less than 12 hours. Severe abdominal AEs were not correlated with severe skin AEs and were not reported in the placebo group. Respiratory symptoms indicative of pulmonary infiltration were not reported.

A

	Intervention			Placebo			
	Mild	Moderate	Severe	None	Mild	Moderate	Severe
I1	67	33	0	88	12	0	0
I2	60	40	0	88	12	0	0
I3	20	53	27	100	0	0	0
C1	29	42	29	0	80	20	0
C2	7	57	36	0	40	40	20





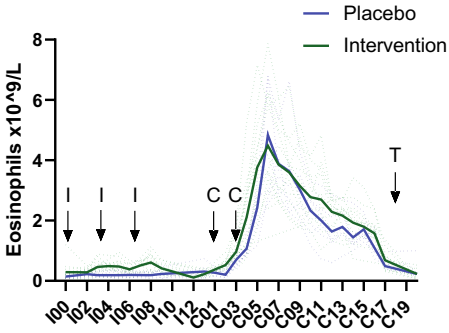


Figure 2. Skin adverse events and eosinophils. Percentage of volunteers with mild, moderate and severe itching after each exposure (A). Duration of rash per group in days after each hookworm exposure (B), individual data in green (intervention) and blue (placebo) with means (black) and SD. I1=first immunisation, I2=second immunisation, I3=third immunisation, C1=first challenge, C2=second challenge. Representative pictures of skin rash at two weeks after second challenge, example of severe rash (C) and mild rash (D).Eosinophil counts in peripheral blood over time (E) in the intervention group (green) and placebo group (blue), solid lines represent group mean, dotted lines represent individual volunteers. X-axis: week of the trialI=immunisation, C=challenge, T=treatment with albendazole

During the immunisation phase intervention group volunteers showed a mild elevation of eosinophils to a maximum of 0.9×10^9/L which was not found in the placebo group ($p<0.001$) and returned to baseline levels at check-up three weeks after immunisation. Eosinophil counts peaked around week 6 after challenge in all volunteers (figure 2E). There were no differences in peak eosinophil count after challenge between the intervention and placebo group (mean intervention group 4.7, placebo 5.2 $\times 10^9$/mL, $p=0.60$). However, counts at 12 and 16 weeks after challenge were higher in the intervention group as compared to the placebo group (week 12: mean intervention 2.3, placebo 1.6 $\times 10^9$/mL, week 16: 1.7 vs 1.1 $\times 10^9$/mL), although this did not reach statistical significance ($p=0.07$ and $p=0.1$ respectively).

Volunteers with severe skin rash after challenge showed higher eosinophil counts than those without, particularly at week 16 after the challenge (mean severe skin rash 2.3 $x10^9$/mL, mild-moderate rash 1.5 $x10^9$/mL, p=0.005). Severe abdominal adverse events were not associated with the height or duration of peak eosinophilia (mean 4.8 $x10^9$/mL for severe AEs, 4.9 for non-severe, p=0.86), nor were severe skin AEs (mean 4.5 $x10^9$/mL for severe skin rash, 5.1 $x10^9$ for mild-moderate rash, p=0.57).

Kato-Katz and PCR for *N. americanus* on stool were performed on the per protocol population (n=13 for immunisation group, n=5 for placebo group) and were all negative at week 8, 9 and 12 of the immunisation phase, proving complete abrogation of the infection by repeated albendazole treatment. All volunteers showed detectable secretion of eggs in faeces by Kato-Katz, detected for the first time at week 7 (n=2), week 8 (n=15) or week 9 (n=1) after challenge.

Egg load after challenge was lower in the intervention group (geometric mean 571 epg, range 372-992) as compared to the placebo group (873 epg, range 268-1484), however this did not reach statistical significance, possibly due to the small sample size with large variability in the placebo group (p=0.10) (Figure 3A).

Volunteers with severe rash had a markedly lower egg load compared to volunteers with mild to moderate rash with a 40% reduction in egg burden (geomean 441 epg vs 742 epg, p=0.003, figure 3B). A difference in egg load between those with and without grade 3 abdominal AEs was not found (GM for severe AEs 549 epg, for non-severe 728 epg, p=0.23). Egg detection by PCR showed similar trends as the microscopy data (mean Ct value for placebo group 27.9, for intervention group 29.1, p=0.08), however the difference between mild to moderate and severe rash was not detected (mean Ct-value for mild to moderate rash 28.6, for severe rash 29.2, p=0.36, Figure 3C and 3D). The hatching assay showed no differences between groups, both intervention and placebo group and those with or without severe rash (supplementary table 2).

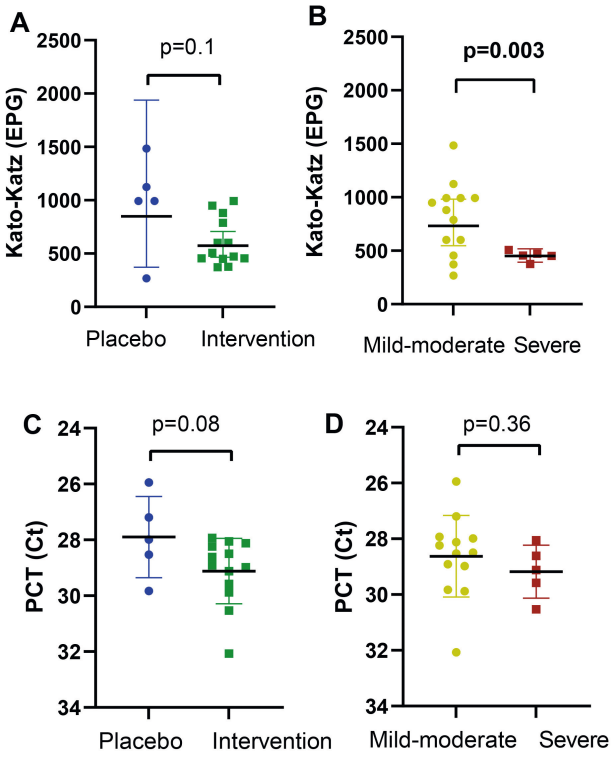


Figure 3. Parasitological analyses after challenge. Hookworm eggs detected in faeces by microscopy, reported as eggs per gram (EPG) (Kato-Katz, A+B) or real-time qPCR (Ct-values, C+D), for volunteers from the intervention group (green) and placebo group (blue) in left panels and volunteers with mild-to-moderate skin rash (yellow) and severe skin rash (red) in right panels. Symbols indicate individual values for egg count or DNA load, lines (black) indicate group GM with 95% CI bars.

IgG1 titers at challenge were significantly increased compared to baseline in those with severe skin rash (fold increase 4.5 vs 1.2, p=0.03). Furthermore, IgG1 titers after challenge peaked to much higher levels in the intervention as compared to placebo group (fold change at weeks 12 after the challenge: 4.0 vs 0.8 AU/mL, p=0.002 for intervention and placebo group respectively; week 16: 3.6 vs 0.9 AU/mL, p=0·03). Similarly, those volunteers with severe rash had higher peak IgG1 after challenge as compared to those with mild-to-moderate rash (week 12: 6.7 vs 1.7 AU/mL, p=0.003; week 16: 6.2 vs 1.5 AU/mL, p=0.02). (figure 4A+B).

Six volunteers showed IgG1 seroconversion after challenge, all were in the intervention group. Four of these had severe skin rash. Seroconversion was significantly more frequent in those with severe rash versus non-severe rash (67% vs. 8%, p=0.02) and was related to duration of rash after second challenge (mean 57.5 days in those who seroconverted vs 27.7

days in those who did not, p=0.003) but not to severe abdominal adverse events (p=1.00) or peak eosinophil count (p=0.24) (supplementary table 3). In the seroconverted group, the mean egg load trended to be lower (GM 507 vs 838 epg, p=0·09) (supplementary table 3).

Changes in hookworm-specific IgG4 were insignificant, e.g. only one volunteer seroconverted. There were no differences between placebo and intervention group in IgG4 titers or between those volunteers with mild-moderate and severe skin rash (Supplementary figure 1). IgE titers did not increase in any of the volunteers over the course of the study.

Circulating cytokines measured in serum showed considerable interindividual variation (supplementary figure 2). No statistically significant differences between groups could be detected. Both IL-4, a Th2-cytokine, and IL-1b, a pro-inflammatory cytokine, showed a decreasing trend after the challenge, whereas the pro-inflammatory cytokine IL-8 showed an increasing trend in all groups.

To integrate analyses of measured parameters (eosinophils, antibodies, cytokines, adverse events) and identify those which most strongly associated with protection in this study, we performed a sparse Partial Least Squares (sPLS) regression.[20] This method finds the combination of measured parameters that has a maximum covariance with the outcome (egg load). Seven features were associated with low egg counts after challenge, all from the challenge phase (figure 4C).

Eosinophil counts during egg production (at challenge phase weeks 12-15) were associated with protection. We moreover confirmed that only adverse events of the skin but not other AEs were associated with protection, as were increases in IgG1 subclass at its peak 12 weeks after challenge (figure 4C and 4D). Correlation analysis of the selected features revealed 3 main clusters of correlated features: egg load with IFNg at two weeks after first challenge (although these were not significantly correlated), the eosinophil levels during egg production and the skin adverse events with IgG1 at week 12 after challenge (Figure 4E). An AUC was calculated for eosinophil numbers in the challenge phase weeks 12-16, which correlated significantly with lower egg loads (rho=-0.59, p=0.012, Pearson test) (Figure 4F). Skin adverse events and eosinophil numbers together separated those with high egg load with those from lower egg loads.

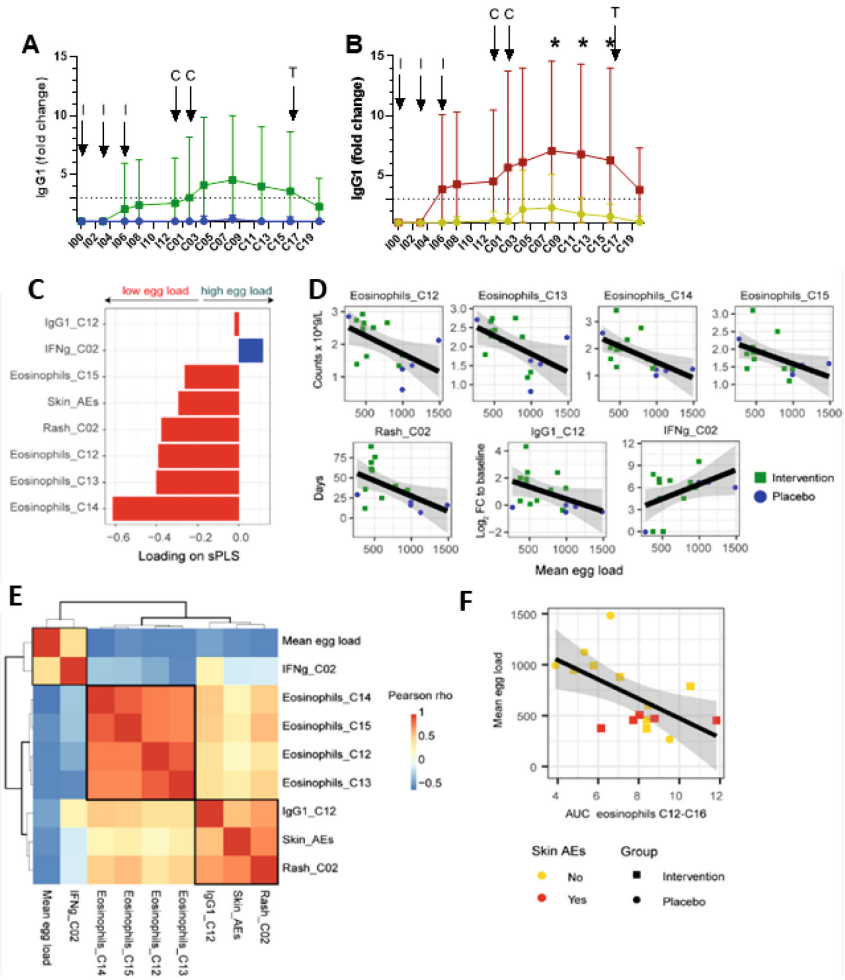


Figure 4. IgG1 titers and sPLS analysis Increases in hookworm-specific IgG1 plotted as fold-change over baseline for placebo (blue circles) versus intervention group (green squares) (A), and volunteers with mild-moderate skin rash (yellow circles) versus severe skin rash (red squares) (B). Symbols indicate mean, error bars SD, dashed line indicate threshold for seroconversion, set at 3-fold rise from baseline. *=p<0.05, I=immunisation, C=challenge, T=treatment with albendazole (C): Features associated with decreased or increased egg load in the sPLS regression model. Loading on the first principal component is shown per feature, with red bars associated with low egg load and blue bars associated with high egg load. (D) Correlation of egg load on the x-axis with selected variables on the y-axis. Green squares indicate participants in the intervention group, blue dots indicate placebo group. Black line and shaded area represent linear regression result and 95% confidence intervals. (E) Correlation matrix of selected features by sPLS regression. Colors indicate the strength and direction of Pearson rho value. (F) Correlation of egg load on the y-axis with the area under curve (AUC) of eosinophil counts during the egg production phase weeks C12-C16. Individuals are depicted with (red) or without (golden) severe skin adverse events. Individuals in placebo and intervention group are depicted by circles and squares, respectively. Black line and shaded area represent linear regression result and 95% confidence intervals. Timepoints are indicated as week of the trial. C: challenge phase

Discussion

To our knowledge, this study is the first to describe the protective effects of immunisation with short-term infections using hookworm larvae. We demonstrated that protection from subsequent challenge is associated with severe skin reactions, eosinophilic response and parasite specific IgG1 production. These results suggest that antibody-mediated effector mechanisms in the skin may play an important role in the protection induced by short-term, abrogated larval infection.

In our study, IgG1 was the predominant immunoglobulin subclass directed to parasite antigen, particularly prominent in those with severe skin rash. This contrasts with natural infections, where IgG4 is the most prominent immunoglobulin subclass, with higher levels of IgG4 being observed with higher worm burdens. [21] IgG4 is less pronounced after experimental infection, indicating this subclass may be associated with chronic trickling infections rather than infrequent, high-dose short-term exposures. The integrative analysis confirmed that IgG1 and eosinophilic responses were correlated with lower egg loads. This points to a mixed Th1 and Th2 response mediating protection, although the exact contribution of each component remains to be further elucidated.

Although not significant, we observed more frequent abdominal adverse events in the intervention group. In our previous study, we noticed an association between eosinophilic response and abdominal adverse events and a non-significant trend to lower egg counts in those with more abdominal adverse events. [15] Although the observation in the current study is less clear, these combined findings warrant speculation about a possible eosinophilic enteric response to hookworm antigens. In our previous study[15] using repeated controlled *Necator americanus* hookworm infections with 50L3, where we abrogated the infection at a later stage (week 20), we did not observe the severe skin responses. This indicates that the early destruction of larvae, much alike the radiation-attenuated larvae used in animal models and UV-irradiated larvae in the controlled human infection model described by Chapman et al.,[13] is critical in inducing protective immunity, which then attacks the invading larvae in the skin stage upon subsequent infections. The involvement of eosinophils and IgG1 in such a response is supported by earlier in vitro studies showing their ability to kill schistosomula. [22] The skin eruptions and severe nightly itching are reminiscent of the symptoms seen after human infection with canine hookworms (*Ancylostoma braziliense* and *caninum*), which can be similarly erythematous, vesicular and serpentine[23] and are thought to occur when larvae get trapped in the human skin.

The central role of the human skin in protective immune responses to hookworms has not been described before. Rather, the lungs were thought to be the primary site for immune induction in models using irradiated hookworm larvae in dogs and murine infection experiments.[7 24] In other human helminths such as schistosomes, we have previously found regulatory rather than inflammatory responses in *ex vivo* human skin models, with the

increased expression of IL-10 and PD-L1 by antigen-presenting cells in the skin.[25] These initially regulatory responses were thought to be the reason why cercarial dermatitis is usually mild.[25] It is interesting to observe that the induction of immunity can reverse such natural immune tolerance in the skin. In repeated helminth infection models using the murine helminth *Nippostrongylus braziliensis*, entrapment of larvae in skin has also been demonstrated after repeated infections [26] In these models, a large number of neutrophils were observed to swarm *Nippostrongylus braziliensis* in murine skin,[27] forming neutrophil extracellular traps to capture and aid the killing of larvae, although the larvae themselves could escape the traps by releasing deoxyribonucleases, resulting in survival in some. Taking skin biopsies after controlled infections can elucidate whether the effector cells in human hookworm infection models are the same.

Although not as pronounced as in our study, skin adverse events were also found in one prior study where the immunising effects of uv-irradiated larvae were tested in a controlled human infection model described by Chapman et al.[13] Specifically, the attenuation process was targeted to have the larvae cause a mild to moderate rash, which therefore may have induced a weaker immunological response than that seen in our study. Based on a prior dose escalation study, [15] we decided to select higher challenge doses. Our primary endpoint included multiple samples taken over several weeks when egg excretion is relatively stable instead of a single measurement, which greatly enhances the power of the challenge model. [14 15] The higher challenge dose resulted in twenty-fold higher larvae per grams of faeces recovered in the hatching assay and due to multiple sampling a more robust outcome that takes the variability in egg excretion into account.

Although an attenuated larvae approach to vaccination is not feasible on a large scale, the skin and associated initial larval stages may open up novel avenues for target discovery. The only vaccine currently in clinical development (Na-GST-1/Na-APR-1) targets adult worms.[28] A previous larval antigen candidate, Na-ASP-2, showed potential to inhibit larval migration in the skin[29] underscoring the possibilities of vaccines that target early larval stages. However, this vaccine failed in early clinical development due to the induction of IgE-mediated allergic responses in a pre-exposed population.[30] Our study now shows that early larval-stage antigens do not induce specific IgE responses in non-immune populations but may be efficacious in inducing protective immunity, which is why we would argue that these should be considered as vaccine candidates. Serological studies in endemic areas, to be performed prior to initiating phase 1b studies for any larval antigen vaccine, could be done to exclude the presence of pre-existing IgE and support its continued clinical development. Interestingly, the occurrence of strong eosinophilic responses after challenge suggests that antigen specific allergic responses may occur particularly to later stage antigens.

The repeated infection-treatment protocol was specifically designed to enhance the development of protective responses which may be diluted in natural infections due to interfering co-infections or prior infections. The controlled infection setting thus allows for

a more robust characterization of immune responses to early infection, thereby elucidating a hitherto uncharacterized response that cannot be studied in endemic areas. We have shown that this work is feasible and safe and can move to endemic areas to further assess immune responses in pre-exposed populations.

Limitations

Due to a high loss to follow-up the placebo group was reduced from an original eight to five participants. This has significantly impacted the study power to detect differences between the intervention and placebo group. Moreover, the very apparent skin eruptions in some volunteers broke the blinding for both trial physicians and volunteers. However, all laboratory evaluations, including Kato-Katz slides, PCR and ELISA measurements were performed by blinded personnel, minimizing bias. The findings in this study are specific to *Necator americanus*, the most prevalent hookworm species, but may not be generalizable to *Ancylostoma* infection.

In conclusion, this study is the first to describe protective skin-mediated IgG1 responses against infection with hookworm larvae. This finding supports the investigation of larval antigens as possible vaccine targets and confirms IgG1 as reliable correlate of protection for vaccine efficacy.

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Conflict of interest statement

The authors have no conflict of interest to declare.

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Supplementary material

Supplementary material 1. In- and exclusion criteria

Inclusion criteria

In order to be eligible to participate in this study, a participant must meet all of the following criteria:

1. Participant is aged ≥ 18 and ≤ 45 years and in good health.
2. Participant has adequate understanding of the procedures of the study and agrees to abide strictly thereby.
3. Participant is able to communicate well with the investigator and is available to attend all study visits.
4. Participant agrees to refrain from blood donation to Sanquin or for other purposes throughout the study period.
5. For female participants: participant agrees to use adequate contraception and not to breastfeed for the duration of study.
6. Participant agrees to refrain from travel to a hookworm endemic area during the course of the trial.
7. Participant has signed informed consent.

Exclusion criteria

A potential Participant who meets any of the following criteria will be excluded from participation in this study:

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, 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:

- positive HIV, HBV or HCV screening tests;
- the use of 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;
- having one of the following laboratory abnormalities: ferritine <10ug/L, transferrine <2.04g/L or Hb <6.5 mmol/L for females or <7.5 mmol/L for males.
- 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 one year prior to study onset;

- inflammmatory bowel syndrome;
- regular constipation, resulting in bowel movements less than three times per week.

2. Known hypersensitivity to or contra-indications for use of albendazole, including co-medication known to interact with albendazole metabolism (e.g. carbamazepine, phenobarbital, phenytoin, cimetidine, theophylline, dexamethasone).
3. Known allergy to amphotericin B or gentamicin.
4. For female participants: positive urine pregnancy test at screening.
5. Positive faecal qPCR for hookworm at screening, any known history of hookworm infection or treatment for hookworm infection.
6. Being an employee or student of the department of Parasitology of the LUMC.
7. Current or past scars, tattoos, or other disruptions of skin integrity at the intended site of larval application.

Supplementary material 2. ELISA procedure

Hookworm antigen: Necator americanus L3 extract.

Cultured Hookworm L3 larvae were collected in sterile water and stored in 50ml tubes at -80°C after which they were freeze dried and stored at -80°C again. After thawing, the larvae were suspended in PBS, transferred to a glass homogenizer and kept on ice whilst washing 4 times. The solution in the homogenizer was crushed for 15 minutes and left for one hour on ice. Crushing and resting was repeated three times. Crushed hookworm larvae were transferred to a glass tube, and the homogenizer was washed once with PBS. The crushed hookworm larvae were sonified (Branson Sonifier) 6 times for 30 seconds with an interval of 20 seconds. The samples were kept overnight at -80°C. The frozen samples were thawed and centrifuged for 25 minutes, 13.000 rpm at 4°C. The supernatant as well as the resuspended pellet were centrifuged again, after which supernatants from both tubes were collected and pooled. Antigen concentration was determined using a BCA kit (ThermoFisher 23225) following manufacturers instruction.

<u>IgG1 and IgG4 ELISA</u>: High-binding C96-wells maxisorp plates (Nunc-Immuno™ 430341) were coated with 5ug/ml hookworm antigen in 0.1M Na-carbonate pH 9.6. After overnight incubation at 4°C, plates were washed 4 times with 0.05% Tween 20 (Sigma-Aldrich, 27, 434-8) in PBS wash buffer. Plates were blocked with 5% BSA/PBS for one hour at 37°C. After washing, a mix of positive serum was used in a serial dilution of 1:2 as positive controls, samples were at least 1:2 diluted in PBS 0.05% Tween 20 assay buffer. Plates were incubated overnight at 4°C for IgG1 and IgG4, and 1 hour at 37°C for total IgG. For IgG1 detection, conjugated monoclonal antibody of HRP-labelled anti human IgG1 (Fc) (clone MH161-1, HP6188, Sanquin; cat no: M1328) was added and for IgG4 detection HRP-labelled anti human IgG4 (CH3) (clone MH164-1, HP6196, Sanquin; cat no: M1331) was added, both at a concentration of 1.8uL diluted in 5.5 ml PBS 0.05% tween and both 50 uL/well. For total IgG detection, 50 uL/well 1.8 uL alkaline phosphate conjugated anti-human IgG (Sigma A9544,

4°C) in 5.5 ml PBS 0.05%Tween-4% BSA was added. Plates were incubated at 37°C (one hour for IgG, four hours for IgG1 and IgG4). After washing, TMB substrate was added (TMB Microwel substrate system(KPL, 50-76-00) for IgG1 and IgG4, 6mg p p-nitrophenylphosphate (p-NPP) in 6 ml diethylanolamine (DEA) buffer for IgG)) and development was stopped with 18M H2SO4 in water, after which plates were read at by 450 nm at the ELISA plate reader. Total IgG plates were incubated in the dark for one hour at room temperature and then read at wavelength 405 nm.

IgE ELISA: Polysorp F96-wells plates (Nunc-Immuno™ 475094) were coated with 5ug/ml hookworm antigen in 0.1 M Na-carbonate buffer, pH 9.6. After overnight incubation at 4°C wells plates were washed 4 times with 0.05% Tween 20 (Sigma-Aldrich, 27, 434-8) in PBS wash buffer. Blocking with 5% BSA/PBS was performed for one hour at room temperature. After washing four times, a mix of positive sera were added in a serial dilution of 1:2 in PBS 0.05% Tween 20 assay buffer and serum samples were diluted at least 1:2 in assay buffer. Plates were incubated overnight at 4°C. After five washings 100uL/well of 22uL goat anti human IgE HRP (Invitrogen A18793, 1 mg/ml) diluted in 11 ml PBS 0.05%Tween was added and plates were incubated for three hours at 37°C. Samples were flicked off and washed five times. After washing, TMB substrate was added (TMB Microwel substrate system(KPL, 50-76-00) incubated in the dark for one hour at room temperature and development was stopped with 18M H2SO4 in water, after which plates were read at by 450 nm at the ELISA plate reader. All Ig analyses: OD values are converted to AU/ml using a standard curve with unknown concentration, enabling comparison of a rise or fall in serum concentration within the same subject.

Supplementary table 1. Overview of study visits and sampling

Week	Timepoint	Study procedures	Samples collected for:
Immunisation phase			
Week 0	I00	First immunisation	Eosinophils, Ig, cytokines
Week 1	I01		
Week 2	I02	Albendazole treatment	Eosinophils
Week 3	I03	Second immunisation	Eosinophils, Ig, cytokines
Week 4	I04		
Week 5	I05	Albendazole treatment	Eosinophils
Week 6	I06	Third immunisation	HW PCR, Eosinophils, Ig, cytokines
Week 7	I07		
Week 8	I08	Albendazole treatment	Kato-Katz, HW PCR, Eosinophils, Ig, cytokines
Week 9	I09		Kato-Katz, HW PCR, Eosinophils
Week 10	I10		
Week 11	I11		
Week 12	I12		Kato-Katz

Supplementary table 1. Overview of study visits and sampling *(continued)*

Week	Timepoint	Study procedures	Samples collected for:
Challenge phase			
Week 13	C00	First challenge	Eosinophils, Ig, cytokines
Week 14	C01		Eosinophils
Week 15	C02	Second challenge	Eosinophils, Ig, cytokines
Week 16	C03		Eosinophils
Week 17	C04		HW PCR, Eosinophils, Ig, cytokines
Week 18	C05		HW PCR, Kato-Katz, Eosinophils
Week 19	C06		HW PCR, Kato-Katz, Eosinophils
Week 20	C07		HW PCR, Kato-Katz, Eosinophils
Week 21	C08		HW PCR, Kato-Katz, Eosinophils, Ig, cytokines
Week 22	C09		HW PCR, Kato-Katz, Eosinophils
Week 23	C10		HW PCR, Kato-Katz, Eosinophils
Week 24	C11		HW PCR, Kato-Katz, Eosinophils
Week 25	C12		HW PCR, Kato-Katz, Eosinophils, Ig, cytokines
Week 26	C13		HW PCR, Kato-Katz, Eosinophils
Week 27	C14		HW PCR, Kato-Katz, Eosinophils
Week 28	C15		HW PCR, Kato-Katz, Eosinophils
Week 29	C16	Albendazole treatment	HW PCR, Kato-Katz, Eosinophils, Ig, cytokines
Week 30	C17		HW PCR, Kato-Katz, Eosinophils
Week 32	C19		HW PCR, Kato-Katz, Eosinophils
Week 37	C24		Eosinophils, Ig, cytokines

Ig: Immunoglobulins, HW PCR: fecal hookworm PCR

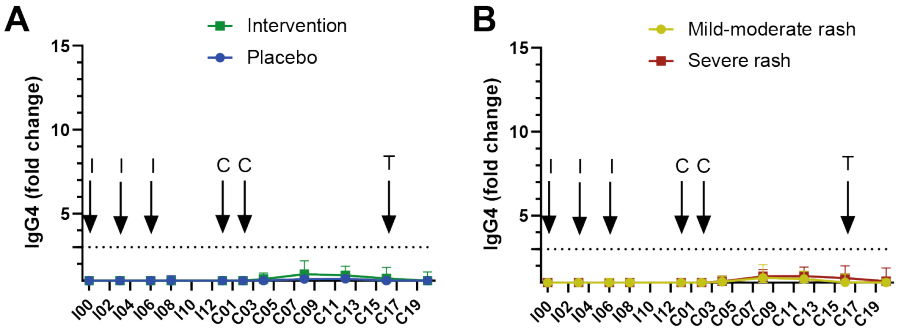
Supplementary table 2. Hatching assay

	Hatching week 12 (mean (SD), larvae per gram faeces)	Hatching week 16 (mean (SD), larvae per gram faeces)
Placebo group (n=5)	164 (146)	248 (122)
Intervention group (n=15)	96 (68)	207 (116)
	p=0.51	p=0.72
Severe skin AEs (n=5)	109 (102)	191 (136)
Non-severe skin AEs (n=12)	119 (100)	230 (110)
	p=0.72	p=0.57

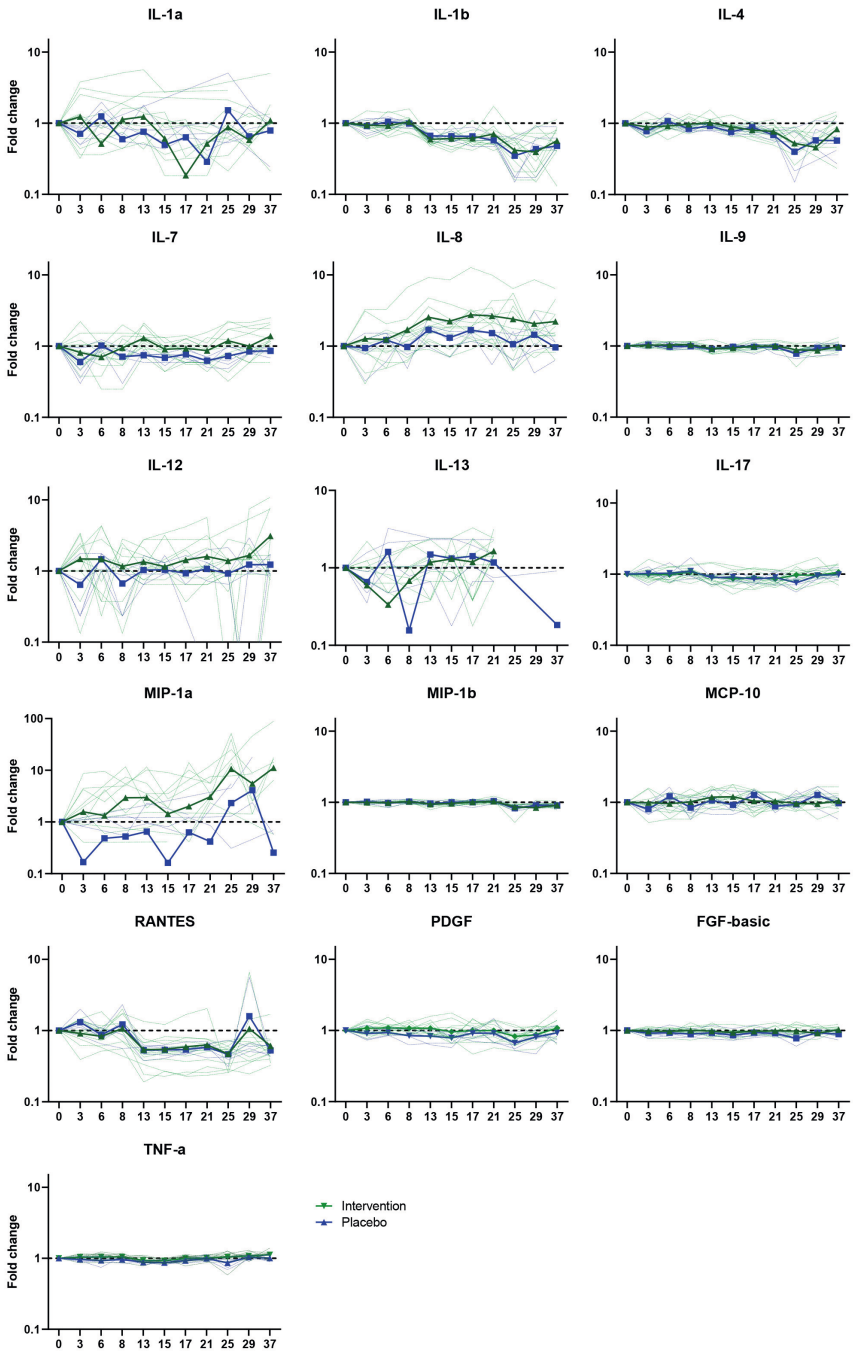
Supplementary table 3. Outcomes for volunteers with and without seroconversion for IgG1.

	IgG1		
	Seroconversion⁺ (n=6)	**No seroconversion (n=12)**	
Placebo Intervention	0 6 (100%)	5 (42%) 7 (58%)	p=0.09
Non-severe skin rash Severe skin rash	2 (33%) 4 (67%)	11 (92%) 1 (8%)	**p=0.02***
Eggload (GM epg, SD)	507 (186)	838 (369)	p=0.09
Grade 3 itching - Yes - No	2 (33%) 4 (67%)	5 (42%) 7 (58%)	p=0.32
Grade 3 abdominal AEs - Yes - No	3 (50%) 3 (50%)	6 (50%) 6 (50%)	p=1.00
Duration of rash after third immunisation (days) (mean) (SD)	30.9 (9.4)	14.0 (18.9)	**p=0.02***
Duration of rash after first challenge (days) (mean) (SD)	32.6 (13.4)	19.6 (17.5)	p=0.13
Duration of rash after second challenge (mean) (SD)	57.5 (20.8)	27.7 (18.8)	**p=0.01***
Peak eosinophil count (x10^9/L, mean (SD))	5.1 (1.9)	4.8 (1.6)	p=0.75

⁺Seroconversion is defined as fold change >3 compared to baseline *p<0.05



Supplementary figure 1. IgG4 titers. Increases in hookworm-specific IgG4 plotted as fold-change over baseline for placebo (blue circles) versus intervention group (green squares) (A), and volunteers with mild-moderate skin rash (yellow circles) versus severe skin rash (red squares) (B). Symbols indicate mean, error bars SD, dashed line indicate threshold for seroconversion, set at 3-fold rise from baseline. I=immunisation, C=challenge, T=treatment with albendazole



Supplementary figure 2. Cytokine measurements by Luminex. Bold lines indicate mean per group, dotted lines indicate individual participants. Green triangles = intervention group, blue circles = placebo group. Data expressed as fold change from baseline.

Chapter 7

Money-oriented risk-takers or deliberate decision makers; a cross-sectional survey study of participants in controlled human infection trials

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Abstract

Objective
To quantitatively investigate the motivations, decision-making and experience of participants in controlled human infection studies.

Design
Cross-sectional descriptive survey study.

Setting
Previous participants of controlled human infection studies at the Leiden Controlled Human Infection Center, control group of students from Leiden University.

Participants
61 previous participants and 156 controls.

Measurements
Ranking of motivational and decisional factors, risk-propensity score and multiple-choice questions on experience of trial participation and ethical aspects of controlled human infection studies.

Results
Motivating factors for participants were contributing to science (80%), contributing to research that may benefit developing countries (72%) and the financial compensation (62%). For 51% of participants a reason other than financial compensation was the most important motivational factor. Participants considered trust in the study team (70%), time investment (62%), severity of symptoms (52%), chance of developing symptoms (52%) and whether it is an easy way to make money (52%) in their decision to participate. Most controlled human infection participants (84%) were proud of their participation, would advise others to participate (89%) and would participate in a similar trial again (85%). Controlled human infection participants had a higher risk propensity score than students (4.37 vs 3.5, $p<0.001$).

Conclusions
Although financial compensation is important, the motivations for participants in a controlled human infection study are diverse and participants make a balanced appraisal of risks and burden before participating.

Introduction

Controlled human infection (CHI) trials are increasingly used in the development of novel vaccines and drugs against a variety of pathogens. [1] In these trials, volunteers are purposely infected with a pathogen in order to test the efficacy of new vaccines or medicines and to study host-pathogen interaction. [2] CHI trials have boosted vaccine development against for example malaria[3] and cholera, [4] and generated valuable information on host-pathogen interactions in many other diseases. [2] Currently over 40 000 volunteers have participated in these studies, [1] with exponentially increasing numbers over the past decades. Like phase 1 drug trials also including healthy volunteers CHI-studies lack individual benefit to the volunteer, requiring a thorough review of the balance of risks and burden to the participant versus the social and scientific benefits. Literature on the ethical debate of CHI-trials is growing, with particular emphasis on informed consent, undue influence by financial compensation and the right to withdraw. [5-7]

Like the debate concerning phase I drug trials[8] there is suspicion that volunteers are only driven by money[9 10] and as a result do not adequately weigh the risk and burden of participation[11], the 'money-orientated risk-taker'. Participants in phase I trials score higher on questionnaires examining sensation-seeking behaviours compared to age- and sex-matched controls, adding to the notion that these volunteers are more prone to take, possibly ill-considered, risks in their lives. [12 13] However, recent research shows that phase I participants consider other arguments besides the financial compensation, such as curiosity, contributing to medical research, helping future patients and the risks involved[14 15]. In response to a recent publication[16] public discussion, particularly on social media, scrutinised voluntariness of participation, since studies often include medical students as participants who were presumed to have felt pressure to participate, next to the ongoing discussion about acceptability of risks and burdens. Qualitative data on motivation of participants was recently collected in two studies with volunteers in controlled human malaria infection trials in the United States and Kenya. These showed that participants had other motivations next to the financial incentive.[17,18] However, these studies only included small groups of participants (16 and 36 respectively) in a malaria trial, and quantitative data on motivations and experiences is lacking. Given the ongoing debate on the ethics of CHI-trials, a more quantitative assessment of the experiences and motivation of participants in a broader group of volunteers is needed to gain better insight into the profile of the CHI-volunteer, their motivations and experiences.

In order to investigate whether participants in CHI-trials are different from the general population it is valuable to compare the participants to a control group. This also enables a longitudinal comparison of motivations and thought-processes of potential participants with those who have actually participated, providing a better insight into how volunteers come to their decision. An additional benefit of a control group from the general population is there will be a proportion unwilling to participate. These controls provide a comparator

in decisional factors and can give information on the acceptance of aspects of controlled human infections even by those unwilling to take part.

The Leiden Controlled Human Infection Center has conducted multiple CHI-trials in malaria, schistosomiasis and hookworm. This unique setup offers an ideal opportunity to fill the abovementioned knowledge gaps. We therefore conducted a survey study in former participants of these trials, using students from the local university as a control group. The aim of this study is to quantitatively investigate the motivation, decision-making process and risk propensity of participants in CHI-trials compared to a control group. Furthermore, this study explores participants' views on ethical questions in CHI-trials.

Methods

This cross-sectional descriptive survey was conducted amongst participants of CHI-trials performed at the Leiden Center for Controlled Human Infections and students of the Leiden University in October 2018.

Participants

Participants of previously conducted CHI-trials with malaria, hookworm or schistosomiasis were invited to participate in an anonymous survey. Inclusion criteria were having undergone controlled human infection and previous consent to be contacted again for further studies. There were no exclusion criteria. All 66 previous participants were eligible for inclusion. CHI-trials were conducted between November 2016 and September 2018. Surveys were distributed and collected via e-mail through data management program Castor EDC. [19] Participants who did not respond to the e-mail were sent one reminder. CHI-participants received a 10€ voucher as reward.

As control group students from the local university were included. This group has been selected as the majority of participants in CHI-studies at the study centre is recruited from this population. Before lectures at the medical faculty the anonymous paper survey was distributed to all students present and collected afterwards. Surveys were furthermore distributed during two meetings of local (non-medical) student societies, where the researchers handed students present the survey and collected them after completion. Controls did not receive compensation.

With an expected response rate of 80% we estimated that around 50 previous participants would return the survey. Based on experiences in recruiting we estimated that one-third of students would be willing to participate in a CHI-trial, so in order to include an equal number of controls willing to participate to actual participants we aimed to include 150 controls.

Survey

The survey was designed by the researchers, based on previously published research[14 15] and topics of ethical debate.[5] Motivational and decision-making factors were chosen based on the research by Grady et al[15] and by identification of potential motivational factors through discussion with researchers involved in screening and recruitment of trial participants. Participants were allowed to add their own factors. Motivational factors in the survey were "curiosity", "contributing to science", " contributing to developing countries", " financial compensation", " interest in the subject" and " personal experience". Factors in the decision making process were " Severity of possible symptoms", "chance of developing symptoms", "time investment", " an easy way to make money", " trust in the study team" and "it's research about parasites". Questions on ethical acceptability were formulated based on issues identified in literature as key concepts in CHI-trials [5-7] (surveys in supplement A).

CHI-participants (from here referred to as PP) reflected on their own experiences, whereas the control group (CC) were asked to consider participation in a malaria trial and a trial with hookworm to reflect the different types of studies conducted. CC were provided descriptions of the trials detailing study procedures, possible adverse events, number of visits and sample collections and the financial compensation (descriptions in supplement A). PP and CC were asked to rate motivational factors and factors considered in their decision about participation. Each factor could be rated as not important, slightly important, considerably important or very important. Next to this ranking CC and PP were also asked to identify the single most important factor.

Attitudes towards risk-taking were investigated using the Risk Propensity Scale (RPS)[20], a seven-item questionnaire consisting of statements on taking risks in daily life that are rated between 1 and 9 (supplement B). Higher scores represent a higher propensity to take risks. This questionnaire was selected as this is a concise questionnaire focussing on general risk-taking propensity in daily life.

Experiences of PP and opinions on ethical issues were examined using multiple-choice questions. Wherever relevant, CC were presented with similar questions.

Statistical analysis

A ranking order of motivational and decision-making factors was compiled, ranking from the factor with the highest percentage of 'important' or 'very important' to the lowest. Differences between CC and PP were calculated using a Fisher's exact test.

RPS scores were analysed as described by Meertens.[20] Differences in mean scores were analysed using a linear regression model, adjusting for age, sex and health-care related education or job Frequencies were calculated for the multiple-choice questions on the experiences of PP and ethical issues. Differences in demographical characteristics were calculated using a Chi-square test, differences between CHI-models were calculated using a

one-way ANOVA for continuous parametric data and Kruskall-Wallis test for non-parametric data, and a Chi-square test for categorical data. A p-value <=0.05 was considered statistically significant.

Calculations were made using SPSS v23.[21] The institutional review board of the Leiden University Medical Center where the study was performed reviewed the protocol and provided ethical approval (P18.203).

Patient and public involvement

No patients were involved in this study. This study was designed to investigate healthy volunteers' opinions and preferences. Volunteers were not involved in the design or recruitment process. Interested participants were presented the results during a meeting, participants will be provided the research article after publication.

Results

61 of 66 CHI-participants and 156 of 156 students returned the survey. There were no missing answers in the questionnaires of PP, although many CC did return incomplete questionnaires. Nevertheless, since all questions were answered by at least 85% of controls, all questionnaires were included in the analysis (All survey outcomes are provided in Supplement C).

Baseline characteristics and demographics for both PP and CC are in Table 1. The majority of PP (67%) were students while participating in their trial. Most PP had not previously taken part in medical research (72%) and 53% were employed or studying in a healthcare-related field. In both groups the majority were female. CC were younger than PP (p<0.0001) and most were recruited from the medical faculty.

Of the CC, 69% would not participate in any of the CHI-trials (referred to as CN), 22% would only participate in the malaria trial, 3% only in the hookworm trial and 6% in both trials (CP).

Table 1. Demographic characteristics of study participants

	CHI participants (n=61)	Controls (n=156)
Participation in trial for:		
Schistosomiasis (n=17):	16 (26%)	N/A
Hookworm (n=26):	22 (36%)	
Malaria (n=23):	23 (38%)	
Sex		
Male:	24 (39%)	35 (22%)
Female:	37 (61%)	98 (63%)
Missing:		23 (15%)

Table 1. Demographic characteristics of study participants *(continued)*

	CHI participants (n=61)	Controls (n=156)
Age		
< 18 yrs	0	3 (2%)
18-24 yrs:	38 (62%)	145 (93%)
25-30 yrs:	11 (18%)	8 (5%)
>30 yrs:	12 (20%)	0
Employment		
Student:	41 (67%)	156 (100%)
Working:	19 (31%)	
Other:	1 (2%)	
Previously participated in research		
Yes:	17 (28%)	N/A
No:	44 (72%)	
Employed in healthcare or healthcare related study?		
Yes:	32 (53%)	126 (81%)
No:	29 (47%)	30 (19%)
Would you participate in one of these controlled human infection trials?		
Yes, both	N/A	9 (6%)
Yes, only malaria		35 (22%)
Yes, only hookworm:		4 (3%)
No:		108 (69%)

Motivation

Motivation was investigated both by ranking factors of importance and by identifying the single most important factor. PP considered “contributing to science” as an important (43%) or very important (38%) motivating factor, followed by “contributing to developing countries” (41% important, 31% very important) and the financial compensation (25% important, 38% very important) (figure 1). However, when asked the single most important motivation, PP most often noted the financial compensation (49%) followed by “contributing to developing countries” (29%) . There were no apparent differences in motivation for participants from different CHI-models.

For CP the financial compensation was most often important (39% important, 52% very important, p=0.001 for comparison between PP and CP), followed by “contributing to science” (33% important, 39% very important, p=0.48) and “contributing to developing countries” (46% important, 26% very important, p=0.9). The single most important motivation was financial compensation for 41% of CP and “contributing to science” and “interest in the subject” for 15% each. The single most important factors were not distributed significantly different between PP and CP.

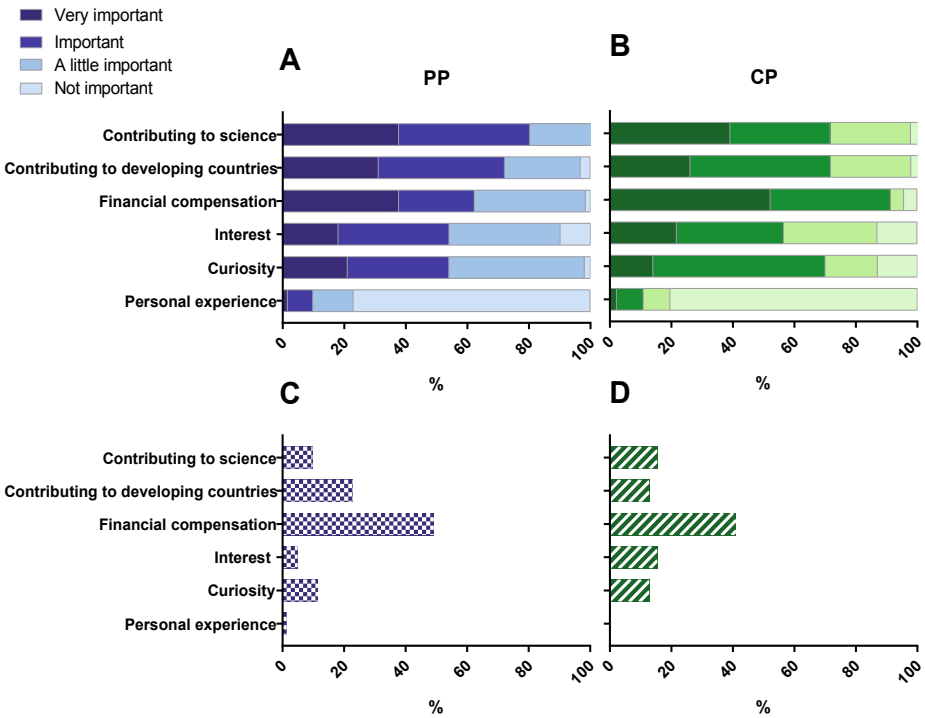

Figure 1. Ranking of motivational factors to participate in a CHI trial for PP (panel A) and CP (B). Single most important motivation factor for PP (C) and CP (D).

Decision to participate

PP most often found trust in the study team important in their decision to participate (34% important, 36% very important) followed by the time investment (43% important, 20% very important), severity of symptoms (36% and 18%), chance of developing symptoms (31% and 23%) and "an easy way to make money" (31% and 23%). The single most important factor in the decision to participate was highly variable, including the chance of developing symptoms (23%), severity of symptoms (21%) and time investment (20%).

CC most often considered the chance of developing symptoms and severity of symptoms important ($p<0.001$ for comparison between PP and CC), with CP also considering the time investment and "an easy way to make money". The severity of symptoms was the single most important factor (47% for CP, 53% for CN) (Figure 2), which is significantly more often than for PP ($p<0.001$).

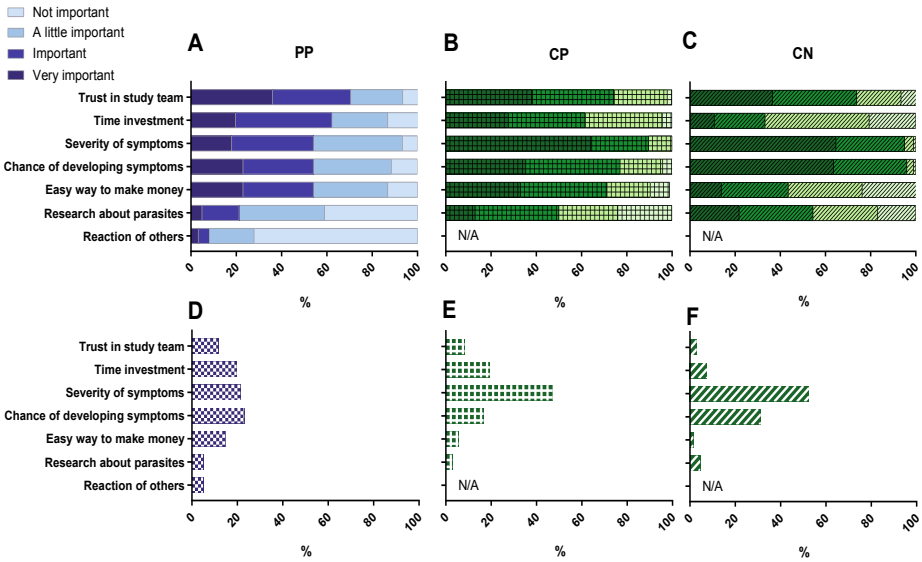

Figure 2: Ranking of factors considered in the decision to participate by PP (A), CP (B) and CN (C). The single most important factor in the decision to participate for PP (D), CP (E) and CN (F).

Assessment of symptoms and risks

The majority of PP (57 out of 61, 93%) considered the trial to be of no or little risk and the majority were not afraid of symptoms before the start of the trial (49 of 61, 80%). For 10 PP their fear of symptoms increased during the trial, mainly because they saw other volunteers with symptoms or as one volunteer stated "we were working each other up the day of the malaria infection about the mosquito bites and what would happen". For the others, fear of symptoms declined (n=8) or remained the same (n=43). PP scored the symptoms they experienced during the trial on a scale of 0-10, with 0 being no complaints at all, 10 complaints so severe they had to withdraw from the trial. The mean score was 2.85 (SD 2.7, range 0-10) for all models, with no significant differences between CHI-models (p=0.228).

Reaction of others

Many (80%) PP reported negative reactions about their trial participation, quoting reactions like: "Are you getting worms in your body?" or "You are taking a risk with your health". However, 64% also received positive reactions, such as "That's an important thing to support", "That is very interesting research to participate in" and "That's good money for little effort". The responses of third parties largely did not influence their decision to participate (93%). All PP but one reported no outside pressure to participate in the study; the one exception was a participant who, while describing no pressure to initially participate, reported some during the study when the participant was unable to meet some of the logistical demands of the study. In response, the participant was offered the option of missing out on certain follow-up procedures in order to remain in the study for the primary

endpoint, rather than dropping out altogether. This participant described being glad to have been offered that proposition and was proud to have completed the study after all.

Opinion on ethical issues

PP and CC were asked their opinion about the concept of deliberate infection and the right to withdraw. For 77% of PP it was considerably or very important to always be able to withdraw. However, 95% replied that they found it understandable that in a CHI-trial immediate withdrawal is not always possible if this was done for their own safety or that it was acceptable if explained during the informed consent procedure. PP also found it acceptable for a physician to deliberately make them ill for the benefit of the trial (100%). Some added that this was what they voluntarily signed up for, as long as possible symptoms were explained before the trial. CC generally had similar views: 94% felt it was understandable that it is not always possible to withdraw and 82% found it acceptable for a physician to deliberately make a person ill for the trial.

Financial compensation

Of the PP, 10 out of 61 would have participated without any financial compensation. The majority of PP (84%) considered the compensation as good, and 3 considered it too high. PP most often spent the financial compensation on a holiday (41%), followed by costs of daily life (20%) and savings (18%). PP view the compensation as an incentive to participate (56%), compensation for costs (50%) and payment for risk and burden (49%). The majority of CN could not be convinced to participate for double the compensation (86%) and only 3 (3%) would change their mind about participation if both the compensation and the risks were doubled. CP were also unwilling to take more risk: only 5 of the 44 (11%) would still participate if the risk was twice as high but compensation also twice as high (Figure 3).

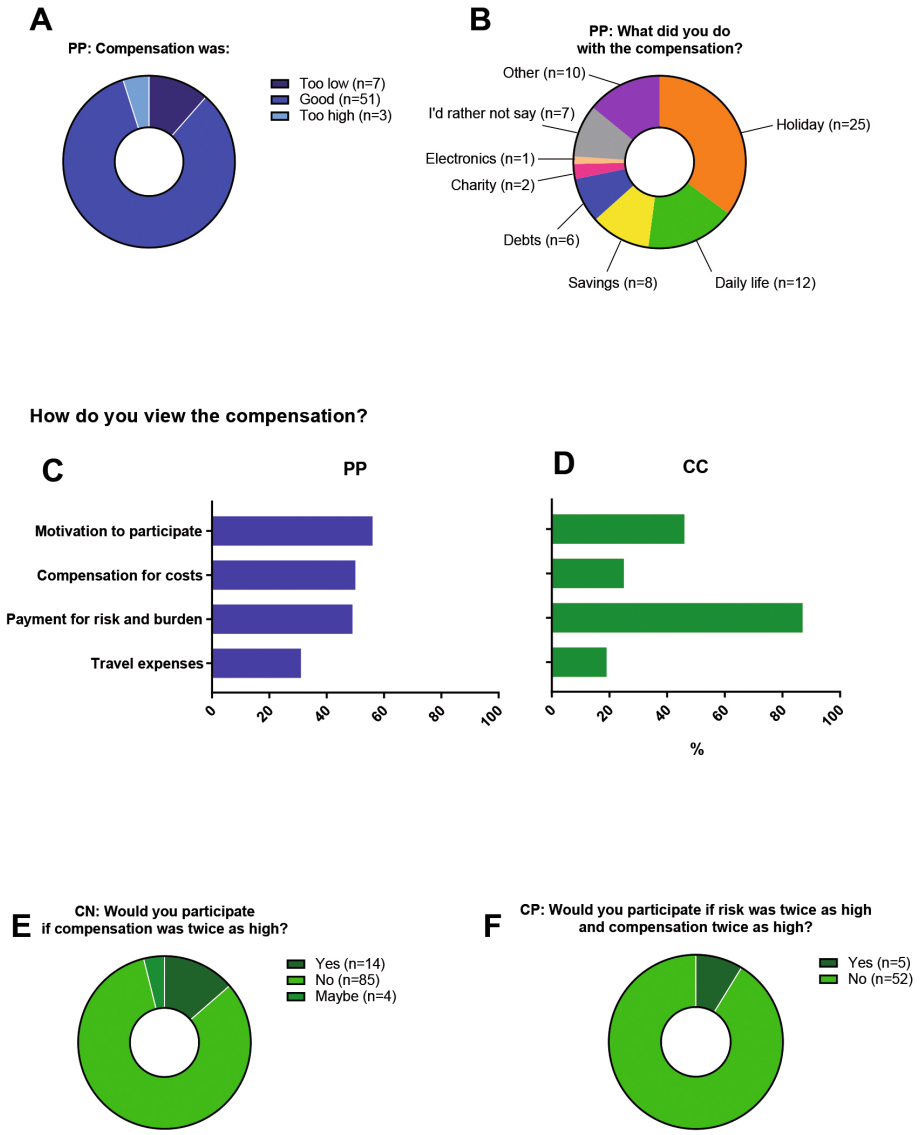

Figure 3. Opinion of PP (n=61) on the amount of financial compensation (A) and how they used the compensation (B). View of PP (C) and CC (D) on why financial compensation is offered (multiple answers could be given). Opinion of CN (n=103) to change their mind if compensation was twice as high (E) and opinion of CP (n=57) if the compensation was twice as high and risk was twice as high (F).

Looking back at participation

Remarkably, a large proportion (59%) of PP felt they had gained benefits from their participation other than the financial compensation, like increased knowledge about the conduct of clinical trials or the disease for which they participated, the pride of having contributed to important research and the experience of going through a trial with the other participants and the study team. One volunteer stated that he had 'learned to get up early in the morning and improve my daily rhythm'. Most (84%) were proud of their participation, would advise others to participate (89%) and would participate in a similar trial again (85%) (Figure 4A). In retrospect, 80% felt that the benefits of the study outweighed the burden they experienced, and of the 20% who did not, 3 out of 12 stated they had experienced so little discomfort they did not have any burden. For 46% of volunteers the symptoms met their expectations, 36% experienced fewer symptoms than expected and 20% experienced more (Figure 4B). Even those participants who had more symptoms than expected evaluate their participation positively: 8 out of 12 felt proud of their participation and would advise others to participate, 10 out of 12 would themselves participate again (Figure 4C).

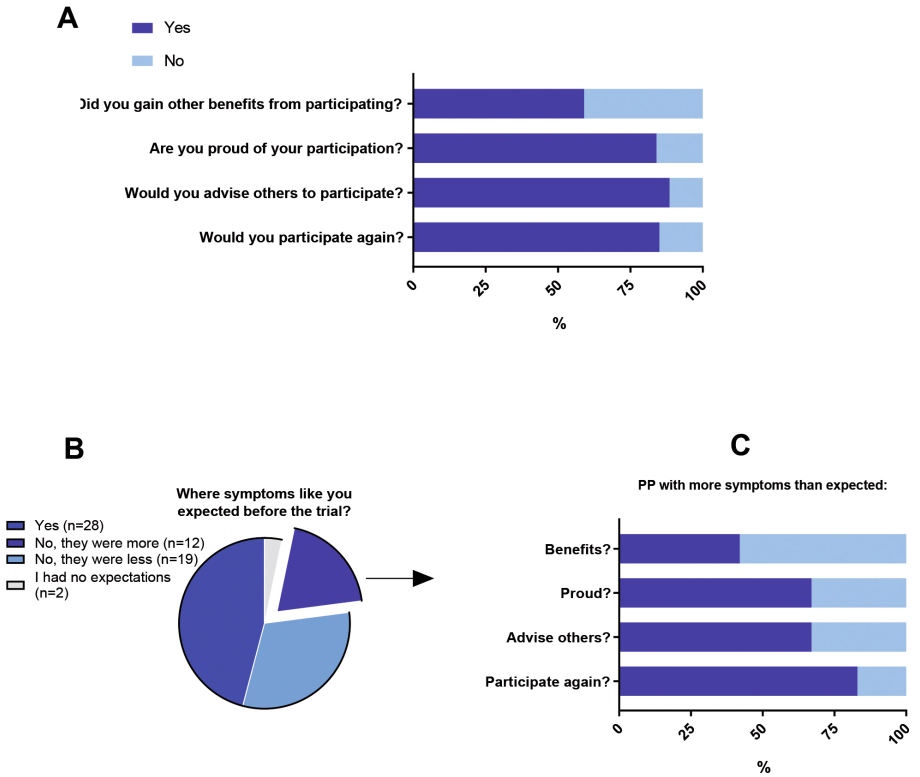

Figure 4. General evaluation of PP (n=61) looking back at their participation (A), assessment of symptoms when looking back (B) and general evaluation of PP who experienced mores symptoms than expected (C).

Risk propensity scale

PP had a significantly higher risk propensity score than CC (estimated difference 0.9, p<0.001)) (Figure 5). CP also scored significantly higher than CN (estimated difference 0.9, p=0.001). No evidence for differences between participants from different CHI-models, males or females or those with a health-care related job or education were observed.

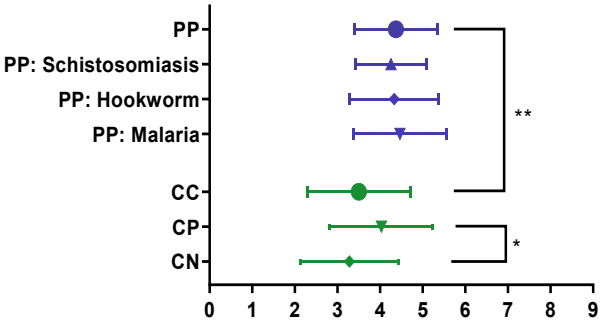

Figure 5: Risk Propensity Scale. Higher scores indicate a higher propensity to take risks. Symbols indicate mean, errors bars indicate standard deviation. ** p<0.001, * p=0.001

Discussion

This survey study is the first to quantitatively investigate the motivations and experiences of participants in CHI trials. These findings shed light onto the experiences and opinions of participants on issues that have been subject of extensive ethical debate.

We have found that, contrary to commonly mentioned fears, [10 22] the largest group of volunteers felt that contributing to science and to research benefitting developing countries was an important motivation. For 51% of PP the financial compensation was not the most important reason to take part. Interestingly, for 38% of PP financial compensation was not or only of little importance, and 10 (16%) would have participated without any compensation. Our data convincingly shows that factors other than financial compensation are important motivators which are considered in the decision to participate.

A larger group of CC found the compensation important compared to PP, although as a single most important motivation for participation proportions were similar. CC also gave more importance to the symptoms compared to PP. Possibly, the compensation is initially most important for a potential participant to be interested in the study, with motivations becoming more diverse after receiving more information about the study and through actual participation. In the decision-making process CC gave more importance to the symptoms, which may reflect that during the first deliberations about participations the symptoms are an important decider, whereas with more information other factors are taken into account.

The motivations of CHI-participants seem to be concurrent with findings in volunteers of phase I drug trials. Stunkel and Grady describe in a 2011 systematic review[8] that although the financial compensation is usually necessary, it is not sufficient for participation, and note that risk is the deciding factor in participation. However, other large-scale studies in phase I drug-research participants, [15] noted that money is the most important motivator in 60% of individuals, which is clearly more than we found. Possibly, the population (students, gender and age) might play a role in motivating factors as well as the nature of the trial. A survey of the motivations of individuals participating in Ebola and influenza vaccines is a good example of the latter, whereby almost 90% of participants found contributing to the health of others important. [23] It is possible that both CHI-trials, especially those researching vaccines for Neglected Tropical Diseases and phase 1 trials for vaccines with similar expected public health benefits may attract volunteers with more altruistic motivations compared to phase I drug research in general.

Differences in population may also be reflected within CHI-studies in different countries. Our Dutch PP were motivated by other factors than Kenyan participants of a controlled human malaria infection (CHMI) trial, who were most often driven by the financial compensation and the health care provided by the trial staff. [18] The Kenyans were rewarded the wage of a day's work for each day of participation to make up for lost income. This was different for the Dutch PP, who have universal access to healthcare and receive compensation for time spent and travel expenses. Participants from both countries, however, showed little concern about trial risks and showed high levels of trust in the study team. In a qualitative study amongst US CHMI participants[17] the participants similarly describe little concerns about the risks, trust in the study team as important and mixed motivations for participation. The differences between the American, Kenyan and Dutch CHI-participants illustrate the influence of cultural differences and healthcare organization that remain important to address and separately investigate.

This study also provides more insight into the presence of undue influence by the financial compensation. We have found that a majority of PP has used their received compensation for leisure activities such as a vacation or put the money in their savings accounts. This indicates they do not have a direct financial need in daily life to take part but could spend the money for more luxury expenses. The control group also provides evidence that potential participants cannot be persuaded to participate for more money if they are not inclined to do so in the first place, or accept more risk for more money, even though the compensation is an important motivation to participate for them. We acknowledge that without any compensation many PP would probably not participate but do conclude that the motivations of participants are varied and that the role of the financial compensation is not as important as presumed.

Another important issue in current debate is the acceptable risks and burden to participants and the risk-taking attitude of trial participants. This survey cannot answer what acceptable

risks and burdens are, but can give important insight into what participants actually consider acceptable.

Both PP and CP scored higher on the RPS as compared to CN. Interestingly, the scores in both groups were lower than those of the original validating study for the RPS who had a mean score of 4.63 (SD 1.23, range 2.00-07.00),[20] suggesting that the RPS varies considerably between different populations. Possible symptoms and risks were an important reason for CN to decline participation, whereas CP and PP apparently weigh the symptoms but find them acceptable. This higher acceptance of possible risks matches the higher risk-taking propensity, but does not mean that risks and burden are not considered. Even the majority of participants who experienced more symptoms than expected look back positively on their participation, are proud of their participation and would participate again. Combined with the finding that the large majority of PP felt the benefits outweighed the burdens of the study, the majority would participate again and would advise others to do so too and that many reported to have gained more benefits than the financial compensation alone, we conclude that at least for these studies the balance of burdens and risks was acceptable to the volunteers.

This study did not specifically assess understanding and informed consent by the PP, however some conclusions on the success of informed consent and voluntariness can be drawn. All participants but one reported no pressure to participate. Although a reporting bias cannot be excluded PP were a heterogenous group of volunteers with diverse backgrounds, none of which connected to the research department. Most participants also indicate that the symptoms experienced were as expected or less, showing they had adequate expectations before starting with the trial. This is confirmed by the fact that most PP reported no change or a decrease in their fear of developing symptoms during the study. We have found no suggestion of pressure to participate and generally conclude PP were well informed about participation, although a more targeted survey would address this question more directly.

This survey also illustrates PP's and CC's views on other issues of ethical debate in CHI-trials. The right to withdraw is considered very important by both groups, however most, including CN, agree that it is acceptable to put restrictions on this if done for the safety of the volunteer and agreed beforehand. The majority of CC did not express ethical concerns about the concept of deliberate infection as they believe that the research will be performed in a safe manner and that risk and benefits are adequately weighed, showing an apparent acceptance of this kind of research even by those who would not participate. This shows that if properly informed, participants are willing to accept some restrictions on the right to withdraw, highlighting the importance of complete and thorough informed consent procedures.

Recall bias may have distorted some of the answers to the questionnaires because of the long lag time between completion of the CHI-trial and filling out the survey for some

volunteers. Some answers to questions in the PP group may also have been influenced by participation in the trial. In addition, social desirability and missing answers may have confounded the results, although surveys were processed anonymously and missing answers were evenly distributed among the questions. Notwithstanding, this study has included a reasonably large number of CHI-participants compared to previous studies and covers several different CHI-models, thereby improving generalizability.

The use of the control group has several limitations. The control group of students may not be a complete representation of the participant population as it is more homogenous in age, education and healthcare background than the actual participants which impairs generalizability. Controls were furthermore offered a hypothetical participation, which may not be comparable to the actual decision to take part. However, participants are largely selected from the same population and this control group represents two-thirds of trial participants. We thus believe that the comparison is still of value.

Conclusion

As the first study to quantitatively investigate the motivations and perceptions of participants, this survey is a crucial addition to the ongoing debate on CHI-trials. This study is amongst the first to add the voice of participants to the current debate. We found that the motivation of CHI-participants is highly varied with significant importance for altruistic motivations. Participants are able to make a balanced appraisal of risks and burdens that results in a mostly satisfactory experience of participation for them. Based on these findings we propose that the current image of the CHI-participant as 'money-oriented risk-taker' is not accurate and may have to be nuanced to the CHI-participant as 'deliberate decision-maker'.

Acknowledgements

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Supplementary material

Supplement A: Surveys

A. Questionnaire for participants in controlled human infection trials

General:

1. In which study did you participate? Malaria/Schistosomiasis/Hookworm
2. Are you male or female?
3. What is your age? 18-24/25-30/>30
4. At the time of your participation in the trial were you: Student/Working/Unemployed
5. Had you participated as a subject in medical research before? Yes/No
6. Do you work in healthcare or do you follow a health-care related study? Yes/No

Motivation:

7. On a scale of 0 to 5 indicate how important the following factors were for your decision to participate (0=not important at all, 5=very important)
   a. Curiosity 0 1 2 3 4 5
   b. Contributing to science 0 1 2 3 4 5
   c. Helping people who are less well-off than me 0 1 2 3 4 5
   d. The financial compensation 0 1 2 3 4 5
   e. I'm interested in the subject 0 1 2 3 4 5
   f. Personal experience with the disease 0 1 2 3 4 5
   g. Other, namely ……
8. On a scale of 0 to 5 indicate how much did you weigh the following factors before deciding to participate?
   a. Severity of possible symptoms 0 1 2 3 4 5
   b. Chance to get symptoms 0 1 2 3 4 5
   c. How much time the study will cost 0 1 2 3 4 5
   d. Easy to make money 0 1 2 3 4 5
   e. Trust in the study team 0 1 2 3 4 5
   f. The fact that this is a study about parasites 0 1 2 3 4 5
   g. Reaction of people around you 0 1 2 3 4 5
9. Did you discuss your participation with people around you? Yes/No
   a. If no: why not (open question)
   b. If yes: with whom? Parents/partner/friends/roommates/class mates/colleagues/others……
   c. Did you receive positive reactions on your participation? Yes/No (space for open answers)
   d. Did you receive negative reactions on your participation? Yes/No (space for open answers)
10. Did you feel pressurised to participate? Yes/No

a. If yes: why? Needed the money/did not want to say no after signing up/pressure from the study team/other……

How was the infection experienced?

11. How did you estimate the risk of this study before participating? (0=very low, 5=very high) 0 1 2 3 4 5
12. Before the infection took place, were you afraid of getting symptoms? Yes/No
13. Has this changed during the course of the trial? Yes/No
    a. If yes, has your fear of symptoms increased or decreased?
14. How did you experience the moment of the infection itself? Positive/neutral/exciting/fearful/other
15. On a scale of 0 to 5, indicate how you experienced being infected for this study (0=not at all, 5=very much)

1. Exciting 0 1 2 3 4 5
2. Interesting 0 1 2 3 4 5
3. Fearful 0 1 2 3 4 5

Symptoms and trust in study team

16. On a scale of 0 to 5 how would you rate your symptoms during this trial? (0=no symptoms, 5=so bad I had to quit the trial)
17. Were the symptoms as you had expected before the start of the trial? Yes/No, space for open answer
18. Did you feel the symptoms and risks of this study weigh up to the possible benefits for you and for science? Yes/No, space for open answers
19. Do you think it is acceptable that a doctor might make you ill as part of research?
    a. Yes, I trust that I will we well taken care of and that the research is safe
    b. Yes, if it contributes to science and to finding a cure or treatment for a severe disease the benefits outweigh the disadvantages
    c. No, this goes against the principle that a doctor should do no harm
    d. Other, namely…….

Informed consent

20. How important was the screening and presentation you received for your decision to participate? (0=not at all, 5=very important) 0 1 2 3 4 5
    a. What's the most important thing you remember from the screening? Possible symptoms/risks of participation/when and how often to visit the trial centre/rules surrounding life style during the trial/other
    b. Did your opinion about the study change after talking to the trial physician about possible risks and symptoms?

4. Yes, afterwards I was relieved, I thought the symptoms would be more severe
5. Yes, I thought the complaints were less severe
6. No, the information in the letter was enough

7. Other …….
   c. Can you briefly describe the purpose of the study you participated in? Open answer

Right to withdraw

21. An important part of a study protocol is that volunteers can always withdraw from a study.
    a. How important do you feel it is to be able to withdraw from a study at all times? (0=not at all, 5=very important) 0 1 2 3 4 5
    b. In a controlled human infection trial it is often not possible to immediately withdraw from the study, because there needs to be a treatment and final check-up even after withdrawal, to ensure the safety of the volunteer. How do you feel about this?
8. That's logical: this is done for your own safety and you know this before participation
9. That feels as a restriction to my freedom to withdraw from the trial
10. Other, namely ……

Compensation

22. Would you participate in this trial is there was no financial compensation? Yes/No
23. How do you view the compensation?
    a. As a compensation for time spent and travel costs
    b. As a compensation for the risk and discomfort of participation
    c. As motivation to participate
24. What did you do with the money you received? (multiple options) Holiday/Electronics/Paid debts/Used it in daily life/Gave to charity/I'd rather not say/Other…….
25. What did you think of the amount of the compensation? Alright/too high/too low
26. If the risk of severe symptoms was twice as high, but the compensation was also twice as high, would you participate? Yes/No
27. Other than the financial compensation, do you feel you have benefitted from your participation? Yes/No If Yes, how?.....

Concluding

28. Are you proud of your participation?
29. Would you advise others to take part in a trial like this? Yes/No space for open answer
30. Would you participate in another trial? Yes/No
    a. If no: why? Takes too much time/symptoms too severe/compensation too low/other

A. Questionnaire – version for students

1. What is your age?
   - <18 years old
   - 18-25 years old

- >25 years old

2. I am male/female
3. Would you participate in a study investigating a new drug? Yes/no

Malaria study:

Wanted: healthy volunteers for a study into the efficacy of a new vaccine against malaria. Earlier research has shown that this vaccine can be administered safely to humans. Now, the effect on protection against malaria will be studied. After three vaccinations, volunteers are exposed to bites of a malaria mosquito. After these bites volunteers visit the trial centre daily for 14 days for check-up visits. At each visit volunteers are checked if they have developed malaria. If a volunteer becomes positive he or she is immediately treated. Possible side effects include itching after vaccination and after mosquito bites and headaches, fever, myalgia and a flu-like syndrome if a volunteer gets malaria. Including vaccinations and all check-up visits volunteers have to come to the trial centre 25 times, for 15 minutes each. Compensation: €1200,-

Hookworm study:

Wanted: healthy volunteers for a study into hookworms. Hookworms are parasites measuring 1-2 cm that live in the intestine. In children this infection can cause anaemia, protein deficiency and impaired cognitive and physical development. In order to treat this infection and develop a vaccine more research is needed. For this study volunteers are infected with hookworm. This is done by placing a gauze with water containing the larvae on the skin. The larvae cannot be seen with the naked eye. Possible symptoms are itching and a rash on the site of infection and abdominal complaints, such as abdominal pain and diarrhoea. Volunteers have to come to the trial centre weekly for 16 weeks for a check-up visit of 15 minutes and have to hand in a stool sample every week. After the 16th week all volunteers are treated so the worms go away. Compensation: €1500,-

4. Would you participate in (one of) these studies?
   - No, with neither of these ➔ go to Q5, skip Q6
   - Yes, but only with the malaria trial ➔ go to Q5, then to Q6
   - Yes, but only with the hookworm trial ➔ go to Q5, then to Q6
   - Yes, with both studies ➔ go to Q6

5. If you do not want to participate in this study or these studies, how important are the following factors in your decision? (0=not at all, 5=very important)
   - Takes too much time 0 1 2 3 4 5
   - I think the risk is too great 0 1 2 3 4 5
   - I'm afraid to get symptoms 0 1 2 3 4 5
   - Compensation is too low 0 1 2 3 4 5
   - The idea to be infected with a worm 0 1 2 3 4 5
   - The idea to be infected with a parasite 0 1 2 3 4 5
   - Other, namely .....

6. If you do want to participate in (one of) these studies, how important are the following factors for you? (0=not at all, 5=very important)

- Curiosity 0 1 2 3 4 5
- Contributing to science 0 1 2 3 4 5
- Helping people who are less well-off than me 0 1 2 3 4 5
- The financial compensation 0 1 2 3 4 5
- I'm interested in the subject 0 1 2 3 4 5
- Personal experience with the disease 0 1 2 3 4 5
- Other, namely ……

7. When considering participation, how important are the following factors to you? (0=not at all, 5=very important)

- Severity of possible symptoms 0 1 2 3 4 5
- Chance to get symptoms 0 1 2 3 4 5
- How much time the study will cost 0 1 2 3 4 5
- Easy to make money 0 1 2 3 4 5
- Trust in the study team 0 1 2 3 4 5
- The fact that this is a study about parasites 0 1 2 3 4 5

8. Do you think it is acceptable that a doctor might make you ill as part of research?

- Yes, I trust that I will be well taken care of and that the research is safe
- Yes, if it contributes to science and to finding a cure or treatment for a severe disease the benefits outweigh the disadvantages
- No, this goes against the principle that a doctor should do no harm
- Other, namely…….

9. An important part of a study protocol is that volunteers can always withdraw from a study. How important do you feel it is to be able to withdraw from a study at all times? (0=not at all, 5=very important) 0 1 2 3 4 5

10. In a controlled human infection trial it is often not possible to immediately withdraw from the study, because there needs to be a treatment and final check-up even after withdrawal, to ensure the safety of the volunteer. How do you feel about this?

- That's logical: this is done for your own safety and you know this before participation
- That feels as a restriction to my freedom to withdraw from the trial
- Other, namely ……

11. Would you participate in this trial is there was no financial compensation? Yes/No

12. How do you view the compensation?

- As a compensation for time spent and travel costs
- As a compensation for the risk and discomfort of participation
- As motivation to participate

13. If the compensation was twice as high, would you participate in the trial? Yes/No

14. If the risk of severe symptoms was twice as high, but the compensation was also twice as high, would you participate? Yes/No

Room for additional remarks

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Supplement B: *Risk Propensity Scale*

Adapted from: Meertens RM and Lion R. Measuring an individual's tendency to take risks: The Risk Propensity Scale. *J Appl Social Psychol* 2008;38(6):1506-20.

Risk Propensity Scale

Please indicate the extent to which you agree or disagree with the following statement by putting a circle around the option you prefer. Please do not think too long before answering; usually your first inclination is also the best one.

1. Safety first.

totally disagree	1	2	3	4	5	6	7	8	9	totally agree

2. I do not take risks with my health.

totally disagree	1	2	3	4	5	6	7	8	9	totally agree

3. I prefer to avoid risks.

totally disagree	1	2	3	4	5	6	7	8	9	totally agree

4. I take risks regularly.

totally disagree	1	2	3	4	5	6	7	8	9	totally agree

5. I really dislike not knowing what is going to happen.

totally disagree	1	2	3	4	5	6	7	8	9	totally agree

6. I usually view risks as a challenge.

totally disagree	1	2	3	4	5	6	7	8	9	totally agree

7. I view myself as a . . .

risk avoider	1	2	3	4	5	6	7	8	9	risk seeker

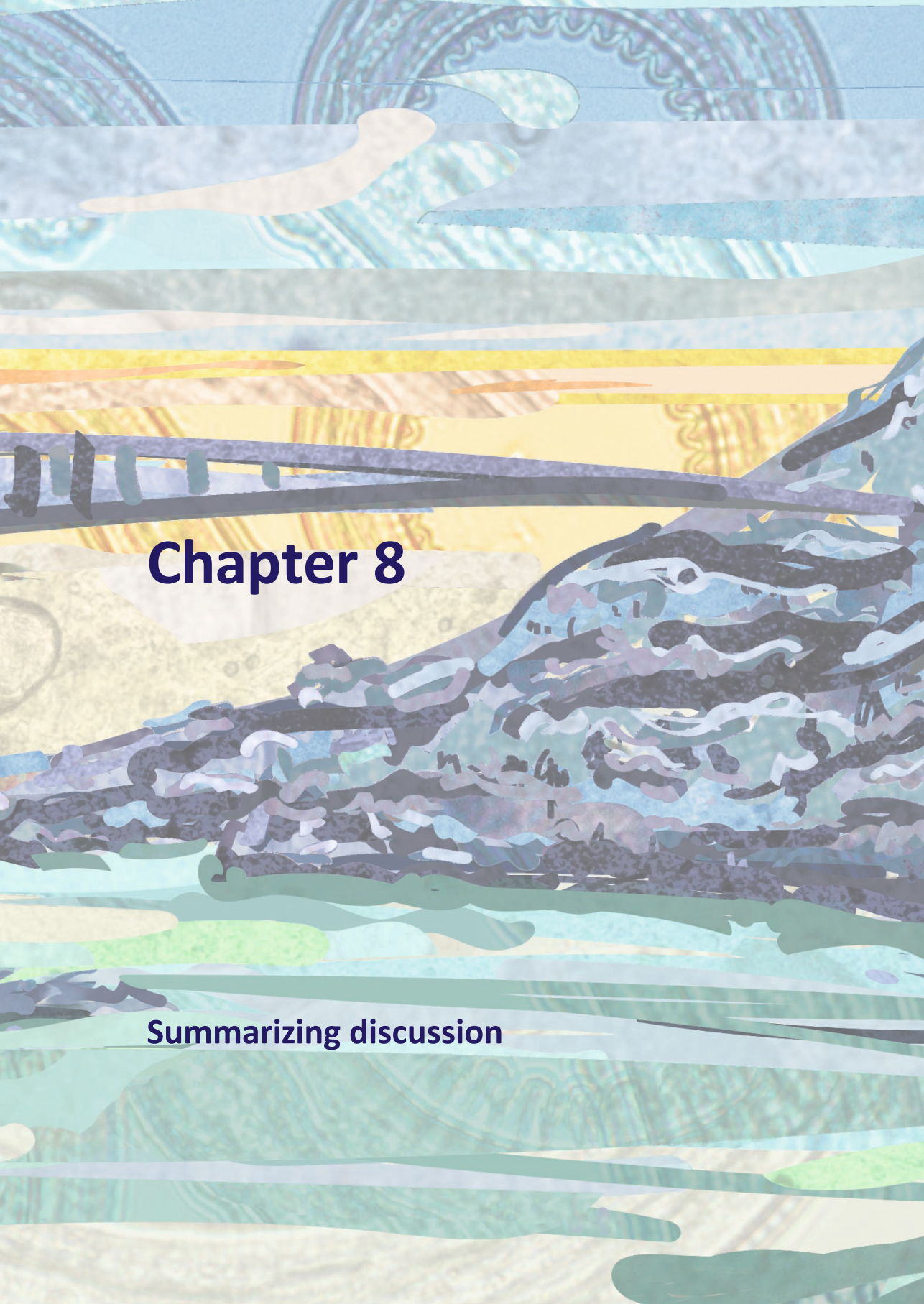
Supplement C: Complete Survey results

Results for motivation and decision to participate are presented in figures 1 and 2 in the original article.

Question		Participants	Students
Did you talk about your participation with other?	Yes No	56 (92%) 5 (8%)	N/A
Did you receive positive reactions?	Yes No	36 (64%) 20 (36%)	N/A
Did you receive negative reactions?	Yes No	45 (80%) 11 (20%)	N/A
Were you influenced by the reactions?	Yes No	4 (7%) 57 (93%)	N/A
Did you feel pressure to participate?	Yes No	1 60	N/A
How did you assess the risk before participation?	No risk Little risk Moderate risk High risk	11 (18%) 46 (75%) 3 (5%) 1 (2%)	N/A
Were you afraid of symptoms before the infection?	Yes No	12 (20%) 49 (80%)	N/A
Did this change during the research?	Yes No	18 (30%) 43 (70%)	N/A
In what way?	Increased Decreased	Increased: 10 Decreased: 8	N/A
How did you experience moment of infection?	Positive Neutral Exciting Frightening Other	15 (24.5%) 16 (26%) 26 (42.5%) 1 (2%) Other: 3 (5%)	N/A
Exciting	Not A little Considerable Very	22 (36%) 28 (46%) 10 (16%) 1 (2%)	N/A
Interesting	Not A little Considerable Very	5 (8%) 16 (26%) 29 (48%) 11 (18%)	N/A
Frightening	Not A little Considerable Very	42 (69%) 19 (31%) 0 0	N/A
Severity of symptoms (scale 0-10) (SD)	All Malaria Schistosomiasis Hookworm	2.85 (2.7) 2.0 (1.7) 2.8 (2.7) 3.8 (3.3)	N/A

Question		Participants	Students
Were symptoms like you expected before the trial started?	Yes No	28 (46%) 33 (54%)	N/A
Did you feel the burden of the study weighs against the possible benefits?	Yes No	49 (80%) 12 (20%)	N/A
Do you think it is acceptable a doctor might make you ill for this study?	Yes No Missing	61 (100%) 0 0	124 (82%) 27 (18%) 5
How important was the screening and information appointment in your decision to participate?	Not A little Considerable Very	11 (18%) 26 (43%) 12 (20%) (12 (20%)	N/A
What was the most important thing you took from the screening? (Multiple answers possible)	Possible symptoms Risks of participation How often are visits Rules for daily life Other	31 (51%) 31 (51%) 28 (46%) 17 (28%) 4 (7%)	N/A
Did your opinion about the study change after the screening?	Yes, I had worries that were answered Yes, I thought symptoms would be more severe No, the letter was sufficient Other	19 (31%) 4 (7%) 35 (57%) 3 (5%)	N/A
How important is it to you to always be able to withdraw from a study?	Not A little Considerable Very Missing	3 (5%) 11 (18%) 25 (41%) 22 (36 %) 0	0 12 (8%) 48 (31%) 94 (61%) 2
In CHI-trials it's not always possible to immediately withdraw. How do you feel about this?	That's logical, it's done for your own safety Feels like hampering freedom to with draw Other	58 (95%) 2 (3%) 1	146 (94%) 7 (4.5%) 1 (0.5%)
If there was no compensation, would you have participated in this trial?	Yes No	10 (16%) 51 (84%)	4 (3%) 150 (97%)
How do you see the compensation? (multiple answers possible)	Compensation for costs Travel expenses Payment for risk and burden Motivation	31 (50%) 19 (31%) 30 (49%) 34 (56%)	38 (25%) 29 (19%) 134 (87%) 71 (46%)
What did you do with the compensation? (multiple answers possible)	Holiday Electronics Debts Daily life Charity I'd rather not say Other	25 (41%) 1 (2%) 6 (10%) 12 (20%) 2 (3%) 7 (11%) 18 (30%)	N/A

Question		Participants	Students
The received compensation was:	Too low Good Too high	7 (11%) 51 (84%) 3 (5%)	N/A
Other than the financial compensation, did you have other benefits from participation?	Yes No	36 (59%) 25 (41%)	N/A
Are you proud of your participation?	Yes No	51 (84%) 10 (16%)	N/A
Would you advise others to participate in a trial like this?	Yes No	54 (88.5%) 7 (11.5%)	N/A
Would you participate again in a similar trial?	Yes No	52 (85%) 9 (15%)	N/A
Would you participate if compensation was twice as high?	Yes No Maybe	N/A	50 (33%) 96 (64%) 4 (3%)
CN	Yes No Maybe	N/A	14 (13%) 85 (83%) 4 (4%)
CP, only malaria	Yes No Maybe	N/A	25 (71%) 10 (29%) 0
CP, only hookworm	Yes No Maybe	N/A	4 (100%) 0 0
CP, both	Yes No Maybe	N/A	7 (87,5%) 1 (12,5%) 0
Would you participate if the risk was twice as high but the compensation also twice as high?	Yes No Maybe	N/A	8 (5%) 143 (94%) 1 (1%)
CN	Yes No Maybe	N/A	3 (3%) 101 (97%) 0
CP, only malaria	Yes No Maybe		3 (9%) 31 (91%) 0
CP, only hookworm	Yes No Maybe		0 4 (100%) 0
CP, both	Yes No Maybe		2 (22%) 7 (78%) 0



Chapter 8

Summarizing discussion

Building bridges: connecting disciplines to improve controlled human infections

In this thesis we have approached the controlled human hookworm infection model and the concept of controlled human infection from multiple disciplines and angles.

We started with presenting an overview of controlled human infections. We have developed an improved controlled human hookworm infection challenge model and applied Bayesian statistical modelling to describe the egg output with higher precision. This model has been applied as challenge model in an immunization study investigating the protective efficacy of short-term exposure to hookworm larvae. Additionally, changes in the gut microbiome after controlled human hookworm infection were explored. We furthermore investigated the motivations and experiences of participants and participants' views on ethical questions surrounding controlled infection studies. In this discussion we will summarize findings, merge the different trials together and will argue why it is our ethical responsibility to approach controlled human infection in such a multidisciplinary way.

Controlled human hookworm infection model

In this thesis we have described the results of three controlled human hookworm infection trials. In the first study, the Controlled Human Hookworm Infection Leiden (shortened to CHHIL) described in **chapter 3**, we report a pilot study to establish the hookworm culture in Leiden and investigate long-term kinetics of egg excretion. This pilot has led to the development of a Bayesian statistical model to describe egg excretion. Using this model we described the development of a plateau phase around 12-13 weeks after infection and discovered the use of multiple samples from the plateau phase as a more reliable outcome measure for future trials. We furthermore found that although variability in egg excretion was reduced for samples of the same volunteer on the same day due to the homogenization of faeces, variability between individuals and within individuals on different timepoints was still considerable. We therefore wanted to further reduce variability in egg excretion by introducing a repeated infection. This was investigated in the ReCHHI study (Repeated Controlled Human Hookworm Infection). The effects of one, two or three doses of 50 L3 were described in **chapter 4**. Using the previously established Bayesian statistical model we found that a double infectious dose indeed lowers variability relative to the mean and increased egg load compared to a single dose without aggravating adverse events. A third dose however did not further improve the model. Power calculations based on the statistical modelling showed that the number of samples taken is the most important determinant for study power. The combined results from these two studies leads us to propose a challenge model of 2x50 L3, with a follow-up of 16 weeks after first infection and taking samples from week 12-16 to determine egg excretion in repeated samples. This model was subsequently applied to investigate the protective efficacy of short-term exposure to infective larvae against challenge infection in the ITCHHI trial (Immunisation, Treatment and Controlled

Human Hookworm Infection) in **chapter 6**. Here, we found that those volunteers with severe skin reactions showed significantly lower egg load after challenge, demonstrating a possible relation between the skin phase and the development of protective immunity. Concluding, we have established an improved controlled human hookworm infection model and shown its application in an immunization trial that for the first time showed a protective effect of short-term exposure to infective larvae against hookworm challenge.

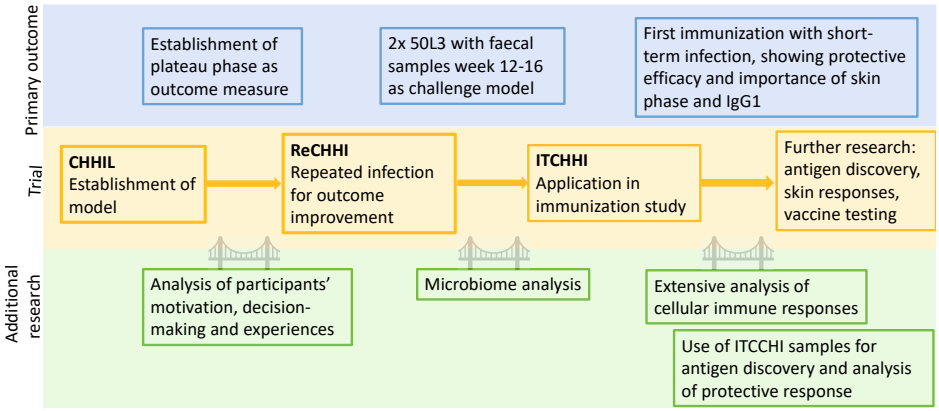


Figure 1. Schematic depiction of controlled human hookworm infections conducted in this thesis with primary research developments resulting from the trial, and examples of additional research conducted.

Multidisciplinary approach

Next to the involvement of advanced Bayesian statistics to improve the controlled human hookworm infection model, we have maximized the scientific output of the studies by taking an interdisciplinary approach. **Chapter 5** describes the analysis of the gut microbiome in relation to the controlled human hookworm challenge. Here, we found that gastro-intestinal symptoms following hookworm infection were associated with increased microbiome instability, which after establishment of the infection stabilized over time. In **chapter 7**, we have explored ethical aspects of controlled human infections. We described how volunteers' motivations for participation are highly varied and that volunteers take many factors into account in their decision to partake. The concept of controlled human infections and critical questions surrounding it, such as restrictions on the right to withdraw and the necessity for quarantining were widely accepted by both participants, students interested in participation and students who would not participate. This led us to conclude that participants in controlled human infection trials are not, as often feared, money-oriented risk-takers, but rather deliberate decision-makers who have made a multi-facetted decision to take part.

Connecting disciplines to improve controlled human infection models

Through this thesis we have aimed to establish improvements to the controlled human hookworm infection model though collaboration with several other disciplines.

Improving outcome measurements in CHI-models

Accuracy in outcome measures is a major challenge in the development of clinical trials. Firstly, the outcome measure needs to be reliable in order to be able to correctly interpret the study results. Secondly, precision in the outcome measure, e.g. the standard deviation, is a crucial component of sample size calculations and therefore affects study power. In any clinical study sample size calculations require a fine balance between including a sufficient number of participants for a meaningful outcome but not exposing an overly large group of participants to the possible harms of the study. Improved precision in outcome measures can reduce sample sizes whilst retaining study power.[1]

In controlled human hookworm infection (CHHI) models, faecal egg output is the main outcome used. Some studies have used endoscopic evaluation of worm establishment in the gut.[2 3] However, this is not a feasible study outcome in larger scale trials because of the burden to the volunteer, high costs and remaining uncertainty about the reliability of this measure. Therefore, examining egg output in the faeces is the best proxy outcome to establish infectious burden, as is done in the field.[4]

In initial controlled human hookworm trials, egg output was significantly lower compared to natural infection and could not be achieved in all donors.[5] This hampers comparability to infections in the field and necessitates large sample sizes in order to establish infection in a sufficient number of participants. In our CHHIL-study, we have shown that it is possible to increase the infectious dose with a tolerable safety profile resulting in the highest egg counts then described in literature. The repeated infection further increased the egg output to a posterior mean at plateau level of around 1500 epg, hereby resembling infection in mild-endemic settings.[6]

Egg excretion is a highly variable outcome, with high sampling errors and dependent on day-to-day variation in egg excretion.[7] In our studies we have used Kato-Katz as measure for egg excretion, as microscopy is a field-applicable technique and mostly used in field studies. Other studies have used culture data as primary outcome.[8] This outcome measure is dependent on culture techniques, egg survival in storage and due to its laborious process cannot be used for multiple sampling. Recent studies are focusing on the use of PCR in field settings. PCR studies are complicated by lack of field-applicability and may suffer from confounding in the outcome measure as the amount of DNA in eggs can differ depending on the cell stage of the egg, which does not reflect the actual egg count but rather the maturity of the eggs. Although some correlation between PCR and egg counts is found this is not repeated in all studies.[9] PCR will probably gain more importance in future as techniques

improve, however PCR similarly suffers from to day-to-day variation in egg excretion, which is considerable.

This high variability hampers the precision of egg counts as outcome measure. Single measures on one day do not give a representative impression of hookworm infection burden. Using 'traditional' statistical methods such as means or medians is possible but has the downside of either missing a lot of variation or being easily skewed due to high variability if using a single measurement of faecal egg load. We have therefore incorporated expertise from advanced Bayesian statistical modelling into the analysis of egg counts. This has led to the development of a statistical model that is able to calculate a posterior estimated mean and give estimates for variability on different levels. This model identified high variability between daily measures in the same volunteer and the development of the plateau phase upon which further studies were based. Application of this model to power calculations further refined the established outcome measure, where it showed that using multiple sequential samples decreased the variability and improves study power. In data with many factors of variability this Bayesian model-based power calculations take into account all sources of variability and uncertainty in the data, thereby generating a more reliable power estimate. The Bayesian model can also generate insight into where most variability in outcomes can be found, targeting possible interventions to reduce variability. This shows that crossing the bridge to involve more elaborate statistical analysis than perhaps is common in this type of trials significantly impacted the reliability and usefulness of this controlled infection model.

Applications in other controlled human infection models

Bayesian modelling has also been applied to controlled human malaria infection, describing the kinetics of parasitemia using qPCR.[10] Power calculations based on this model found that a vaccine targeting the liver stage requires a large sample size compared to an erythrocytic vaccine stage due to the large inter-individual variation in first generation parasites and that needle-based CHMI may result in improved power for hepatic vaccines. Other CHI-models that use quantitative measures of infectious load that may be highly variable, such as viral load in nasal swabs (e.g. influenza, respiratory syncytial virus) or excretion of oocysts in stool (cryptosporidium) may also benefit from a similar approach.

Another approach to improve outcome measures can be done by standardizing the monitoring of clinical symptoms. Some studies use mainly clinical symptoms as outcome of their study as add-on or even instead of microbiologic parameters. In influenza trials, questionnaires have been used to capture participant-reported symptoms.[11] This questionnaire was added to traditional anti-H and anti-N antibody measurements, allowing for better comparison with natural infection and better understanding and tracking of symptom development. This gives a better indication of direct patient benefit of drugs or vaccines in terms of quicker symptom resolution or diminished symptoms than viral shedding or antibody measurements, and therefore results in a clinically more relevant outcome.

An example of the value of the systematic collection of adverse events and the application of statistical techniques for outcome measure improvements is the development of a disease severity score for the *Shigella* and enterotoxic E. Coli (ETEC) CHI-model. Here, available participant data has been gathered for the individual models and through multi-correspondence analysis a disease severity score was developed.[12 13] This disease score has several advantages over the current outcome measures. Most *Shigella* and ETEC CHI-models use stool-based outcome measures. However, these are not field-applicable, suffer from different definitions used in different trials and miss other important disease information. The disease severity scores incorporated objective disease signs, subjective symptoms and stool output. This resulted in an outcome measure that was more in line with the classification of clinical disease and more efficient in capturing disease in participants. Using this new score, the attack rate in the control group was increased. This has the advantage of being able to decrease sample sizes in future studies. There are still several limitations to this score, including a large variation between challenge strains used and these scores which need to be validated in new trials,[14 15] however its development is a good example of the possibilities of combined data analysis.

Adverse events

When performing studies where volunteers can expect little to no personal benefit, as with all CHI-studies, one of the major ethical issues is the possible risk and burden to the volunteers. Adverse event reporting is a major factor in this assessment. Furthermore, as discussed above adverse events may be an important outcome measure of the trial. Understanding which adverse events develop, why and in which volunteers is therefore very important to further improve controlled human infections.

Adverse events in controlled human hookworm studies

In our hookworm studies we have seen a remarkably large variation in the number and severity of skin and abdominal adverse events, both between studies and between individuals. In all trials, there have been volunteers with very few, mild adverse events and others with more severe events. In the ReCHHI study abdominal events were most severe, with three volunteers requiring rescue treatment. No association between infectious dose and abdominal events was found in this study. In the ITCHHI study skin adverse events were most severe, which was found to be related to egg load after challenge. Previous studies had found that exposure to large numbers of larvae at the same site resulted in more severe skin adverse events.[5] We therefore divided the infectious dose over several sites, which was successful in reducing skin adverse events after first exposure. Although there was interindividual variability in severity in our studies, skin adverse events were generally related to repeated exposures and the immunization process. Clear predisposing factors for the abdominal adverse events however were not found. Gastro-intestinal symptoms were not dose-related in our studies. Previous studies with much smaller inocula have described a similar unpredictable pattern in adverse events, although inocula up to 20

larvae do not seem to produce severe adverse events.[5 16 17] In the ReCHHI study we did find a correlation between peak eosinophilia and severity of abdominal adverse events. It can be speculated that this is a reflection of an eosinophil driven enteritis upon establishment of the worms in the gut. Without biopsies it is however not possible to confirm this. In the ITCHHI study the relation between adverse events and eosinophils was less clear although a similar non-significant trend was observed. Identifying factors that predispose volunteers to more severe adverse events would be of great value to improve the risk-benefit balance of the CHHI model. These factors may include a previous history of abdominal symptoms such as irritable bowel syndrome, factors in the gut microbiome or immunological characteristics. In the microbiome study (**chapter 5**) we did find that volunteers with more severe abdominal adverse events had less stable gut microbiome after infection. Whether this instability in microbiome composition was a predisposing factor to, or a consequence of, more severe (eosinophilic) enteritis remains uncertain. More data needs to be gathered to investigate if and which microbiome factors predispose to more severe abdominal adverse events.

If we could better predict which volunteers develop severe adverse events this would greatly improve the safety profile of a CHI-trial. In the controlled human malaria model some research has been performed comparing adverse event profiles using different inoculation methods.[18] However this analysis only looked at immunological factors after infection. No research has currently been done comparing pre-infection parameters to post-infection adverse events. Systematically recording adverse events and recording baseline characteristics together could provide an interesting opportunity to investigate risk factors for the development of severe adverse events. At this moment trial protocols are not developed for this purpose. As very little is known about pre-disposing factors this would also involve the analysis of large amounts of data, necessitating particular expertise in analysing and interpreting large datasets.

Advanced data integration can also be applied to better investigate correlates of protection. We show an example of this in the integrative analysis performed in **chapter 6**. Although the sample size was too small to be used as a predictive tool, the analysis confirmed findings on correlates of protection. In larger data sets, this can be further developed as a prediction tool and may aid in investigating factors associated with adverse events. As this will need to focus on factors that can easily be measured before start of a trial, much more data particularly on easy to assess clinical characteristics is needed for this analysis.

Standardized method of reporting adverse events

As we have argued above a thorough understanding of adverse events is vital for a good assessment of risks and burdens to study participants and could contribute significantly to a better understanding of which volunteers are at risk of developing more severe adverse events. An attempt to review the safety profile of controlled human infections has recently been published, showing serious adverse events are rare and confirming an overall good safety profile.[19] A more in-depth analysis of adverse events however was hampered by the

highly diverse reporting between different studies. Many CHI-studies are relatively small scale and therefore in themselves not powerful enough to identify risk factors for the development of severe adverse events or develop meaningful clinical outcome scales. If adverse event data would be more uniformly documented and reported this would provide highly valuable information and enable conduct of for example meta-analysis of safety outcomes. More standardized reporting would also enable datasets to be combined in integrative analysis for which CHI-studies on their own are usually too small. This is one instance where we may not need to bridge to a different discipline but to other research groups in a similar discipline, harmonizing reports to improve both research quality and participant safety.

Questioning participants and engaging participants in study design

The questionnaire study described in **chapter 8** is one of the first studies to quantitatively study participants' motivations and experiences. Recently Kamuya et al. have described in detail qualitative interviews with participants in malaria controlled human infection trials performed in an endemic setting, providing an important addition to the knowledge of participant experiences in these studies. Although there were differences with the findings in our study, particularly due to the different setting, all studies thus far have described that although the financial compensation is important, participants consider many different aspects before taking part in a study. Volunteers also generally report a satisfactory experience in participating. [20-22]

This knowledge is an important contribution to the ethical debate surrounding controlled human infection. There has been extensive discussion on ethical issues such as social value, acceptable risks and burdens, concepts such as quarantine, the right to withdraw and the influence of payment. [23-25] These are issues that can gain from assessment of participant and public opinion.

The COVID-19 pandemic has greatly increased the voice of the public in the discussion surrounding participation particularly in CHI-trials. The 1DaySooner movement started out as a collective of people willing to participate in COVID-19 challenge studies and is now developing into an important platform of participants speaking out on many aspects of controlled human infections and driving research development in CHI-studies.[26]

When developing controlled human infection models in endemic settings, community engagement is one of the key factors to be taken into account and gains particular attention in set-up of trials. [22 27-29] It is recognized that community acceptance and involvement is vital in the success of these trials. This engagement however is largely lacking in already established CHI-models in the non-endemic areas where most CHI-studies are still conducted. Initiatives such as 1DaySooner aim to mitigate this, but there is still a considerable lack of participant questioning and engagement.

Participant involvement can furthermore aid in the ethical debate surrounding healthy volunteer research. CHI-studies have been ethically scrutinized particularly on the aspects of deliberate infection and perceived breach of the right to withdraw due to quarantine restrictions for transmissible pathogens.[23] Our questionnaire study however found that most participants were happy to agree with these restrictions, as long as it was well explained in the informed consent procedure. Involving participants in trial set-up can also inform researchers about which aspects participants themselves consider to be too high a burden or ethically unacceptable. This can result in a better ethical appraisal of a proposed trial. Routinely adding questionnaires to any participation in a CHI-trial would result in a balanced representation of participants' views that can be used to further improve follow-up studies. An excellent opportunity to build a bridge between the biomedical and social sciences, and society itself.

Maximising scientific output of CHI-studies

The CHI-model has mostly been propagated as a model to test novel drugs and vaccines. However, CHI-trials have much more potential uses than only vaccine- and drug studies, as we have highlighted in **chapter 2** and the studies in this thesis further underscore. The microbiome study presented in **chapter 5** is an example where a more fundamental research question was addressed with samples of a CHHI trial. The ITCHHI study (**chapter 6**) obviously does not immediately lead to a vaccine that is field-applicable, but has generated highly novel insights into the development of protective immunity against hookworm infection, insights which may impact vaccine development in future. Similar more fundamental studies include trials investigating the interplay of influenza infection with pneumococcal nasal carriage[30 31] and the chemo-attenuated malaria model.[32]

CHI-models are not only a vehicle for testing vaccines and drugs, but their controlled nature offers important opportunities to better understand the pathogen-host interactions. These more fundamental questions can be answered either as an add-on to a clinical trial researching a drug or vaccine, or through a separate study. Many clinical CHI-trials have led to multiple publications, one describing the clinical result and others reporting a more fundamental analysis of for example microbiome factors or an extended immunologic analysis. For any clinical trial additional research questions can be formulated. In order to make maximal use of the investments of volunteers' contribution, materials and research funding, already in early planning stages careful consideration must be given to all possible scientific outcomes other than the primary research question. Involvement of researchers from different specialties than the principal investigator aids to find and address these questions. This add-on approach to fundamental questions significantly increases the scientific value of a CHI-study. In fact, it can even be argued that it is our ethical duty as researchers to maximise the output from a given trial to make the maximal use of the investment of the volunteer and specifically look for the fundamental questions that can be answered next to the primary question.

Concluding remarks

In this discussion we have suggested several improvements to be made to CHI-models, including the refining of outcome measures, systematic registration of adverse events, engagement of participants and addressing fundamental questions alongside vaccine- and drug studies.

For these improvements to be implemented a multi-disciplinary approach to CHI is inevitable. In this thesis we have presented the results of collaboration with multiple disciplines which have led to the conclusions presented here. We have demonstrated the added value of advanced statistics in the modelling of hookworm egg excretion and in-depth analysis of all available data to establish correlations. We have also shown the importance of adding an ethical and/or social science approach and have provided an example of how one CHI-trial can be analyzed by several disciplines each bringing up their own novel findings. In addition, we argued for the inclusion of multiple specialties in designing CHI-trials to maximise the output of not only the clinical trial but also fundamental questions that can be answered by a CHI-trial.

Building the bridge

The bridge on the cover of this thesis forms a connection not between two islands but between the two ends of a fjord in Norway. It brings together two pieces of land that, although a connection exists, require a long way to meet. Standing on one coast, it is easy to view the other side as an island without connection. This bridge links the two ends together in a shortcut, greatly reducing travel times along the coast. This is symbolic for the aims in this thesis: scientific disciplines are all connected, we are not islands. However, we cannot always immediately see this connection and may feel like an island. Reaching the same output may then feel like a very long road to travel. Reaching out and bridging the fjord brings the two ends together and allows us to much more rapidly continue our journey to the communal aim: fighting infectious diseases and improving health care for all.

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Appendices

Nederlandse samenvatting

Samenvatting van dit proefschrift getiteld: 'Bruggen bouwen: een multidisciplinaire aanpak van het gecontroleerd humane mijnworm infectiemodel'.

Gecontroleerde humane infectiestudies worden al sinds de 19e eeuw gebruikt als vaccin, therapeuticum en steeds vaker als platform voor het ontwikkelen van vaccins en geneesmiddelen tegen infectieziekten. Bij dergelijke studies worden gezonde vrijwilligers blootgesteld aan een zorgvuldig gemonitorde infectie, waarna effecten van geneesmiddelen, vaccins, immunologische reacties en andere infectie-gerelateerde processen nauwgezet kunnen worden onderzocht. Belangrijke doorbraken in het onderzoek binnen de infectieziekten, zoals de overdraagbaarheid van gele koorts, besmettelijkheid van luchtwegvirussen, de ontwikkeling van het eerste geregistreerde malaria vaccin RTS'S en de registratie van het orale cholera vaccin voor reizigers, zijn (mede) mogelijk gemaakt door gecontroleerde infecties. Met name voor pathogenen die het armste deel van de wereld treffen, waar geld voor onderzoek vaak schaars is, is de efficiëntie van gecontroleerde infectiestudies van groot belang voor het ontwikkelen van mogelijke behandelingen en vaccins en voor het beter begrijpen van de onderliggende immunologische processen.

Een van die armoede-gerelateerde infecties wordt veroorzaakt door mijnwormen. Dit zijn kleine wormen, waarmee zo'n 300 miljoen mensen wereldwijd zijn besmet. Deze wormen vestigen zich in de dunne darm, waar ze door chronisch bloed- en eiwitverlies zorgen voor bloedarmoede en ondervoeding, met name bij kinderen en vrouwen in de vruchtbare leeftijd. De wormen produceren eieren, die via de ontlasting in de omgeving terecht komen als er onvoldoende sanitaire voorzieningen zijn. Vanuit deze eieren ontwikkelen zich larven, die via de huid opnieuw mensen kunnen besmetten. In tegenstelling tot veel andere infecties, ontwikkelen mensen geen beschermende afweer tegen mijnworminfecties en kunnen dus voortdurend opnieuw besmet worden, ook na behandeling. Waarschijnlijk wordt dit veroorzaakt doordat de volwassen wormen een remmende werking hebben op het afweersysteem, zodat zij zelf beter kunnen overleven. Hierdoor wordt echter ook geen goede bescherming opgewekt tegen nieuwe infecties. Er is nog weinig bekend over welke mechanismes eventueel wel tot bescherming kunnen leiden. Wel is bekend dat de huidige bestrijding van mijnworminfecties middels grootschalige behandelcampagnes onvoldoende effectief is door de voortdurende herbesmettingen die plaatsvinden. Een vaccin zou hierbij van grote waarde zijn. Er zijn echter nog weinig aanknopingspunten om effectieve vaccins te ontwikkelen. Het gecontroleerde humane infectiemodel biedt door de gecontroleerde opzet veel mogelijkheden om immunologische responsen beter te ontrafelen, en als er een vaccin wordt ontwikkeld de effectiviteit hiervan snel en efficiënt te onderzoeken.

Het concept gecontroleerde humane infectiestudie roept wel ethische vragen op: gezonde vrijwilligers worden blootgesteld aan een infectie, is dat wel ethisch toelaatbaar? Om deze vraag te beantwoorden zijn diverse ethische raamwerken opgesteld, om te definiëren

waaraan zo'n studie dan moet voldoen. Hierbij worden de ethische principes die gelden voor al het onderzoek met gezonde vrijwilligers aangehaald: onder andere de balans tussen de risico's en belasting versus de verwachte wetenschappelijke en maatschappelijke opbrengst, de rol van de financiële vergoeding en de autonomie van de deelnemer. De auteurs van deze raamwerken stellen dat mits aan deze ethische principes wordt voldaan gecontroleerde infectiestudies ethisch te verantwoorden zijn.

In dit proefschrift wordt de ontwikkeling van een gecontroleerd humaan mijnworm model beschreven, waarna wordt samengewerkt met diverse disciplines. Daarna wordt ingegaan op enkele ethische aspecten van gecontroleerde infectiestudies door middel van onderzoek naar motivatie en ervaringen van deelnemers.

In **hoofdstuk 2** beschrijven we het landschap van gecontroleerde infectiestudies. Hierin laten we zien dat het aantal studies de afgelopen decennia fors is toegenomen, met een zeer divers palet van verschillende infectieziekten waarvoor een gecontroleerd infectiemodel is ontwikkeld.

In **hoofdstuk 3** beschrijven we de eerste opzet van het gecontroleerde mijnworm infectiemodel. We laten hier zien dat een dosis van 50 larven voldoende is voor een effectieve infectie die goed werd verdragen. Daarnaast laten we zien dat naarmate de infectie langer duurt de uitscheiding van eieren stabiliseert in een plateaufase. Dit plateau kan gebruikt worden als uitkomstmaat voor toekomstige onderzoeken. Er wordt echter nog wel veel variabiliteit gezien, wat de bruikbaarheid van het model als uitkomstmaat in vaccin- of geneesmiddelstudies vermindert.

In **hoofdstuk 4** beschrijven we verdere verbeteringen van het humane mijnworm infectiemodel waarbij we hebben geprobeerd om de uitkomstmaat betrouwbaarder te maken. Dit hebben we gedaan door te onderzoeken of herhaalde infecties zorgen voor een stabielere uitscheiding van eieren in de ontlasting. Vervolgens hebben we Bayesiaanse statistiek toegepast op de gemeten ei-uitscheiding, om de variabiliteit hierin beter te kunnen beschrijven. Hierbij vonden we dat een herhaalde infectie inderdaad zorgt voor zowel hogere als stabielere ei-uitscheiding. Een derde infectie voegde hier echter niets aan toe. Een belangrijke factor in het verbeteren van de betrouwbaarheid van de ei-uitscheiding als uitkomstmaat, bleek het combineren van herhaalde metingen op verschillende tijdspunten tot een gemiddelde. Door gedurende een aantal weken in de plateaufase ei-uitscheiding te meten en hiervan een gemiddelde te nemen, nam de bruikbaarheid van deze uitkomst belangrijk toe. Hiermee stelden we vast dat het gecontroleerde mijnworminfectie model waarschijnlijk het beste werkt met een dosis van tweemaal 50 larven.

Het model dat we ontwikkelden in hoofdstuk 3 en 4, is vervolgens toegepast op een immunisatiestudie beschreven in **hoofdstuk 6**. In dit onderzoek werden vrijwilligers blootgesteld aan een kortdurende infectie met larven, waarna de infectie weer werd

geklaard door behandeling met albendazol. Hierdoor ontstond alleen blootstelling aan de larven-fase van infectie. Na drie van dergelijke immunisaties werden alle vrijwilligers geïnfecteerd met mijnwormen, die deze keer wel het volwassen stadium bereikten. Hierbij bleek dat de vrijwilligers die geïmmuniseerd waren met larven, en dan met name degenen die hierop een sterke reactie in de huid vertoonden, een lagere ei-uitscheiding hadden dan vrijwilligers die een placebo-immunisatie hadden ondergaan. Deze vrijwilligers lieten ook een meer uitgesproken respons in met name IgG1 zien. Hiermee toonden we aan dat het mogelijk is om een beschermende respons op te wekken door kortdurende blootstelling aan larven, waarbij de huid potentieel een belangrijke rol speelt in het opwekken van die bescherming.

In **hoofdstuk 5** beschrijven we hoe een brug werd geslagen naar het microbioom onderzoek. Het microbioom van de deelnemers aan de studie beschreven in hoofdstuk 4 werd onderzocht voor, tijdens en na hun deelname aan het mijnwormonderzoek. Dit onderzoek liet zien dat vrijwilligers met meer maag-darmklachten gedurende de eerste fase van de infectie een meer instabiel microbioom van de darm hebben. Dit herstelde weer in de chronische fase van de infectie. Of deze instabiliteit wordt veroorzaakt door de komst van de larven en de daardoor veroorzaakte enteritis of dat personen met een meer instabiel microbioom ook sneller klachten krijgen kon niet worden onderscheiden.

In **hoofdstuk 7** werd een andere discipline geëxploreerd: die van de onderzoeksethiek. Middels vragenlijsten werd onderzocht wat de motivaties waren van de deelnemers aan gecontroleerde humane infectiestudies; hoe ze hun deelname hebben overwogen en hoe ze hun deelname hebben ervaren. Hieruit bleek dat hoewel de financiële vergoeding belangrijk was, dit voor bijna de helft van de deelnemers niet de belangrijkste motivatie voor deelname was; het bijdragen aan onderzoek, aan de behandeling van ziektes en interesse in het onderwerp bleken ook belangrijke motivators voor deelname. Ook wogen vrijwilligers veel factoren mee in hun beslissing om deel te nemen, waaronder vertrouwen in het studieteam, de tijdsinvestering en de kans op het ontwikkelen van klachten. Het overgrote deel van de deelnemers was trots op hun deelname, zou nogmaals meedoen en zou het anderen ook aanraden.

In de samenvattende discussie in **hoofdstuk 8** bespreken we welke verbeteringen er verder mogelijk zijn aan de gecontroleerde humane infectiemodellen en hoe deze verbeteringen door interdisciplinaire samenwerking tot stand kunnen komen. Zo kan samenwerking met geavanceerde statistiek zorgen voor het preciezer maken van uitkomstmaten, waarmee deelnemersaantallen kunnen worden verkleind en kleinere effecten beter kunnen worden opgespoord. Door meer samenwerking kan een betere registratie van ongewenste effecten worden vastgelegd, waarbij door het combineren van data en geavanceerd modelleerwerk mogelijk beter kan worden voorspeld welke vrijwilligers ernstiger klachten gaan krijgen. Hiermee kan inclusie en veiligheid van studies worden verbeterd. Ook wordt het belang van het betrekken van deelnemers bij het opzetten van studies onderstreept, waarbij

de inbreng van deze deelnemers belangrijke informatie kan geven over wat vrijwilligers daadwerkelijk een last of risico vinden en waar voor hen die grens kan liggen. Door het includeren van verschillende specialismes bij het opzetten en uitvoeren van studies kan de wetenschappelijke opbrengst van een individuele studie worden vergroot. Omdat het hier studies betreft met vrijwilligers, kan zelfs worden beargumenteerd dat een dergelijke multidisciplinaire samenwerking ethisch verplicht is om het maximale te halen uit de investering die de vrijwilliger belangeloos doet. Door multidisciplinaire samenwerking kan de afstand tussen onderzoeksdisciplines worden overbrugd, tot het gezamenlijke doel om gezondheidszorg voor iedereen te verbeteren.

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2023

Hoogerwerf MA, Janse JJ, Kuiper VP, van Schuijlenburg R, Kruize YC, et al. *Protective efficacy of short-term infection with Necator americanus hookworm larvae in healthy volunteers in the Netherlands: a single-centre, placebo-controlled, randomised, controlled, phase 1 trial.* Lancet Microbe. 2023 Dec;4(12):e1024-e1034.

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Curriculum Vitae

Marie-Astrid Hoogerwerf werd geboren op 23 augustus 1988 in Rotterdam. Ze volgde tweetalig vwo aan het Rijnlands Lyceum Oegstgeest, waar zij in 2007 met lof het eindexamen behaalde. Hierna studeerde zij aan de Universiteit Leiden, waar zij in 2010 de bachelor Geneeskunde behaalde en in 2013 cum laude afstudeerde voor de master Geneeskunde. Als onderdeel van de master begon zij haar eerste onderzoeksproject onder supervisie van prof. dr. A. Kaptein naar ziektepercepties bij patiënten met longkanker. Na het afronden van de studie Geneeskunde werkte Marie-Astrid als ANIOS Interne geneeskunde in het Haaglanden Medisch Centrum (HMC). In 2015 startte zij met de opleiding tot internist, tevens in het HMC bij opleider dr. A. Bootsma. Gedurende deze periode bleef een interesse in onderzoek, wat na het voltooien van het perifere gedeelte van de opleiding Interne geneeskunde resulteerde in een onderzoekspositie in de groep van Meta Roestenberg. Dit proefschrift is het resultaat van het aldaar verrichte onderzoek. In 2020 keerde Marie-Astrid terug naar de kliniek voor het vervolg van het opleiding intern geneeskunde in het Leids Universitair Medisch Centrum (LUMC), bij opleider prof. dr. J.W. de Fijter, opgevolgd door dr. N. Appelman-Dijkstra. Sinds september 2021 volgt Marie-Astrid de differentiatie Infectieziekten in het LUMC bij opleider dr. S. Arend welke binnenkort zal worden afgerond. Zij is daar betrokken bij onderzoeksprojecten naar participatie van vrijwilligers in gecontroleerde infectiestudies en vaccinatieonderzoek bij het Leiden University Center for Infectious Diseases (LUCID).

Marie-Astrid is getrouwd met Maarten. Samen hebben zij twee dochters; Eleanore (2019) en Rosemarijn (2021).

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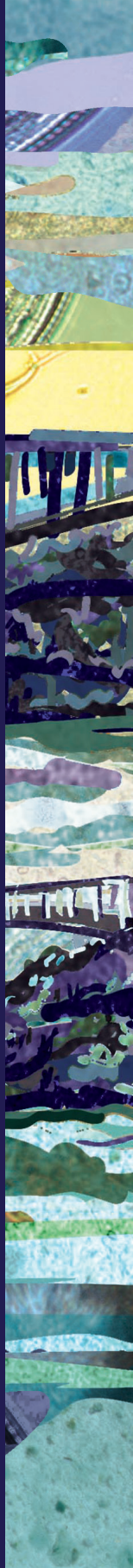
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