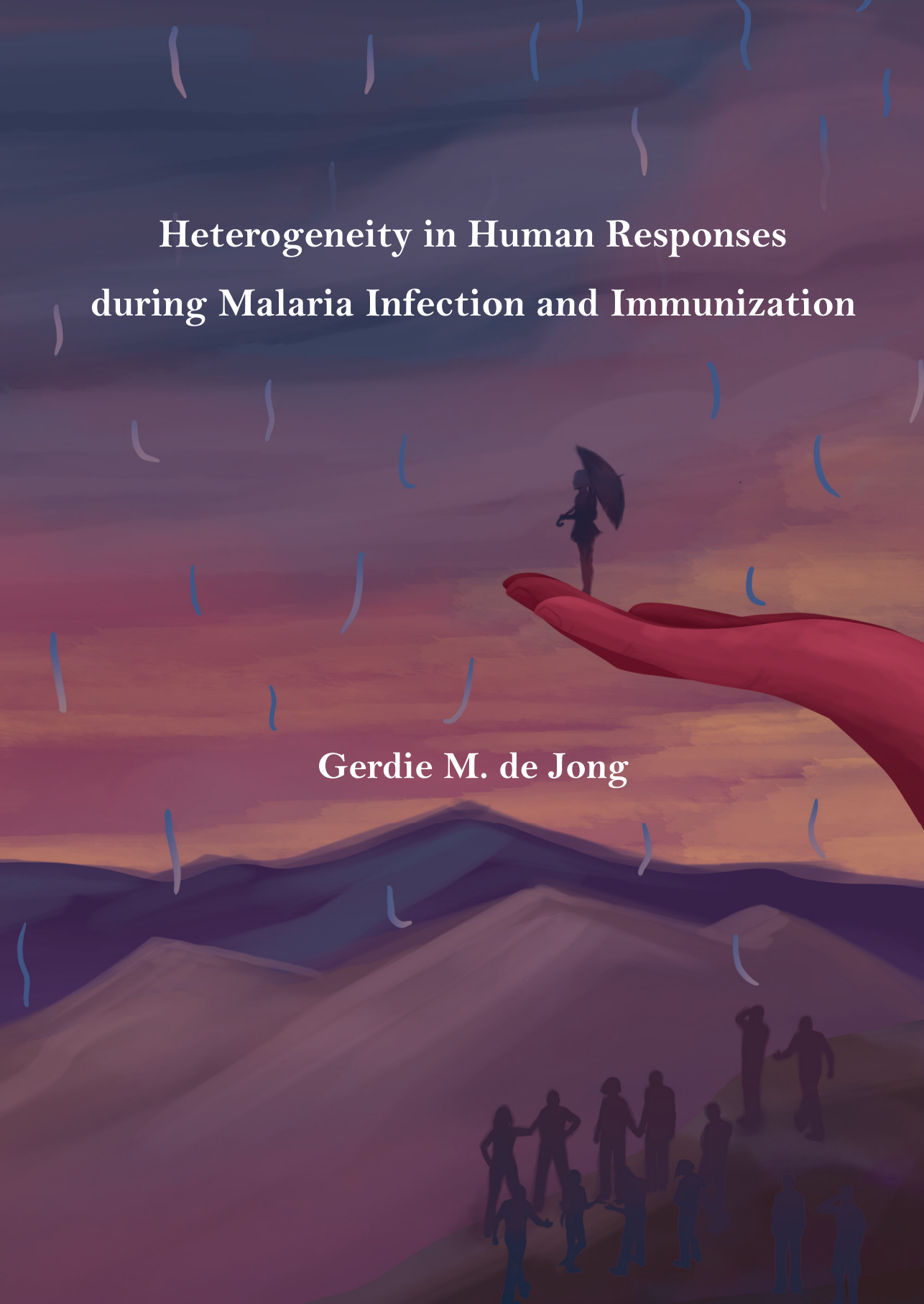
Heterogeneity in Human Responses during Malaria Infection and Immunization



Gerdie M. de Jong

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Heterogeneity in Human Responses during Malaria Infection and Immunization

Heterogeniteit in humane respons tijdens malaria infectie en immunisatie

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Chapter 1

General introduction

Malaria

Malaria is a major global health problem with 219 million new cases and 435,000 deaths in 2017 (1), mostly affecting the poorest populations in sub-Saharan African countries. Although an overall decrease in malaria mortality and morbidity has been observed in the last 15 years, no significant reduction in the number of malaria cases has been reached between 2015-2017 (1). The countries with the highest burden in Africa even had an increase in malaria cases in 2017 compared to 2016 (1) (**Figure 1**). Contributing to the increase in incidence are the emergence of drugs resistant parasites (2, 3) and insecticide resistant mosquitoes (4-6).

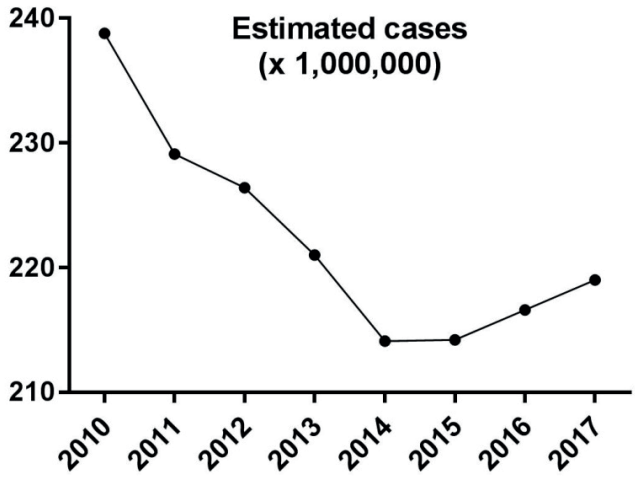

Figure 1 – Estimated malaria cases (World Malaria Report 2018)

Malaria is caused by *Plasmodium* parasites that are transmitted to the human host by a bite of an infected *Anopheles* mosquito. The majority of injected sporozoites migrate through the skin, enter the blood circulation (7) and travel to the liver. Subsequently, the sporozoites invade hepatocytes (liver cells) (8, 9) and start to replicate, resulting in thousands of merozoites (10). Depending on the *Plasmodium* species, the infected hepatocytes, called liver schizonts, rupture 6 to 30 days after infection. The merozoites are then released into the blood stream and invade erythrocytes (11). In the erythrocytes the merozoites mature as part of an asexual life cycle into trophozoites and subsequently into schizonts that comprise new merozoites (12). The infected erythrocytes rupture after infection (13), depending on the *Plasmodium* species this may take 24 hours for *P. knowlesi*, 48 hours for *P. falciparum, P. ovale* and *P. vivax* and 72 hours for *P. malariae*.

During the erythrocyte rupture the newly formed merozoites are released and can infect new erythrocytes.

A small percentage of the asexual parasites develop into a sexual form, gametocytes (14). If these sexual stages are taken up by uninfected *Anopheles* mosquitoes, the male and female gametes can fuse to form zygotes (15). These zygotes become motile, invade the midgut wall of the mosquito and develop in oocysts. As the oocysts grow and rupture, sporozoites are released and migrate to the salivary glands of the mosquito (15). The mosquitoes then become infective and can infect new human individuals (**Figure 2**).

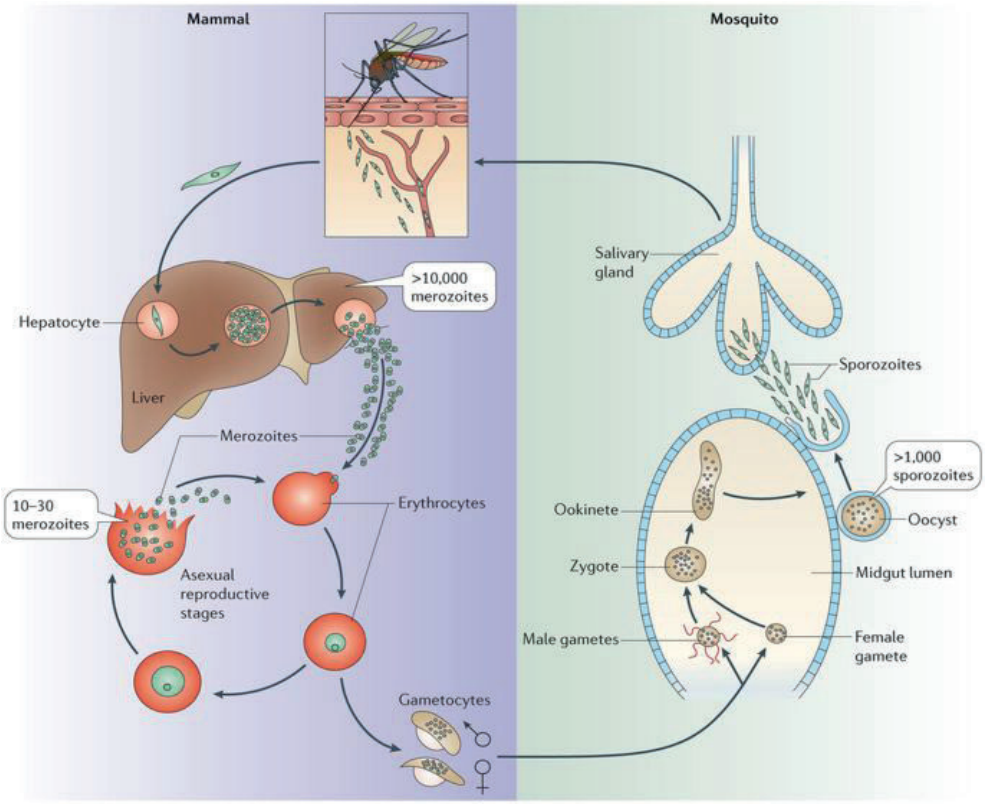

Figure 2 – *Plasmodium* spp. life cycle
Figure reprinted from reference (16) with permission from Springer Nature.

Of all *Plasmodium* species that can infect humans, *P. falciparum* causes the most severe disease. *P. falciparum* infected erythrocytes adhere to the vascular endothelium (sequestration) (17, 18) and to uninfected erythrocytes (rosetting) (19). In contrast to

infection with *P. falciparum*, this occurs only to a very limited extent after infection with one of the other *Plasmodium* species (*P. vivax; P. ovale; P. malariae; P. knowlesi*). Sequestration and rosetting of erythrocytes in vital organs result in obstruction of the blood flow (17), a decline in oxygen delivery to the organs, acidosis and multi-organ failure (20). Whereas the liver stage is asymptomatic, patients become symptomatic during the blood stage infection, experiencing symptoms such as fever, headache and muscle pain. The clinical presentation of malaria depends, at least in part, on pre-existent immunity. Malaria naïve individuals without pre-existent immunity react with a strong pro-inflammatory response and have severe clinical symptoms with a high morbidity and mortality rate whereas individuals that experienced previous malaria episodes have a tendency to have milder clinical symptoms (21, 22). Children under five years of age are most susceptible to malaria (21, 23), as they have not developed an immunological memory response against *Plasmodium* parasites. Only after repeated exposure to *Plasmodium* parasites, clinical immunity (parasitaemia without symptoms) will develop (24). This clinical immunity wanes quickly in the absence of ongoing exposure to *Plasmodium* parasites (25). If timely diagnosed, malaria can be effectively treated with anti-malaria medication (26). However, the diagnosis of malaria in patients in endemic, low-income countries is often delayed since access to health care is difficult due to distance to the clinic or financial restraints (27).

To compare *P. falciparum* infected patients for research purposes, the World Health Organization has determined disease severity criteria, which are now also used in clinical settings (20). Severe falciparum malaria is defined as *P. falciparum* parasitaemia with one or more of the following criteria; impaired consciousness, acidosis, hypoglycemia, renal impairment, anemia, jaundice, pulmonary edema, bleeding shock or parasitaemia >10%. Whereas uncomplicated malaria (absence of mentioned criteria) can be effectively treated, the mortality among patients with severe malaria is high (28, 29). Treatment consists of anti-malarial medication and supportive care in case of organ involvement (20, 26). However, due to the increase in resistance to anti-malarial medication, especially to the most often prescribed drug, artesunate (2, 3), prevention of malaria is becoming more important and an effective vaccine is needed in the fight against malaria. Until now, vaccines have not resulted in sterile immunity (absence of parasitaemia) and further understanding of the highly complex host immune response in the first days after a malaria infection and vaccination is crucial for the development of improved immunization strategies. Due to varying levels of pre-existing immunity in endemic areas, it is difficult to study the host response and vaccine efficacy in endemic areas. Therefore, the Controlled Human Malaria infections (CHMI) model has been developed to study malaria disease in a homogenous group of malaria naïve volunteers (30, 31).

Controlled human malaria infection models

CHMI studies conducted in malaria naïve volunteers are a powerful tool to examine the efficacy of vaccines. In CHMI studies, the high potential, pre-clinical vaccines can be selected from all candidate vaccines. These studies, with a relatively small sample size, may reduce failing large trials in malaria endemic areas and accelerate vaccine development (30). In addition to testing of vaccines, CHMI studies can be used to answer multiple research questions on e.g. acquisition of immunity, the host response after infection and on the pathogenesis of malaria.

During a CHMI, a small group of healthy malaria naive participants is deliberately infected with *P. falciparum* parasites by either bites of infected mosquitoes or by an intravenous inoculum of *P. falciparum* infected erythrocytes or sporozoites. After infection volunteers are intensely monitored and immediately treated with anti-malarial therapy when a predefined threshold of parasitaemia has been reached. CHMI studies are conducted in a highly controlled setting, enable researchers to control infection rates, study distinct aspects of malaria and elucidate the mechanisms of protective immunity. In general, CHMIs have been conducted using standardized protocols and so far over 3000 healthy volunteers have been infected and treated worldwide. The data on parasitaemia during infection show that the disease course can be predicted with only a very limited variation in prepatent period between study participants (30). Additionally, protocols can be adjusted, depending on the study endpoints. To test candidate malaria vaccines, CHMI participants are first immunized after which the safety and dosing of the product is evaluated. When the vaccine is considered to be safe, participants can be immunized by multiple doses and subsequently challenged with *P. falciparum* sporozoites to test the protective efficacy of candidate vaccine. Many candidate vaccines were proposed and tested in the last years, but none of those could establish durable protection (32).

Need for a malaria vaccine

Malaria mortality showed a strong decrease after the year 2000, which has been attributed to increased access to antimalarial therapy and prevention of malaria transmission by bed net distribution in malaria endemic areas (33, 34), due to increased funding for malaria control interventions after the Declaration of the Millennium Development Goals. However, this multitude of still ongoing intervention programs, has not resulted in a lasting (or further) decrease in malaria prevalence. For many viral diseases (e.g. measles), only vaccines proved to be an intervention that achieved a durable reduction of disease specific morbidity and mortality (35-38). Also, reducing asymptomatic forms

of malaria has great clinical impact as asymptomatic malaria increases the susceptibility to other infectious diseases (39-41). Furthermore, the eradication of smallpox (42) is a clear example that immunization of populations can stop spread of an infectious disease. Additionally, vaccination can prevent disease in the individual patient. Development of a malaria vaccine has been a research topic for decades (43, 44). An ideal malaria vaccine will not only result in personal protection, but also reduces disease on the population level. The asymptomatic form of malaria functions as a reservoir for malaria transmission, enabling the parasites to spread in the population (45, 46).

Challenges to overcome

Developing a malaria vaccine has been found to be extremely challenging due to the complex multi-stage life cycle of *Plasmodium* spp. and the interaction of the parasites with the human immune system (47). During each stage of infection, different membrane proteins are expressed and therefore candidate vaccines have been grouped according to the parasite stage and vaccine targets:

1. *Pre-erythrocytic stage vaccines* which elicit an immune response that is targeted to sporozoites and the liver stages.
2. *Blood-stage vaccines* are targeted to the asexual blood stages; after merozoites are released from hepatocytes.
3. *Transmission-blocking vaccines* which prevent transmission of sexual parasites from humans to mosquitoes (48).

In addition to the complex life cycle, other complicating factors for vaccine development are the many different *P. falciparum* strains and antigenic variation (alteration of surface membrane antigens during infection), which enables the parasite to evade the human immune response thereby increasing its survival (49-51). The ideal vaccine will expose the immune system to multiple conserved antigens expressed during the asymptomatic pre-erythrocytic stage, resulting in broad and fully protective immune response which prevents the symptomatic blood-stage. However, humans that are exposed to multiple antigens during a natural malaria infection, can still get re-infected with *Plasmodium* spp. parasites. Therefore, a malaria vaccine-induced immune response should be stronger, more diverse and different from natural infections. Furthermore, in malaria endemic areas acquisition of clinical immunity (asymptomatic parasitaemia) develops slowly and wanes quickly in the absence of repeated exposure to *P. falciparum* parasites (25) whereas vaccine induced protection should be lasting. Moreover, the lack of sterile protection (absence of blood parasitaemia) after a natural infection, due to the induction of immune-

modulatory responses during a malaria infection (52, 53), results in an ineffective host immune response (54, 55). The incomplete understanding of the host response after a natural infection and acquisition of lasting immunity is another hurdle for malaria vaccine development.

Malaria vaccines

The ideal vaccine results in durable sterile protection, and is easy to store and to administer, can be produced on a large scale and is affordable for people living in developing countries. Many candidate malaria vaccines have been tested for protective efficacy in the last years; the *RTS,S* vaccine is the most well-known (55). This pre-erythrocytic subunit vaccine is based on the *P. falciparum* sporozoite surface protein 'circumsporozoite protein' (CSP) which is fused to hepatitis B virus surface antigen virus-like particles. Although this vaccine elicits an immune response that can protect from clinical malaria episodes in immunized individuals in endemic areas, lasting sterile protection was not induced (56, 57). Development of a vaccine that induces sterile protection is favorable as subclinical parasitaemia affects not only the health of an individual but also the global health burden as it remains a competent reservoir for transmission (46). Currently, only studies in which healthy volunteers were immunized by *P. falciparum* while receiving malaria prophylaxis reported sterile protection after a homologous challenge (challenge with the same strain as used for immunization) (58, 59). These studies showed that induction of sterile immunity is possible and that the immune response after development of parasites in the liver is essential. However, the strategy of immunization while receiving malaria prophylaxis is not feasible and safe on population level in endemic countries.

Genetically attenuated parasites (GAP)

A promising novel immunization strategy is the administration of genetically attenuated human or rodent parasites. Deletion of genes specific and essential for liver-stage development, allows development of the parasite in the liver, but program the parasite to die before or during the early blood-stage (60-62). With this approach the immune system is exposed to multiple parasite antigens without a symptomatic and pathogenic blood-stage infection. However, development of a genetically attenuated parasite (GAP) vaccine is still in an early stage: while sterile protection in rodent models was shown (63, 64), breakthrough infections were observed in a phase 1 trial in humans (65). Multiple newly developed GAP vaccines are currently being tested.

Transmission blocking vaccines

Transmission blocking vaccines (TBV) affect the parasite reservoir and increase the herd immunity (66). The interest for this strategy is increasing (67, 68). The transmission of parasites from humans to mosquitoes can be interrupted by antibodies targeting proteins present on sexual parasite stages or proteins that are expressed by the parasite in the midgut of the mosquito, thereby preventing parasite maturation. Vaccines that induce anti-Pfs25 (Pfs25 is a highly important female gametocyte antigen) have been tested in a CHMI in a phase 1 trial focussing on safety, but resulted in systemic adverse events in a subset of volunteers possibly due to the adjuvants used (69, 70). Pfs28, Pfs230 and Pfs48/45 also are under investigation for their transmission blocking capacity (71). However, testing transmission blocking efficacy is still a challenge and occurs only in a laboratory setting by adding antibodies to the infected blood meals before mosquito feeding (72). The infection rate of these mosquitoes is than compared to mosquitoes that were fed on infected blood without antibodies. To establish a more representative transmission-blocking model, a CHMI model is currently being developed. In this model, mosquitoes are infected by healthy infected volunteers after direct skin feeding (73, 74). However, higher transmission rates of mosquitoes are needed to test transmission blocking interventions in this model.

CHMI participants respond differently to an infection

Although the CHMI studies are proven to be save and well tolerated (75), CHMI participants experience malaria symptoms after infection, such as fever, muscle pain, headache, nausea and fatigue. Whereas in some of the study participants these symptoms are mild, others might be unable to perform their normal daily activities. Differences between volunteers are also observed in standard laboratory parameters: a decrease in thrombocyte count, which is a common manifestation during malaria disease, is not observed in all CHMI participants (76, 77), nor are liver function abnormalities (73) and pathological processes like endothelial cell activation and coagulation (78).

Add-on studies in a CHMI cohort showed that onset of malaria symptoms, such as fever can be linked to an increase in pro-inflammatory cytokines during infection (79, 80). However, only a subset of the participants showed an increase in IL1-β, IL-6, TNF-α and/or IFN-γ (80, 81). IFN-γ seems to be the most prominent pro-inflammatory cytokine (82) and enhances the phagocytosis of merozoites and infected red blood cells (83), the

upregulation of MHC class I and II (84) and in the induction of cellular memory responses (84). Various cell types are capable of producing IFN-γ, however, the main source of production depends on the time period since infection (85, 86). The etiology for IFN-γ upregulation in only a subset of CHMI participants is not yet known. To counteract the pro-inflammatory response after infections, a variety of mechanisms are reported that control the inflammatory response of the host, thereby minimizing tissue damage (87-89). During malaria such regulatory pathways (e.g. TGF-β increase) are also up regulated, but only in a subset of CHMI participants (80). Thus, the heterogeneity in immune response observed among CHMI volunteers involves both the pro-inflammatory and regulatory immune response (80, 81). The CHMI model is ideal to further evaluate the differences between the host immune responses to a *P. falciparum* infection in persons that previously have not been infected with *P. falciparum*.

Consequences and importance of understanding the heterogeneity in host response

Understanding the differences in host immune response, pathological processes and clinical presentation is of importance for several reasons. First, the initial immune response correlates with effectivity of immunization (90, 91). Determination of differences after immunization in host response between CHMI participants in relation to study outcome may give further insight in which cytokines and cellular immune responses are beneficial during infection and after immunization. Especially, since studies on correlates of protection after immunization show conflicting results, which may be partly caused by differences between study populations and pre-existent immunity (91). Understanding the etiology of the heterogeneous immune response in a homogeneous CHMI study group is needed for further improvement of malaria vaccines. In addition, this knowledge on the early host immune response might lead to the development of immune-modulating treatment (92, 93). In addition, elucidating differences in adverse events or clinical symptoms, may identify risk factors for the development of severe malaria.

Aims and outline of the thesis

The work described in this thesis can be grouped into two sections with the following aims: *i.* testing a novel immunization strategy for malaria vaccination and optimizing the current CHMI transmission model in order to test transmission blocking vaccines, *ii.* describing and investigating possible causes for the differences in the early human host immune response after a *P. falciparum* infection.

Part *I:* Clinical malaria vaccine development

Chapter 2 describes the results of the first in human clinical trial vaccine study in which a genetically attenuated parasite (GAP) is tested for safety and protective immunity. The genetically modified rodent *Plasmodium berghei* is considered a safe immunization as it may infect human liver cells, but cannot replicate in human erythrocytes. The engineered GAP contain the highly immunogenic CSP-protein of *P. falciparum.* In **Chapter 3** the CHMI model is optimized for testing transmission blocking interventions that interrupt the transmission of *P. falciparum* parasites from humans to mosquitoes.

Part *II:* Heterogeneity in host response after *Plasmodium* spp. infection

In **Chapter 4**, the role of pro-inflammatory and immune modulatory processes during clinical malaria in endemic areas is reviewed. Recommendations are made for parameters that can predict the host immune response and are frequently proposed as biomarkers and therapeutic targets during malaria. In **Chapter 5** we compare liver function abnormalities during malaria between clinical patients and CHMI participants. In addition, we correlate these liver function abnormalities to cytokine profiles. In **Chapter 6**, pro- and anti-inflammatory responses by determination of cytokine profiles during a CHMI are studied. We focus on the relation between different host responses and clinical and parasitological parameters. To further elucidate the differences in host response among CHMI volunteers, in **Chapter 7** the activity of immune cells at baseline is investigated by determination of their ability to produce pro-inflammatory cytokines and to measure the abundance of pro- and anti-inflammatory markers in CHMI participants. These baseline characteristics are correlated to clinical and parasitological parameters during challenge. The findings of this thesis and further perspectives are summarized and discussed in **Chapter 8**.

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Part I

Clinical malaria vaccine development

Chapter 2

Safety and efficacy of a genetically modified rodent malaria parasite against Plasmodium falciparum malaria: An open-label randomized phase 1/2a trial

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Submitted

Abstract

Background

Successful vaccines have been developed using a non-pathogenic counterpart of the causative micro-organism of choice. The non-pathogenicity of the rodent *Plasmodium berghei* (*Pb*) parasite in humans prompted us to evaluate its potential as a platform for vaccination against human infection by *P. falciparum* (*Pf*). We hypothesized that the genetic insertion of the leading protein target for clinical development of a malaria vaccine, *Pf* circumsporozoite protein (CSP), in its natural pre-erythrocytic environment, would enhance *Pb*'s capacity to induce protective immunity against *Pf* infection.

Methods

Hence, we recently generated a transgenic *Pb* sporozoite immunization platform expressing *Pf*CSP (*Pb*Vac), and we now report the clinical evaluation of its biological activity against Controlled Human Malaria Infection (CHMI).

Results

This first-in-human trial shows that *Pb*Vac is safe and well-tolerated, when administered by a total of ~300 *Pb*Vac-infected mosquitoes per volunteer. Although protective efficacy evaluated by CHMI showed no sterile protection at the tested dose, significant delays in patency (2.2 days, p= 0.03) and decreased parasite density were observed after immunization corresponding to an estimated 95% reduction in *Pf* liver parasite burden [confidence interval (CI) 56%, 99%; p=0.010]. *Pb*Vac elicits dose-dependent cross-species cellular immune responses, as well as functional *Pf*CSP-dependent antibody responses that efficiently block *Pf* sporozoite invasion of liver cells.

Conclusion

This translational research study demonstrates that *Pb*Vac immunization elicits a marked biological effect, inhibiting a subsequent infection by the human *Pf* parasite, and establishes the clinical validation of a new paradigm in malaria vaccination.

Introduction

Malaria, a disease caused by *Plasmodium* (*P.*) parasites, remains a severe public health burden. Despite tremendous progress made over the last decade as a result of scaled-up vector control and of improved diagnosis, as well as availability of first line treatments, progress has slowed down over recent years. Malaria still caused ~435.000 deaths in 2017 (1). Besides its direct implications on human health, malaria is a substantial contributor to ongoing poverty in affected countries.

The availability of an effective vaccine against malaria is considered amongst the most important tools for prevention and potential eradication of malaria. The clinical development of a malaria vaccine has been a continuous effort over the past half century (2). Most vaccine candidates so far have shown modest protection or even failed due to the complex biology of malaria parasites and required immune responses (3). The circumsporozoite protein (CSP) of *P. falciparum* (*Pf*), the predominant antigen on the surface of sporozoites, is currently the leading protein target for clinical development of pre-erythrocytic subunit malaria vaccines. Encouraging, but moderate, protection has been obtained with the *Pf*CSP-based RTS,S/AS01e (RTS,S) vaccine (4, 5). RTS,S has been associated with up to 87 percent protection in CHMI and delays patency in non-protected volunteers (6); further, it reduces the number of cases of malaria by 39 and severe malaria by 29 percent over four years in young African children, but the vaccine's efficacy wanes over several years (7). A milestone in itself, as the first malaria vaccine to be given to children through routine immunization, RTS,S is potentially a valuable tool in the fight against malaria when used in combination with other malaria control interventions, and is currently undergoing pilot implementation in various African countries. However, it is clear that malaria vaccines with improved and more durable efficacy are needed (8).

A promising alternative approach are whole-parasite vaccine strategies which are based on attenuated *Pf* sporozoites, However, these novel strategies are still in relatively early stages of clinical development (9). Radiation attenuated sporozoites (RAS) were the first whole-parasite vaccine candidates eliciting high efficacy against a homologous strain during Controlled Human Malaria Infections (CHMI) in malaria-naïve adults (10, 11). However, a markedly lower efficacy was observed in recent field evaluations (12-15). Similarly, a remarkable and highly efficient induction of sterile homologous (16, 17), but not heterologous (18), protection against *Pf* infection was also achieved by immunization using a combination of chemoprophylaxis and sporozoites (CPS). More recently, the first genetically attenuated parasite (GAP) employed as a vaccine candidate, PfSPZ-GA1, showed modest homologous protective efficacy (19). Collectively, these results warrant the evaluation of novel cross-strain or cross-species approaches that might provide protection against genetically diverse human *Plasmodium* strains.

The concept of cross-species vaccination relies on immunization with the non-pathogenic counterpart of the causative micro-organism of disease. The origins of this concept are usually attributed to Sir Edward Jenner, who is claimed to have pioneered the use of a bovine poxvirus to prevent smallpox (20), although Jenner's role in this discovery is the matter of some controversy (21). Nevertheless, the idea of cross-species vaccination has since been employed for several other human diseases, including the Modified Vaccinia Ankara (MVA) virus and other poxviruses against smallpox (reviewed in (22), the bovine bacillus Calmette–Guérin (BCG) vaccine against human tuberculosis (reviewed in (23)), or the selection of rhesus and bovine rotavirus strains to create human rotavirus vaccines (reviewed in (24)). Cross-species immunity between different *Plasmodium* species has been reported in multiple animal studies (25-30), and in clinical studies carried out in the last century as part of an experimental neurosyphilis treatment (31, 32). Live attenuated sporozoite-based cross-species immunity is thought to be dependent on both cellular- and antibody-based responses (30). Experimental evidence indicates that *Pf* sporozoites can protect mice from a *P. berghei* (*Pb*) infection (28), and inoculations of *P. malariae* in humans reduce parasitaemia and symptoms of a subsequent *Pf* infection (31, 32).

While the expression of correctly folded *Pf* proteins in heterologous systems can be challenging, *Pb* parasites have recently emerged as an alternative solution for their structurally integral and conformationally-accurate expression, as exemplified by the transmission-blocking candidate antigen Pfs48/45 (33, 34). Thus, we hypothesized that *Pf*CSP's protective ability against *Pf* could be qualitatively enhanced compared to recombinant subunit CSP vaccine candidates, through its expression in a more native-like conformation, and in its natural pre-erythrocytic environment. This, coupled with the potential for immune responses between different *Plasmodium* species, and the non-pathogenic nature of *Pb* parasites (35), prompted the generation of a transgenic *Pb* sporozoite expressing *Pf*CSP (*Pb*Vac). Pre-clinical studies established the proof-of-principle of *Pb*Vac immunization through the validation of an array of core premises, including the demonstration of (i) its ability to infect and develop inside human liver cells; (ii) its inability to develop in human red blood cells (RBCs), (iii) its high cellular cross-species immunogenicity; and (iv) its ability to induce functional antibodies that block infection of human hepatocytes by *Pf* sporozoites (35). Additional pre-clinical demonstration of *Pb*Vac's safety for human use paved the way to the evaluation of this vaccine candidate in a clinical setting (36).

Here, we performed a first-in-human, dose-escalation study to assess safety and tolerability of *Pb*Vac delivered by infectious mosquito bites and determined its protective efficacy against *Pf* CHMI. We showed that *Pb*Vac is safe and well-tolerated, and induces cross-species cellular immune responses and functional *Pf*CSP-dependent antibody responses,

which can efficiently inhibit *Pf* sporozoite invasion of liver cells. Sterile protection against *Pf* was not observed, but our results demonstrate a strong biological effect elicited by *Pb*Vac and establish the clinical validation of a new approach to malaria vaccination.

Materials and Methods

Study design

This study was a multicenter Phase I/IIa open label, dose escalation trial in malaria-naïve healthy participants to assess safety and tolerability of *Pb*Vac (Phase 1) and to determine the protective efficacy of immunization with *Pb*Vac against CHMI (Phase 2). The study protocol was approved by the Dutch Central Committee on Research Involving Human Subjects (CCMO, NL60019.091.16), the Western Institutional Review Board (WIRB, 20170356), and *Pb*Vac was released for study use by the Gene therapy office in the Netherlands, licenced to the Radboud University Medical Centre (IM-MV 15-010), Erasmus Medical Centre (IM-MV 16-008) and Harbour Hospital (IM-MV 16-010). The trial was conducted according to the principles outlined in the Declaration of Helsinki and Good Clinical Practice standards, and registered at ClinicalTrials.gov, identifier NCT03138096.

In Phase 1, eighteen participants were divided in 3 groups and exposed to bites of 5 (n=3), 25 (n=3) or 75 (n=12) *Pb*Vac-infected mosquitoes. Study subjects of all groups were followed once daily on an outpatient basis from day 1 to 10 and every other day on day 12 to 20 after exposure. During the visits, blood was drawn for routine haematological and biochemical analysis, peripheral Giemsa-stained thick blood smears, and retrospective real-time quantitative polymerase chain reaction (*Pb*18S qPCR) analysis. Signs and symptoms were recorded by a physician and graded as follows: grade 1 (no interference with daily activities), grade 2 (interferes with normal activity) or grade 3 (prevents normal activity). Participants of group 1 and 2 were treated with a curative regimen of atovaquone/proguanil (Malarone®, GlaxoSmithKline) on day 28, and received an end of study visit on day 100 after immunization. Participants of group 3 received three additional immunizations with 75 *Pb*Vac-infected mosquitoes after Phase 1 at four, four, and eight weeks intervals (Phase 2). Three weeks after the last immunization all twelve immunized participants, including six infectivity controls, were subjected to a standard CHMI with 5 *P. falciparum* (strain NF54)-infected mosquito bites (37). All participants were monitored once daily on an outpatient basis from day 6 to 21 for symptoms and signs of malaria, and blood was drawn for routine laboratory tests and for *Pf*18S qPCR analysis, as previously described (38). Participants were treated with a curative regimen of atovaquone/proguanil (Malarone®, GlaxoSmithKline) after a single positive qPCR

(≥100 parasites/mL) after challenge. Additional information can be found in the Clinical Trial Protocol, included as Supplementary Information.

Study participants and eligibility criteria

All 57 volunteers that underwent screening signed an informed consent form after the nature and possible consequences of the studies have been explained. Four of the volunteers included withdrew their informed consent because of change of job and relocation from the area of the study center and, therefore, 4 back-up volunteers were enrolled in the study. A total of 33 participants were excluded after screening. Twenty-four healthy malaria-naïve males and non-pregnant females (aged 18 to 35 years) were recruited at the Harbour Hospital, Rotterdam, after signing informed consent. Extensive screening was performed before inclusion in the Erasmus Medical Centre, Rotterdam, as previously described (39). The complete screening procedures, including the list of inclusion and exclusion criteria, can be found in the clinical trial protocol document, as well as on ClinicalTrials.gov, identifier NCT03138096.

Sample collections

Blood collections for separation and cryopreservation of plasma and peripheral blood mononuclear cells (PBMCs) were performed at the beginning of the trial and the day before each immunization or CHMI using sodium citrate BD Vacutainer® Mononuclear Cell Preparation Tubes (CPT). Further details are provided in the Clinical Trial Protocol, included as Supplementary Information.

Objectives and end point measures

The primary objectives of the study were to assess safety and tolerability of *Pb*Vac and to determine the protective efficacy of *Pb*Vac immunization against CHMI. The endpoints were determined by frequency and magnitude of adverse events, as well as by the presence of parasitemia after exposure to *Pb*Vac as assessed by thick blood smear. The primary endpoint of Phase 2 was to determine the time to parasitemia after CHMI as detected by 18S qPCR. Secondary endpoints were to determine the immunogenicity of *Pb*Vac as assessed by enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA), as well as the composition and function of immune responses after exposure to *Pb*Vac, which were analysed through different assays.

Production and administration of PbVac

The generation and characterization of *Pb*Vac, which consists of genetically modified sporozoites of rodent *P. berghei* parasites (Pb) expressing PfCSP has been previously described in detail in Mendes et al. (35, 36). Briefly, a transgenic Pb parasite line containing a Pfcs expression cassette was generated using the 'gene insertion/marker out' (GIMO) technology. Correct integration of the construct into the genome of transgenic parasites was analysed by diagnostic PCR analysis of gDNA and Southern analysis of pulsed field gel (PFG)-separated chromosomes.

PbVac parasite detection by quantitative PCR

Retrospective *Pb*Vac parasite detection on whole blood samples was performed by the use of a qPCR with *P. berghei* 18S rRNA-specific primers as previously described (36). A standard curve consisting of ten-fold serial dilutions from 10^6 to 1 *Pb*Vac sporozoite was included in all qPCR assays.

Estimation of Pf liver burden reduction

Liver burden reduction calculation was estimated by back-calculating the initial number of first-generation parasites released from the liver and then comparing the means of the logarithms of estimated concentration in controls and immunized individuals with a two-sample T-test. Back-calculations were performed based on a published mathematical model (40, 41) based on the following assumptions: 1) the first generation of parasites are released at 6.87 days after inoculation; 2) the time between parasite generation is 1.84 days; and 3) asexual parasite numbers increase with a factor of 11.8 per parasite generation. Back-calculation was performed using the highest measured parasite concentration for each subject, which yielded the most conservative estimate of the difference between controls and immunized (in contrast to basing the back-calculation on the lowest measured parasite concentration, the first or last measured concentration greater than zero, or a composite estimate based on all concentrations above zero in each person).

IgG purification and PfCSP-specific IgG depletion

Pre- and post-immunizations (before challenge) IgGs from citrated plasma samples were precipitated with a saturated ammonium sulfate solution (Pierce, Thermo Fisher), following the manufacturer's instructions, and resuspended in phosphate buffered saline (PBS) (Gibco, Thermo Fisher). IgGs were purified by affinity chromatography using 1 ml

HiTrap protein G HP columns (GE Healthcare Life Science) and eluted with an amine-based buffer at pH 2.8 (Pierce, Thermo Fisher).

To deplete the IgGs with specificity for *P. falciparum* circumsporozoite protein (*Pf*CSP), post-immunization IgGs were run several times through a 1ml HiTrap NHS-activated HP column coupled to a full length *Pf*CSP (Genova Biotechniques Pvt. Ltd, In Hyderabad, India), as previously described (42). Anti-*Pf*CSP IgGs were eluted from the column with the previously used buffer. After purification and depletion, IgGs (anti- *Pf*CSP fraction and non- *Pf*CSP fraction) were buffer-exchanged with PBS (Gibco) and concentrated using vivaspin 30 concentrator columns (Sartorius). The final concentration of IgGs was measured by the bicinchoninic acid (BCA) assay (Pierce, Thermo Fisher) following the manufacturer's indications.

Anti-P. falciparum circumsporozoite protein enzyme-linked immunosorbent assay

The reactivity of the IgGs from citrated plasma samples at indicated time points and the depletion efficiency were tested against the full-length *Pf*CSP (Genova Biotechniques Pvt. Ltd.) or the repeat region of *Pf*CSP with the amino acid sequence (NANP)4NVDPC using a standardized ELISA, as previously described (42). Briefly, Immunolon polystyrene flat-bottom 96-well plates (Thermo Fisher) were coated overnight at 4°C with 2 µg/ml of *Pf*CSP. The plates were blocked (0,05% Tween20/1x PBS (PBST) with 5 % milk) for 1h at room temperature (RT). Plasma samples were diluted 1:50 in 1% milk PBST and a four-point 1:3 dilution was carried out for each sample. To determine the depletion efficiency, pre and post anti-*Pf*CSP-depleted samples were tested at a final concentration of 1 mg/ml in duplicates. After 3h incubation at RT, plates were washed three times with washing solution (PBST) and incubated for 1h at RT with the secondary antibody, goat anti-human IgG coupled to horseradish peroxidase (HRP) (1:3000, Jackson Immuno research). After six washes the reaction was developed with tetra-methyl-benzidine (TMB) followed by the addition of 0,2M sulfuric acid to stop the reaction and Absorbance was measured at 450nm using an iMark Microplate Absorbance Reader (Bio-Rad) or an Infinite M200 plate reader. Data was analyzed in relation to a plasma pool from 100 Tanzanian hyper-immune adults living in a highly malaria-endemic area (HIT). The plasma pool was diluted 1:200 in 1% milk PBST and a 7 point 1:2 dilution curve generated. Analysis was performed using Auditable Data Analysis and Management System for ELISA (ADAMSEL FPL v1.1) and the data were expressed as arbitrary units (AU), as described elsewhere (18, 42).

In vitro sporozoite invasion inhibition assay

The ability of the induced antibodies to inhibit sporozoite invasion was assessed *in vitro* using the human hepatoma cell line HC-04 (MRA-965, deposited by Jetsumon Sattabongkot). Briefly, HC-04 cells ($5x10^4$ cells/well) were seeded onto rat tail collagen (BD Bioscience) pre-treated flat-bottom 96-well plates (Corning, Merk) for 16-24h. Pre- or post-immunization or anti-*Pf*CSP depleted IgGs were pre-incubated at 6 mg/ml with *Pf*NF54, *Pb*Vac or *Pb*WT salivary gland-dissected sporozoites with 10% heat-inactivated human serum (HIHS). Sporozoites were also incubated with PBS to determine the percentage of invasion and with a mouse anti-CSP antibody, 2A10 (100 µg/ml; MR4, MRA-183A) as invasion inhibition control. In addition, to neutralize possible anti-*Pf*CSP IgGs remaining after the depletion, 500 µg/ml of soluble *Pf*CSP protein was added to the depleted samples and to the 2A10 control. After 30 minutes on ice, $5x10^4$ sporozoites were seeded in triplicates onto HC-04 cells. The plates were centrifuged for 10 minutes at 3000 rpm with low brake and incubated for 3h at 37°C in 5% Co_2. Cells were then washed with PBS (Gibco) to eliminate non-invading sporozoites and trypsinized (0.05% Trypsin-ethylenediamine tetraacetic acid (EDTA); Gibco) for 5 minutes at 37°C to obtain a single cell suspension. 10% HIHS/PBS was added at 1:1 ratio to neutralize the trypsin. Cells were transferred to a V-bottom 96-well plate, washed with PBS (Gibco) and centrifuged at 1700 rpm for 4 minutes at 4°C. Subsequently, a cell viability dye (1:2,000; Fixable viability dye (FVD) eFluor 780, eBioscience, Thermo Fisher Scientific) was added for 20 minutes at 4°C. After washing with PBS, the cells were fixed and permeabilized (eBioscience) for 30 minutes at 4°C and stained to detect intracellular and invading sporozoites with a fluorescently labelled mouse anti-CSP antibody (1:200; 3SP2-fluorescein isothiocyanate (FITC)) for 30 minutes at 4°C. Cells were then washed with 2% fetal bovine serum (FBS)/ PBS and fixed with 1% paraformaldehyde (PFA). The readout was done by flow cytometry using Gallios (Beckman Coulter) and the analysis with FlowJo software (version 10.0.8, Tree Star).

In vitro complement deposition and membrane-compromised sporozoite assay

The capacity of the induced antibodies to fix complement (C3b) and therefore compromise the sporozoite membrane integrity was assessed by flow cytometry, as previously described (42). Briefly, salivary gland-dissected *P. falciparum* (NF54) sporozoites were seeded onto V-bottom 96-well plates ($5x10^4$ sporozoites /well; Costar). Infected salivary glands were washed with PBS and centrifuged at 3200 g for 5 minutes at RT. The supernatant was carefully removed and the sporozoites were then incubated in duplicate with 10 mg/ml of pre- or post-immunization IgGs and 10 % fresh human serum (NHS, active complement),

all components were diluted in 1x veronal buffer (Lonza Bioscience). After incubating 30 minutes at 37°C, the complement was inactivated with 10 mM EDTA/PBS for 5 minutes at 4°C. The sporozoites were then washed with 2% BSA/PBS and stained with a fixable cell viability dye (1:2000; eFluor 780 eBioscience), a fluorescently labelled mouse anti-*Pf*CSP antibody (1:10000; 3SP2-dylight 650) and the anti-C3b complement protein deposition (1:500; C3-FITC Cappel, MP Biomedicals) for 30 minutes at 4°C. The samples were washed and fixed with 1% PFA. Positive cells were quantified by flow cytometry. Data were acquired with Gallios (Beckman Coulter) and analyzed with the FlowJo software (version 10.0.8, Tree Star).

Quantification of plasma IgG and IgM by a flow cytometry-based immunofluorescence assay (IFA)

Quantification of sporozoite-specific antibody titers in the plasma of immunized volunteers was performed by a flow cytometry-based immunofluorescence assay. Cryopreserved *Pf*NF54, *Pb*Vac or *Pb*WT sporozoites were thawed and washed with PBS by centrifuging at 13,200 rpm (16,900g) for 15 minutes at 4°C. Sporozoites were labeled by incubating with 20 µM SYTO 61 red fluorescent nucleic acid stain (Molecular Probes) for 30 minutes at 4°C, centrifuged and resuspended in PBS. Total plasma or purified IgGs (at 5,000ng/mL) were diluted 1:500 in PBS, and 20µL of each dilution were mixed with 20µL of sporozoites in PBS (containing 25-50x10^3 parasites) in a U-bottom 96-well plate. After a 30-minute incubation at 4°C the plate was centrifuged (10 minutes at 3900g and 4°C) and the supernatant removed. Anti-human IgG CF488A (Sigma) and anti-human IgM pacific blue (Biolegend) antibodies were added to the wells for 30 minutes at 4°C. Sporozoites were fixed in 1% formaldehyde (Sigma) before acquisition on a BD LSRFortessa™ X-20 (BD Biosciences). Data was analyzed using FlowJo v10.5.3 (FlowJo, LLC).

Ex vivo phenotyping of peripheral blood mononuclear cells

The impact of *Pb*Vac immunization on circulating immune populations was assessed by flow cytometry. PBMCs were thawed and rested overnight at 37°C in complete RPMI (RPMI-1640 containing 10% heat-inactivated FBS, 2mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 1x Minimum Essential Medium (MEM) non-essential aminoacids, all from Gibco, Thermo Fisher Scientific). 1x10^6 PBMCs were incubated with FVD for 15 minutes at RT, washed with PBS containing 2% FBS and stained with the following anti-human antibodies: CD4 (RPA-T4), CD8 (RPA-T8), CD3ε (UCHT1 or HIT3a), TCRγδ (IMMU510), TCR Vδ2 (B6), CD56 (MEM-188), CD14 (HCD14), all from Biolegend, eBioscience or Beckman Coulter. Cells were washed with PBS containing 2% FBS, fixed in 1% formaldehyde for 10 minutes and

finally washed and resuspended in PBS with 2% FCS before acquisition of approximately 5x10^5 events on a BD LSRFortessa™ X-20 (BD Biosciences). After exclusion of dead cells (based on positive FVD staining) and doublets (based on FSC-A and FSC-H parameters) the following populations were assessed: CD4$^+$ T cells (CD3$^+$CD4$^+$CD8^{neg}TCRγδ^{neg}CD56^{neg}), CD8$^+$ T cells (CD3$^+$CD4^{neg}CD8$^+$TCRγδ^{neg}CD56^{neg}), TCRγδ T cells (CD3$^+$TCRγδ$^+$), TCRγδ Vδ$_2$ T cells (CD3$^+$TCRγδ$^+$Vδ$_2^+$), NKT cells (CD3$^+$TCRγδ^{neg}CD56$^+$), NK cells (CD3^{neg}CD56$^+$) and monocytes (FSC^{high}SSC^{high}CD3^{neg}CD14$^+$). Data was analyzed using FlowJo v10.5.3 (FlowJo, LLC).

Serum cytokine and chemokine analysis

Serum quantities of 9 different cytokines and chemokines were determined using the bead-based LEGENDplex™, (Biolegend) assay, following the manufacturer's instructions. Samples were analyzed at baseline and after the first immunization (days 1, 2, 3, 5, 7 and 10). Flow cytometry data were obtained on a Gallios (Beckman Coulter) apparatus and analyzed with the FlowJo software (version 10.0.8, Tree Star).

Measurement of in vitro T cell responses by intracellular cytokine staining assay

The cellular responses of immunized volunteers to *Pf*NF54, *Pb*Vac or *Pb*WT sporozoites or to *Pf*CSP were assessed using an intracellular cytokine staining assay. 1x10^6 thawed PBMCs were stimulated in a total of 200 µL per well in a U-bottom 96-well plate with 1.5x10^5 purified, cryopreserved and washed *Pf*NF54, *Pb*Vac or *Pb*WT sporozoites, or with media alone as a negative control, for 22h at 37°C. For *Pf*CSP stimulation, PBMCs were rested overnight at 37°C and stimulated for 6h with 1 µg/mL *Pf*CSP (Genova Biotechniques Pvt. Ltd) plus 1 µg/mL BD FastImmune CD28/CD49d costimulation, or costimulation alone as a negative control. Stimulation with 10 ng/ml PMA (Sigma) and 500 ng/mL ionomycin (Sigma) for 5h was used as positive control. In all stimulation conditions 10 µg/ml brefeldin (Sigma) was added to the cultures for the last 5h. After culture cells were incubated with FVD, washed with PBS containing 2% FCS and surface-stained with TCRγδ and CD56 antibodies for 30 minutes at RT. After fixation and permeabilization using the Transcription Factor Staining Buffer Set, according to the manufacturer's instructions (eBioscience, Thermo Fisher Scientific), cells were stained intracellularly with anti-human CD4, CD8, CD3, IFNγ (4S.B3), TNFα (Mab11) and IL-2 (MQ1-17H12) antibodies for 30 minutes at 4°C. Finally, cells were washed and resuspended in PBS with 2% FCS, and approximately 5x10^5 events were acquired on a BD LSRFortessa™ X-20 (BD Biosciences). Data was analyzed using FlowJo v10.5.3 (FlowJo, LLC). Results are presented as the frequency of cytokine-producing cells in the stimulated condition minus in the respective negative control.

Analysis of heterogeneity of individual immune responses

To analyze the heterogeneity of individual responses to immunization, a Z-score was calculated for the magnitude of the cellular and humoral immune responses measured for each volunteer in each assay, by subtracting the population mean prior to immunization from an individual raw score and then dividing the difference by the population's standard deviation prior to immunization. This score reflects the direct comparison of the responses of each volunteer before CHMI with the mean values of each parameter prior to immunization for the entire group, and enables ranking the volunteers according to their responses, for each immune response parameter assessed.

Statistics

Statistical analysis was performed using GraphPad Prism software (version 5, GraphPad Software Inc., California, USA) and Microsoft Excel (version 16.16.8). All volunteers exposed to *Pb*Vac were included in the intention-to-treat analysis. All safety data were descriptive to characterize safety and appearance of parasitemia ('breakthrough' infection). After challenge infection, the prepatent period to *P. falciparum* positivity (time for first positive qPCR >100 parasites/mL) and difference in parasitemia at first positive qPCR were compared between groups by applying the Kolmogorov-Smirnov non-parametric test. Liver burden reduction calculation was estimated as described above. Differences in antibody titers, as well as in cytokine production, upon stimulation across the immunization groups were analyzed by applying the Wilcoxon matched-pairs signed rank test. The non-parametric Spearman correlation test was applied for all analysis of correlation. *P* value of ≤ 0.05 was considered statistically significant.

Results

Study population

Twenty-four volunteers were enrolled in the study from May 2017 to January 2018, of which 3 volunteers participated in group 1, 3 in group 2 and 12 in group 3, receiving 5, 25, and 75 *Pb*Vac-infected mosquito bites, respectively. Group 3 participants received a total of ~300 (4x~75 at 4-, 4- and 8-week intervals) *Pb*Vac-infected mosquito bites prior to *Pf* CHMI by 5 mosquito bites. Six volunteers (group 4) served as non-immunized controls for *Pf* CHMI (**Fig. 1**). All 24 study volunteers (100%) completed all immunizations and/or challenge as scheduled per group. CHMI was administered to 12 immunized participants (group 3) and 6 controls (group 4) on 30 January 2018. Baseline characteristics are shown in **Table S1**.

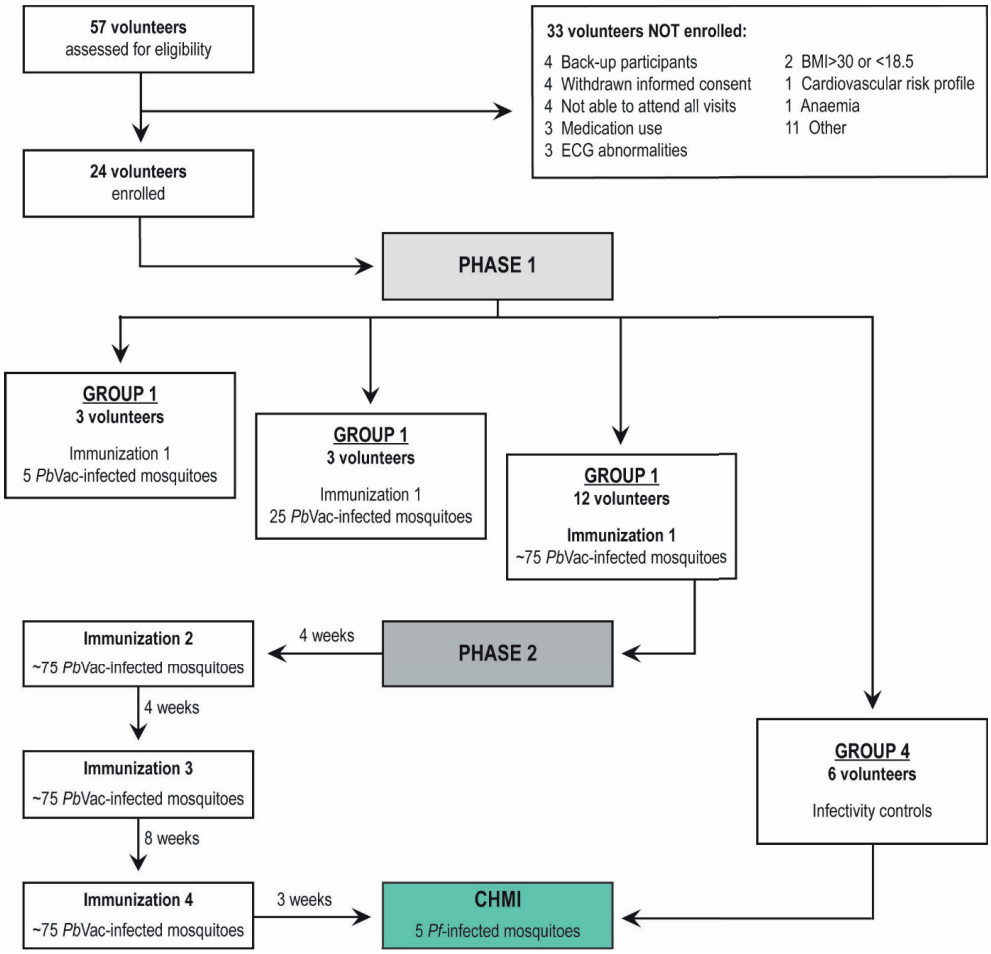

Figure 1 – Trial flowchart
BMI=Body Mass Index, ECG=Electrocardiogram.

Safety and tolerability

Immunization phase: Immunizations were well-tolerated in all study groups without breakthrough infections, as measured by thick smear and retrospective *Pb* quantitative PCR (qPCR) analyses of samples collected daily for 10 days after parasite administration, during Phase 1 of the trial, and on days 1 and 8 after immunization, during Phase 2 of the trial. Only mild to moderate (grade 1 and 2) adverse events, and no grade 3 or serious adverse events, were recorded (**Table S2**). There were no differences in systemic adverse events between study groups, while local reactions related to the mosquito bites were more

frequent in groups 2 and 3. The most commonly reported systemic adverse events were headache (7/18 participants), nausea (5/18 participants) and malaise (3/18 participants). A total of only eight (grade 1 or 2) laboratory abnormalities were observed including decreases in leukocyte, lymphocyte, and platelet counts, and increases in bilirubin, AST/ALT, and creatinine concentrations (**Table S3**).

Challenge phase: Approximately 92% (11/12) of the participants of group 3 and 100% of group 4 reported mild to moderate adverse events (**Table S2**) including headache (6/12 in group 3, 6/6 in group 4), nausea (1/12 in group 3, 4/6 in group 4) and malaise (1/12 in group 3, 2/6 in group 4). The total number of adverse events per participant of groups 3 and 4 was significantly lower (P=0.040) in the immunized subjects than in controls.

Protective efficacy

While sterile protection against a NF54 *P. falciparum* challenge was not observed, a significant delay in time to parasitaemia (prepatent period) was observed in *Pb*Vac-immunized subjects (9.9 ± 2.0 days), compared to controls (7.7 ± 1.6 days) (P=0.026, Kolmogorov-Smirnov test), as detected by qPCR (**Fig. 2A**). Furthermore, there was a significantly 12.8-fold lower parasite peak density on the day of first positive PCR in immunized volunteers compared to the control group (p=0.04, Kolmogorov-Smirnov test) (**Fig. 2B**). Collectively, this corresponds to an estimated 95% average reduction in parasite liver load (CI 56%, 99%; p=0.010, two-sample T-test for logarithm of estimated first-generation parasite concentration) (**Fig. 2C**).

Humoral immune responses

Antibody responses to the candidate vaccine were measured in subjects by IFA against *Pb*Vac sporozoites, as well as against the wild type *Pb* (*Pb*WT) and *Pf*CSP vaccine components. Marked increases in *Pb*Vac sporozoite recognition were observed for both IgG (5.9-fold, P<0.01, **Fig. 3A**) and IgM (3.14-fold, P<0.01, **Fig. S1A**) after immunization. Similar results, albeit of lower magnitude, were obtained for *Pb*WT sporozoite-specific IgG and IgM (2.6-fold, **Fig. 3B;** 2.8-fold, **Fig. S1B**, respectively) and for *Pf*CSP (4.2-fold, **Fig. 3C;** 2.16-fold, **Fig. S1C**).

We subsequently assessed antibody binding and functionality against *Pf*, by IFA, sporozoite membrane integrity and sporozoite liver invasion inhibition assays (**Fig. 3D-F**). Immunization with *Pb*Vac increased the concentration of both IgG and IgM targeting *Pf* sporozoites (3.5-fold, P<0.01, **Fig. 3D**; 1.71-fold, P=0.03, **Fig. S1D**, respectively). Of note, the capacity of plasma IgG to bind *Pf* sporozoites directly correlated with the titers of circulating anti-*Pf*CSP antibodies (**Fig. S1E**). IgG purified post immunization (**Fig. S1F**)

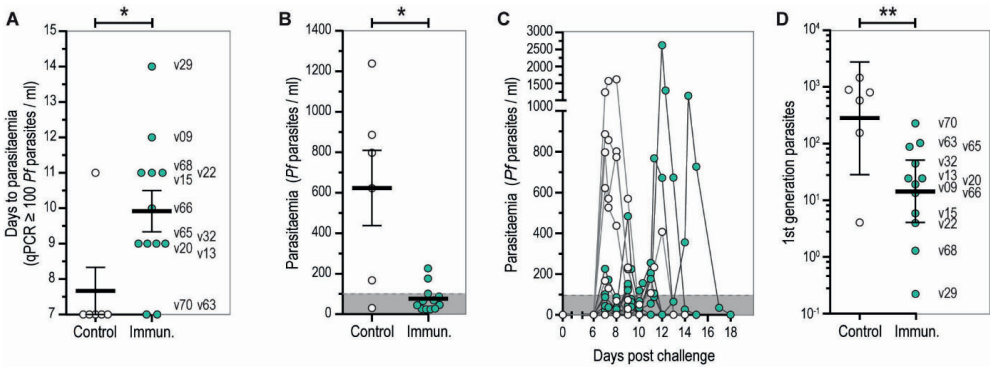

Figure 2 – Clinical efficacy of *Pb*Vac

(A) Prepatent period for non-immunized controls (white circles) and immunized volunteers (green circles), presented as the number of days until parasitemia was at least 100 *Pf* parasites/ml of blood, as measured by qPCR; **(B)** *Pf* parasitemia at first positive qPCR; **(C)** *Pf* parasitemia curves following CHMI; **(D)** Estimation of the number of first-generation *Pf* parasites exiting the liver. Comparisons between controls and immunized volunteers in A) and B) were assessed by employing the Kolmogorov-Smirnov non-parametric test (*≤0.05; **≤0.01) and bars represent mean ± standard error. The shaded area below the dashed line in B) and C) denotes parasitemia values below the 100 *Pf*/ml of blood threshold. In D), significance was calculated using a two-sample t-test and bars represent geometric mean ± standard error. Vxx denotes volunteer number.

enhanced C3 complement protein deposition on *Pf* sporozoites (4.3-fold, $P<0.01$, **Fig. S1G**), resulting in a strong decrease in *Pf* sporozoite viability (**Fig. 3E**).

Finally, purified IgG significantly reduced *Pf* sporozoite invasion of HC-04 cells by a median of 61.1% ($P<0.01$). Depletion of anti-*Pf*CSP-specific IgG completely reversed the inhibitory effect on *Pf* sporozoite invasion (**Fig. 3F)**, as well as the C3 complement fixation on *Pf* sporozoites (**Fig. S1G**). The titers of IgG against *Pf*CSP, the binding capacity of IgG to *Pf* sporozoites, and the enhancement of *Pf* sporozoite lysis, correlated with the cumulative number of *Pb*Vac mosquito bites (**Fig. 3G-I**, respectively and **Table S4**). The combined data suggest that induced antibodies were capable of inhibiting *Pf* sporozoite viability and functionality in a dose-dependent manner. However, these *in vitro* findings did not correlate with the delayed pre-patency nor with the reduced parasite liver load.

Cellular immune responses

Given the largely exploratory nature of the study, a wide-range analysis of cellular immune responses was performed. Peripheral blood mononuclear cells (PBMCs) sampled before immunization (I1-1), 14 days after the first immunization (I1+14) and one day before CHMI (C-1) were analyzed by multi-parameter flow-cytometry with an initial focus on relevant

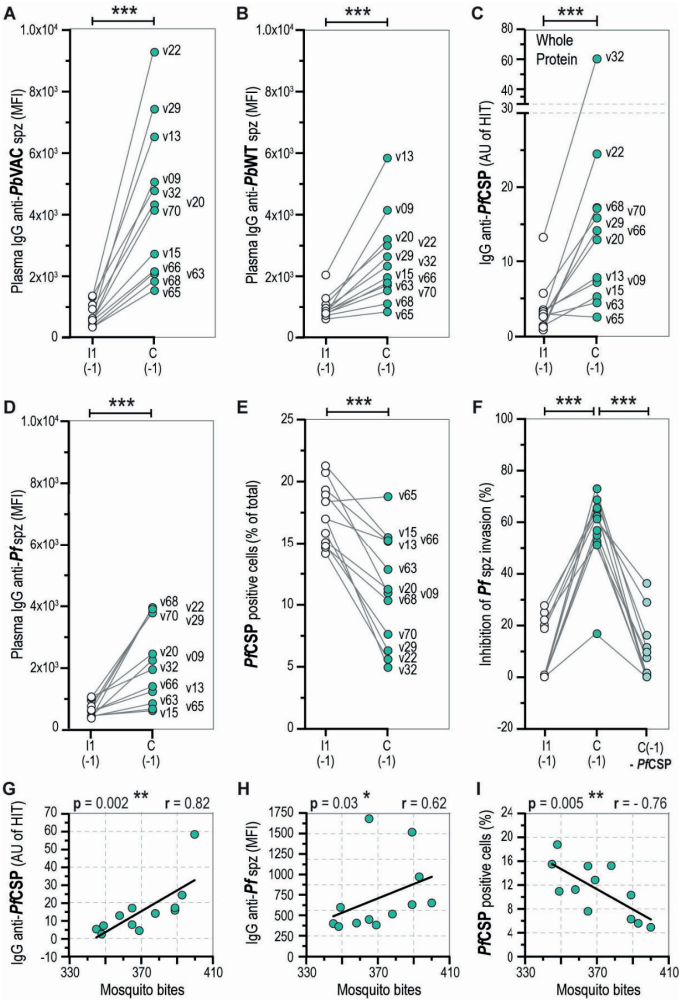

Figure 3 – Humoral responses elicited by *Pb*Vac

(A-B) Plasma IgG binding to *Pb*Vac spz (A) and *Pb*WT (B) spz in samples collected pre-immunization (I1(-1) – white circles) and prior to CHMI (C(-1) – green circles); Median fluorescence intensity (MFI) values are for binding at a 1/1,000 dilution; **(C)** Plasma IgG reactivity against full length *Pf*CSP as measured by ELISA, normalized to a pool of sera from 100 hyperimmune Tanzanians (HIT), which was set at 100 arbitrary units (AU). **(D)** Binding of plasma IgG to *Pf* spz. MFI values are for binding at a 1/1,000 dilution; **(E)** Viable *Pf* spz-infected cells following incubation with purified plasma IgG was assessed as a surrogate for induced *Pf* spz lysis; **(F)** Inhibition of *Pf* spz invasion of HC04 cells by purified plasma IgG from samples collected pre-immunization (I1(-1)) and prior to CHMI (C(-1)), depleted or not of *Pf*CSP-specific antibodies (*Pf*CSP-depleted – light green circles) **(G-I)** Correlation between the cumulative number of mosquito bites and anti-*Pf*CSP antibody titers (G), binding capacity to *Pf* spz (H) and induction of *Pf* spz lysis (I); Differences between groups were assessed by employing the Wilcoxon matched-pairs signed rank test and correlations using the non-parametric Spearman correlation test (*<≤0.05; **≤0.01; ***≤0.001). Vxx denotes volunteer number.

cellular subsets identified in previous clinical challenge studies, including $CD4^+$ T (10, 16, 43), $CD8^+$ T (10, 43, 44), γδ T (45-47), natural killer (NK) (48) cells, and monocytes (48) (**Fig. S2**). An increased frequency of $CD8^+$ T cells and in particular γδ T cells (particularly the Vγ2+ sub-family), and a decrease in circulating monocytes were observed at C-1, unrelated to trial outcome **(Table S5)**. Of note, an increase in circulating NKT cells was found prior to CHMI (at C-1, **Fig. S2**), which associated with the increase in pre-patent period and the decrease in parasite density **(Table S5)**.

No relevant differences were observed between circulating cytokines before and 5, 7 and 10 days after the first immunization (**Fig. S3**). However, a clear association was found between changes in serum cytokine or chemokine concentrations after the first *Pb*Vac administration and the outcome of immunization, including for interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-12, IL-6 and monocyte chemoattractant protein 1 (MCP1/CCL2) (**Table S6**). Such changes also appear to associate with the observed differences in the frequency of circulating immune cells (**Table S7**). For example, serum concentrations of IFN-γ appear to be directly linked to changes in γδT cell frequency before CHMI, while concentrations of TNF-α appear to be more broadly associated with the frequency of not only TCRγδT cells, but also $CD4^+$ T and NKT cells. As expected, changes in the MCP1/CCL2 chemokine are closely linked to variations in the frequency of circulating monocytes, and are accompanied by alterations in other cytokines involved in the same pathway, such as IFN-α and IL-18 (**Table S7**).

Next, PBMCs were incubated with *Pb*Vac or *Pb*WT sporozoites, or with recombinant *Pf*CSP, and the expression of IFN-γ, IL-2 and TNF-α was analyzed. Statistically significant increases were observed in the frequency of *Pb*Vac- and *Pb*WT-, but not *Pf*CSP-specific lymphocytes producing IFN-γ at either I1+14 (P=0.002 and P=0.001, respectively) or C-1 (P=0.002 and P=0.007, respectively) (**Fig 4A-C**). Similarly, *Pb*Vac- (P=0.009 for C-1), and *Pb*WT- (P=0.034 for I1+14), but not *Pf*CSP-specific $CD4^+$ (**Fig. S4-C)** and $CD8^+$ (**Fig. 4D-F**) T cells expressing any combination of IFN-γ, IL-2 and TNF-α were significantly more frequent after immunization (P=0.016 and P=0.042, for C-1, respectively). Interestingly, the frequency of $CD4^+$ T cells simultaneously expressing IFN-γ and TNF-α increased for both *Pb*Vac (**Fig. S4D, S4E)** and *Pb*WT (**Fig. S4F, S4G)** sporozoite-specific responses.

Collectively, these data show that the genetic introduction of *Pf*CSP bears small influence on the cellular responses induced by immunization with *Pb*Vac. In contrast, the *Pb*WT backbone is sufficient to induce an increase in sporozoite-specific cytokine production, but no correlation was found between cytokine production by any of the cell populations analyzed and the clinical outcome. The combined data suggest the existence of a multifaceted response to *Pb*Vac that may comprise different cellular populations or a combination of cellular and humoral immune responses.

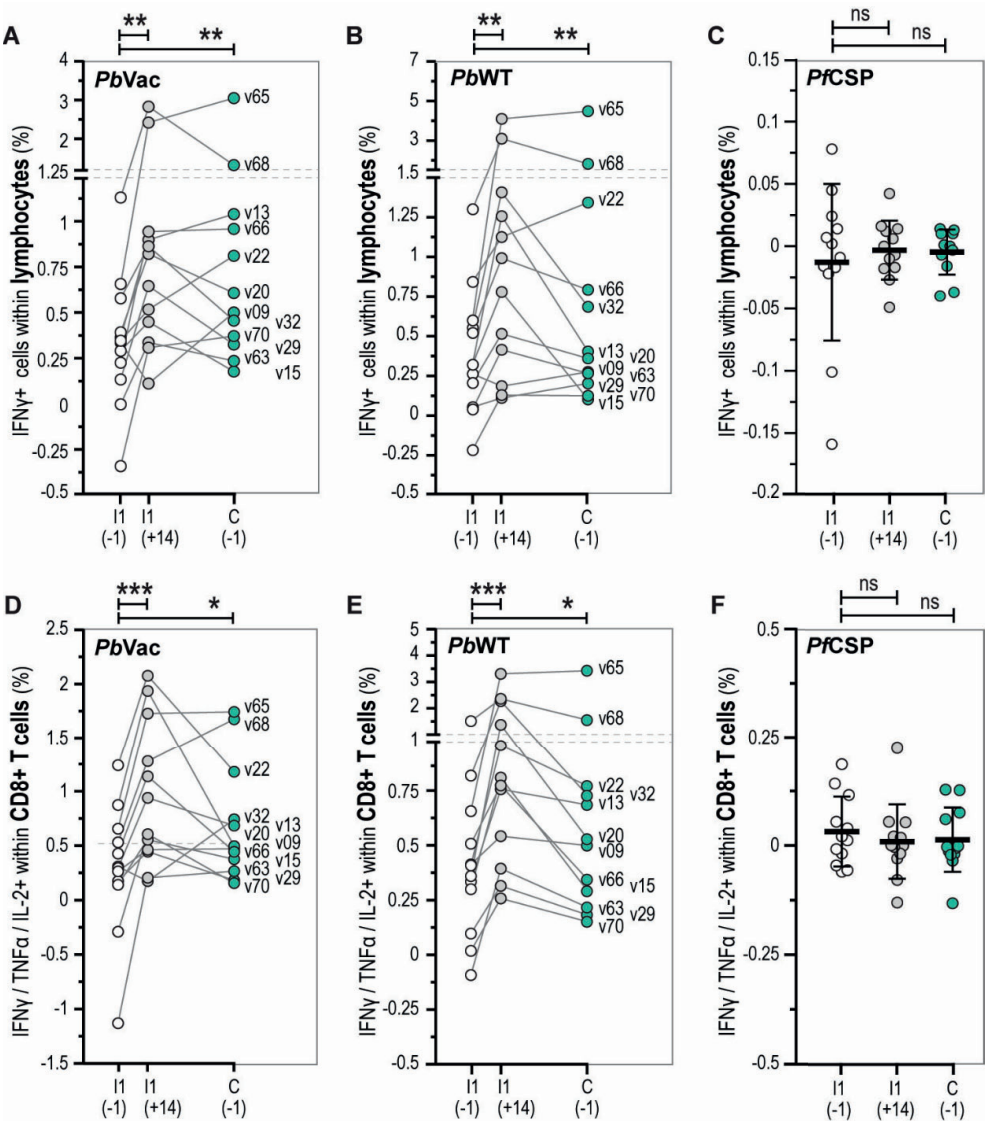

Figure 4 – Cellular immunogenicity of *Pb*Vac

(A-C) Percentage of IFN-γ-producing lymphocytes in peripheral blood samples collected pre-immunization (I1(-1) – white circles), 14 days after the first immunization (I1(+14d) – grey circles) and before CHMI (C(-1) – green circles) following stimulation with *Pb*Vac spz (A) PbWT spz (B) or *Pf*CSP recombinant protein (C); **(D-F)** Percentage of $CD8^+$ T cells producing any combination of IFN-γ, IL-2 and TNF-α in peripheral blood samples, following stimulation with either *Pb*Vac spz (D), *Pb*WT spz (E) or *Pf*CSP (F). Differences between groups were assessed by employing the Wilcoxon matched-pairs signed rank test (*≤0.05; **≤0.01; ***≤0.001). In (C) and (F), bars represent the mean ± standard deviation. Vxx denotes volunteer number.

Cross-species cytokine responses

Finally we studied cytokine responses to *Pf* in PBMCs from volunteers receiving 5, 25 and 75 *Pb*Vac infected-mosquito bites **(Fig. 5A,B, S5A-C)**. IFN-γ production was only statistically increased at the highest *Pb*Vac dosage in total PBMC (**Fig. 5A**), and $CD8^+$ T cells (**Fig. 5B**), but not in $CD4^+$ T cells producing any combination of IFN-γ, IL-2 or TNF-α (**Fig. S5A**). Nonetheless, $CD4^+$ T cells expressing simultaneously IFN-γ and TNF-α were significantly increased after immunization (*P*=0.012) (**Fig. S5B**). There was no statistically significant association between the frequency of cytokine-producing cells and either an increase in pre-patency time or a reduction in liver load of immunized subjects. Our results show that *Pb*Vac immunization elicits dose-dependent cross-species cytokine responses against *Pf*, suggesting that a higher *Pb*Vac dosage might increase protective efficacy.

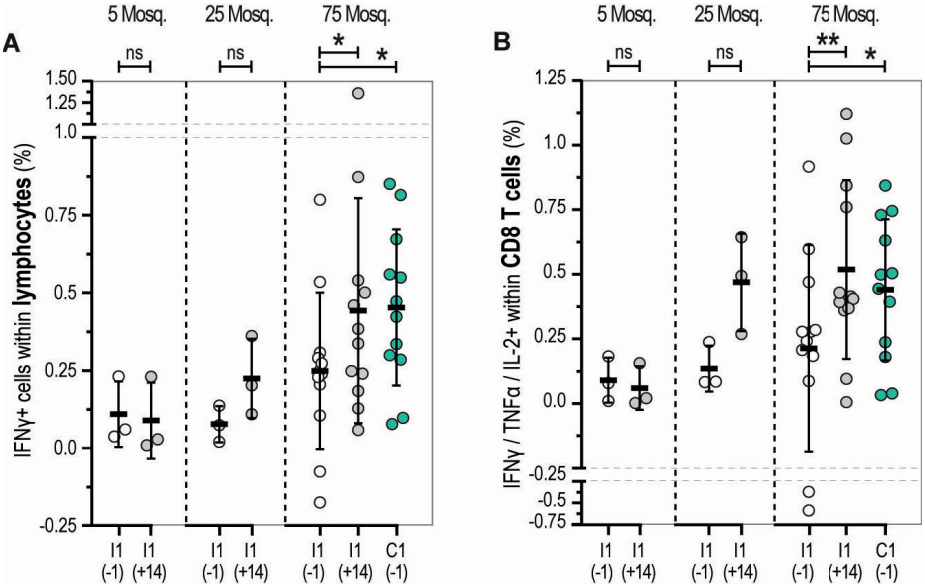

Figure 5 – Cross-species cytokine responses

(A) Percentage of IFN-γ-producing lymphocytes following stimulation with *Pf* spz in peripheral blood samples collected pre-immunization (I1(-1)) and 14 days after immunization (I1(+14)) with 5 and 25 mosquito bites, or collected pre-immunization (I1(-1)), 14 days after the first immunization (I1(+14d)) with 75 mosquito bites and before CHMI (C(-1)); **(B)** Percentage of $CD8^+$ T cells producing any combination of IFN-γ, IL-2 and TNF-α following stimulation with *Pf* spz. Differences between groups were assessed by employing the Wilcoxon matched-pairs signed rank test (*≤0.05; **≤0.01; ***≤0.001).

Discussion

Whole-sporozoite immunization constitutes a promising approach to the much-desired goal to control and eliminate malaria (49). *Pb*Vac, a *Pb* parasite that expresses the *Pf*CSP antigen under the control of the UIS4 pre-erythrocytic stage promoter, belongs to a novel class of whole-sporozoite malaria vaccination approaches based on the use of genetically modified rodent *Plasmodium* parasites expressing target antigens of their human counterparts (35). *Pb*Vac is not only the first whole-sporozoite approach of non-human origin, but also the first employing a genetically modified *Plasmodium* parasite expressing inserted heterologous antigens, evaluated in a clinical study. We show that administration of *Pb*Vac by mosquito bites to healthy volunteers is safe and well-tolerated, exerting, at the employed doses, a pronounced biological effect against a subsequent *Pf* challenge, that appears to be associated with components of both humoral and cellular immune responses.

The efficacy of the protective humoral responses targeting the extracellular, infective sporozoite depend on the concentration and specificity of the response elicited by vaccination (50, 51). In fact, anti-CSP antibodies have been shown to inhibit *Pf* sporozoite infectivity *in vitro* (52, 53) and *in vivo* (54) and their protective role is clearly shown in the context of the subunit vaccine RTS,S (55, 56). Here, we show that *Pb*Vac's *Pf*CSP component is a major contributor to the induced antibody responses, which are sufficient for complement activation, to mediate *Pf* sporozoite lysis, and to inhibit *Pf* sporozoite invasion of hepatic cells *in vitro*. The genetic insertion of *Pf*CSP onto the rodent *Pb* platform enhanced the functionality of *Pb*Vac-induced immune responses, possibly contributing to the biological effect observed upon vaccination.

Our results also suggest a contribution of *Pb*Vac-elicited cellular immune responses to the clinical outcome. In RAS and CPS vaccination, an increase in the frequency of *Pf*-specific $CD4^+$ and $CD8^+$ T cells producing any combination of IFNγ, IL-2 or TNF-α, or the frequency of $V\delta2^+$ T cells associates with sterile immunity in CHMI studies in healthy US/European volunteers (10, 43, 45, 46, 57, 58). In the present study, a marked expansion of not only $CD8^+$ T, γδ T and $V\delta2^+$ T cells, but also NKT cells, was observed post-immunization. Conversely, decreased frequencies of circulating monocytes were observed after vaccination and appear to be linked to early changes not only in MCP1/CCL2 signaling but also in IL-18, an inducer of MCP1 production (59, 60). These observations suggest a possible recruitment of monocytes to the liver, where they may contribute to the development of protective tissue-resident immune responses (61-64). Finally, increased frequencies of IFNɣ-producing lymphocytes and of $CD8^+$ T cells producing any combination of IFNɣ, TNFα or IL-2 were observed upon PBMC stimulation with either *Pb*WT or *Pf* sporozoites, but not with *Pf*CSP.

Collectively, and in agreement with our pre-clinical data (35), these results suggest that *Pb*Vac immunization elicits functional *Pf*CSP-based antibody responses, as well as cross-species, *Pb*WT backbone-dependent, cellular immune responses against *Pf*.

To directly compare the responses of each volunteer with the mean values of each parameter for the entire group prior to immunization, a Z-score was calculated for the magnitude of the cellular and humoral immune responses measured for each volunteer (**Fig. S6**). An analysis of the individual immune responses to immunization, reveals a marked heterogeneity in the magnitude of these responses, with some volunteers developing a predominantly humoral response, and others favoring a cellular immune response (**Fig. S6**). This heterogeneity is not unexpected and has been observed not only in malaria vaccination trials of whole-sporozoite vaccines delivered by mosquito bite, but also in other malaria vaccination trials (65). However, our analysis shows that all individuals display a response to *Pb*Vac in at least one of the immune response parameters assessed. For example, v70, the volunteer which showed the smallest increase in prepatency period and the lowest reduction of liver load following CHMI, appears to be one of the volunteers with the lowest cellular immune responses against either *Pb*Vac (**Fig.S6A**) or *Pf* sporozoites (**Fig.S6B**), but developed a robust functional humoral immunity, capable of inhibiting *Pf* sporozoite invasion of HC04 cells (**Fig.S6C**). On the contrary, v65 is among the top responders of the group in terms of cellular immune responses against either *Pb*Vac or *Pf* sporozoites, but his/her humoral immune responses against Pf sporozoites rank as the lowest of the group. Overall, this analysis highlights the heterogeneity of individual immune responses as well as the lack of a direct correlation between the clinical outcome and a single immune response parameter. A similar absence of clear correlates of protection have been noted for RTS,S (55, 56), as well as for whole-sporozoite immunizations (10, 58), likely due to the multi-factorial and complex nature of the immune signature. Finally, the association observed between the early plasma amounts of the MCP1/CCL2 cytokine, as well as changes in frequency of circulating NKT cells, and the outcome of CHMI warrant further investigation.

Our results show a proportionality between humoral / cellular immune responses and the cumulative number of immunizing bites, as previously determined following RAS and CPS immunizations (10, 43). In fact, in early clinical studies of *Pf* RAS vaccination by mosquito bite, sterile protection was only achieved when volunteers underwent a mean of 9 immunizations and >1000 immunizing bites before CHMI (66). In the present study, the total number of immunizing bites was substantially lower (4 immunizations with ~75 immunizing bites each, for a total of ~300 bites). Therefore, we hypothesize that sterile protection against *Pf* challenge, following *Pb*Vac immunization may be achieved with an increase in the number of sporozoites administered. Since markedly increasing

the dose of parasites used for immunization through increasing the number of mosquito bites appears impractical, achieving this goal will potentially require the inoculation of sporozoites through the injection of sporozoites by intravenous route. This method is amply documented as an efficacious method for delivery of whole-sporozoite RAS and chemoattenuated vaccine candidates (10, 11, 45, 46). Such an endeavor poses several challenges, chief among which is the requirement for a GMP-compliant sterile *Pb*/*Pb*Vac production system, an absolute requisite for the intravenous administration of such parasites to humans.

Whole-sporozoite vaccines against malaria still require relatively high doses of sporozoites to elicit sterile immunity, demanding the production of large numbers of infected mosquitoes reared in highly specialized facilities, with a consequent impact on the cost-of-goods. This is particularly relevant in the case of *Pf*-based vaccines, whose production commands compliance with very stringent safety requirements. The biological effect observed upon *Pb*Vac administration in the present study warrants the exploration of *Pb* as one possible alternative platform for whole-sporozoite vaccination. Besides its inherent safety, *Pb*-based vaccines may offer an alternative to *Pf*-based vaccination, with potential advantages over the latter in terms of cross-species immunization. In fact, we have previously shown not only that *Pb* infectivity of human primary hepatocytes appears to be higher than that of *Pf*, but also that >24,000 *in silico*-predicted $CD8^+$ T cell epitopes, encoded in >60% of both the *Pf* and the *Pb* proteins, are shared between the two parasite species (35). One may further speculate that, given the co-evolution of humans and *Pf* parasites, immune evasion mechanisms may be at play during immunization with the latter that may be absent in the case of *Pb*-based immunization. On the other hand, whereas the expression of *Plasmodium* proteins in heterologous systems for subunit vaccination is notoriously difficult (33, 67), the presence of several genetic loci permissive for gene-insertion modifications on *Pb* (68-70) offers opportunities for the introduction of multiple correctly folded antigens in their native conformation (33). Thus, next-generation *Pb*-based vaccine candidates may include combinations of not only antigens from different human-infective *Plasmodium* spp., but also blood stage or transmission-blocking immunogens, as well as multiple alleles of an antigen (71). Additionally, the hepatotropic nature of *Pb* sporozoites promotes the exposure of antigens in the liver, where tissue-resident immunity is most likely to develop (61-64). Nevertheless, whether sufficiently protective immune responses can be elicited by *Pb*-based immunization needs to be addressed in further clinical studies. Such studies will potentially include both wild-type *Pb*- and *Pb*Vac-immunized volunteers, as well as appropriate *Pf*RAS or *Pf*CPS arms for a comparative analysis of protective efficacy and immune responses elicited by vaccination. These features, along with the presently reported strong biological effect elicited by immunization with *Pb*Vac, even at sub-optimal doses, make the *Pb*-based platform a promising tool for the development of a next-generation malaria vaccine.

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Author contributions
IJR, AMM, CL, YW, CFO, AJB, RWS, and MP designed the trial, which was performed by IJR, GMdJ, RK, JvH, PJJvG, and RWS. RK, and JvH performed the real time PCR analysis and supervised the thick smear reads. G-JvG and WG produced the infected mosquitoes. CL, YW, CFO and AJB provided regulatory and project support during the course of the study. AMM, AFG, and HNC performed the immunological work. IJR, AMM, GMdJ, AFG, HNC, LC, SdV, ASPY, JvH, PJJvG, RWS, and MP analyzed and interpreted the data and results. IJR, AMM, and MP wrote the original draft manuscript, which was critically reviewed and approved by all authors.

Competing interests
AMM and MP are inventors on a patent or patent application issued, allowed or filed internationally ("Genetically modified rodent *Plasmodium* parasites as platforms for a whole-organism malaria vaccine" (WO 2013156949 A1, also published as CN104302313A, EP2838554A1, US20150071966), covering parts of this work. All other authors declare no competing interests.

Data Availability
*Pb*Vac (also known as Pb(PfCS@UIS4), *P. berghei* ANKA (line 2266cl1, *Pfcs@Pbuis4* in GIMO motherline) or *P. berghei* ANKA (line 2266cl2, *Pfcs@Pbuis4* in GIMO motherline) is covered by a Material Transfer Agreement (MTA) between Academish Ziekenhuis Leiden, also acting under the name Leiden University Medical Center ("Provider"), and Instituto de Medicina Molecular (IMM) ("Recipient") dated 28 November 2012 and amended on 1 August 2016.

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Supplementary data

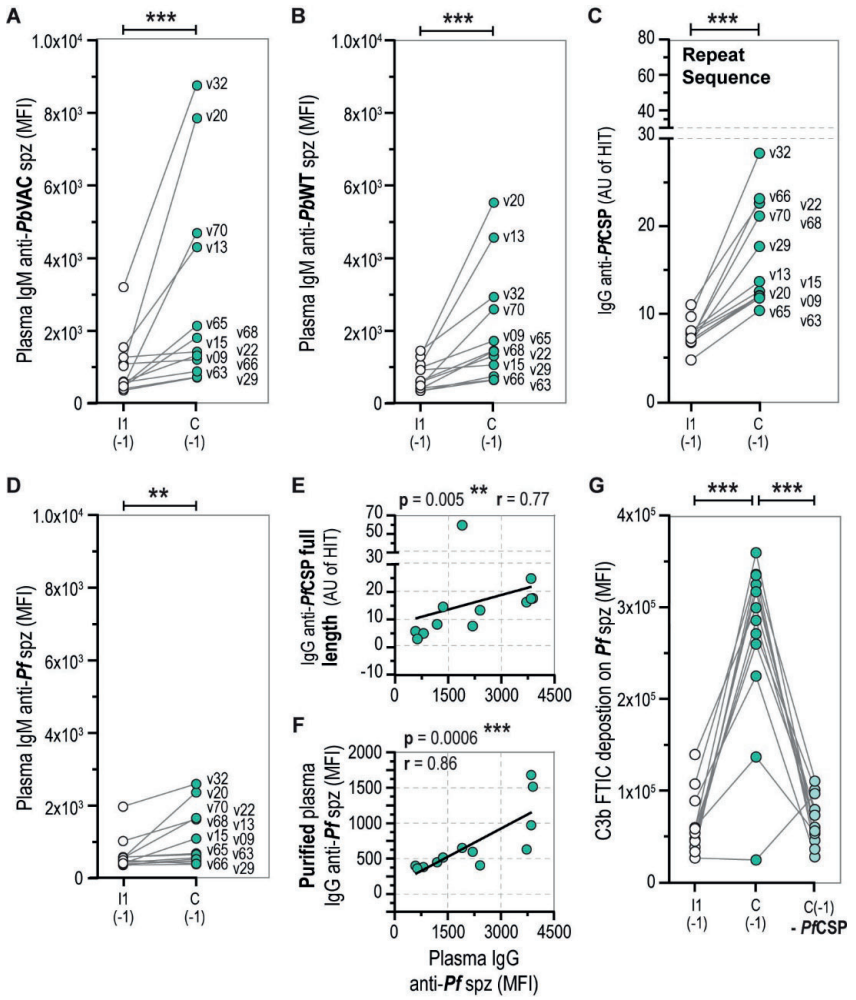


Figure S1 – Humoral responses elicited by immunization with *Pb*Vac

(A-B) Binding to *Pb*Vac spz (A) and *Pb*WT spz (B) of plasma IgM from samples collected pre-immunization (I1(-1) – white circles) and prior to CHMI (C(-1) – green circles); Median fluorescence intensity (MFI) values were registered for binding at a 1/1,000 dilution; **(C)** Plasma IgG reactivity against the repeat sequence of *Pf*CSP as measured by ELISA, normalized to a pool of sera from 100 hyperimmune Tanzanians (HIT), which was set at 100 arbitrary units (AU). **(D)** Binding of plasma IgM to *Pf* spz; **(E)** Correlation between binding of plasma IgGs from samples collected prior to CHMI to *Pf* spz and the titers of anti-*Pf*CSP whole protein antibodies for the same samples determined by ELISA; **(F)** Correlation between plasma IgG binding to *Pf* spz before and after IgG purification for samples collected before CHMI; **(G)** C3b complement deposition in *Pf* spz pre-incubated with purified plasma IgG depleted or not of *Pf*CSP-specific antibodies (*Pf*CSP-depleted – light green circles); Differences between groups were assessed by applying the Wilcoxon matched-pairs signed rank test and correlations using the non-parametric Spearman correlation test (*≤0.05; **≤0.01; ***≤0.001). Vxx denotes volunteer number.

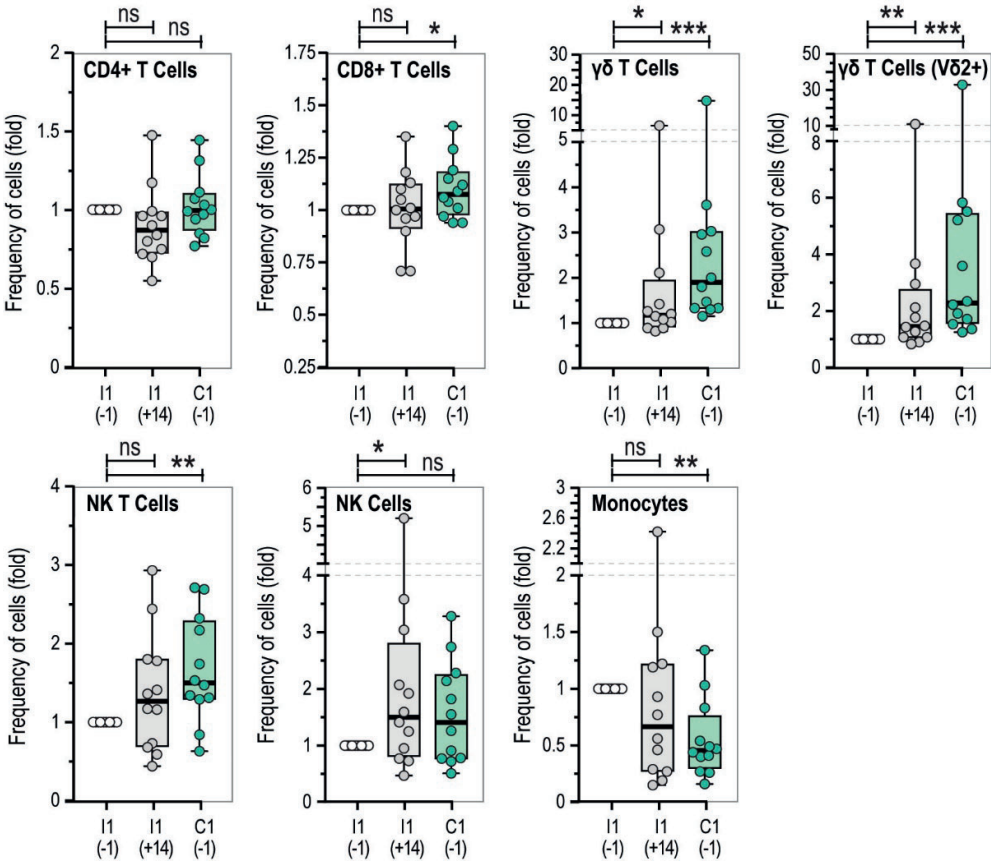


Figure S2 – Frequency of sub-families of circulating immune cells
Fold change in the frequency of sub-families of circulating immune cells between samples collected pre-immunization (I1(-1) – white circles), 14 days after the first immunization (I1(+14d) – grey circles) and before CHMI (C(-1) – green circles). Frequency of sub-families were calculated as a percentage of total lymphocytes. Differences between groups were assessed by applying the Wilcoxon matched-pairs signed rank test (*≤0.05; **≤0.01; ***≤0.001). Boxes represent the 25th to 75th percentiles and whiskers represent the range.

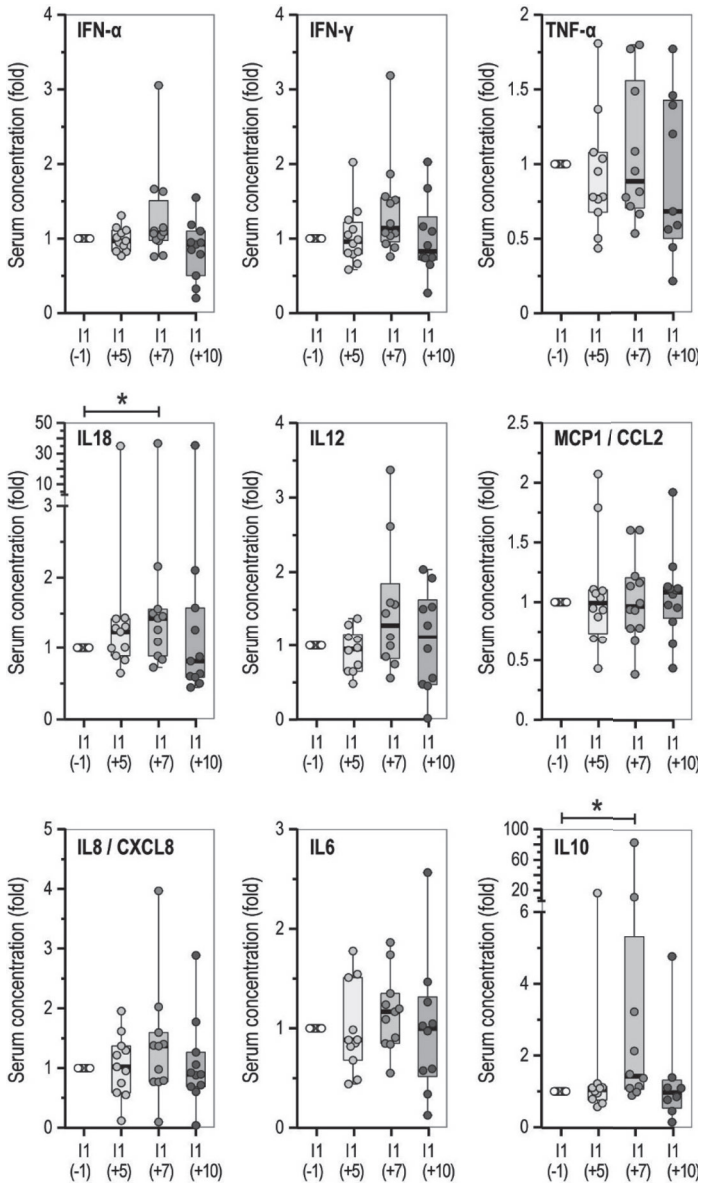


Figure S3 – Serum concentration of cytokines and chemokines after the first *Pb*Vac administration

Fold change in the serum concentration of various cytokines and chemokines between samples collected pre-immunization (I1(-1) – white circles), 5 days after the first immunization (I1(+5) – light grey circles), 7 days after the first immunization (I1(+7) – median grey circles) and 10 days after the first immunization (I1(+10) – dark grey circles). Differences between groups were assessed by applying the Wilcoxon matched-pairs signed rank test (*≤0.05). Boxes represent the 25th to 75th percentiles and whiskers represent the range.

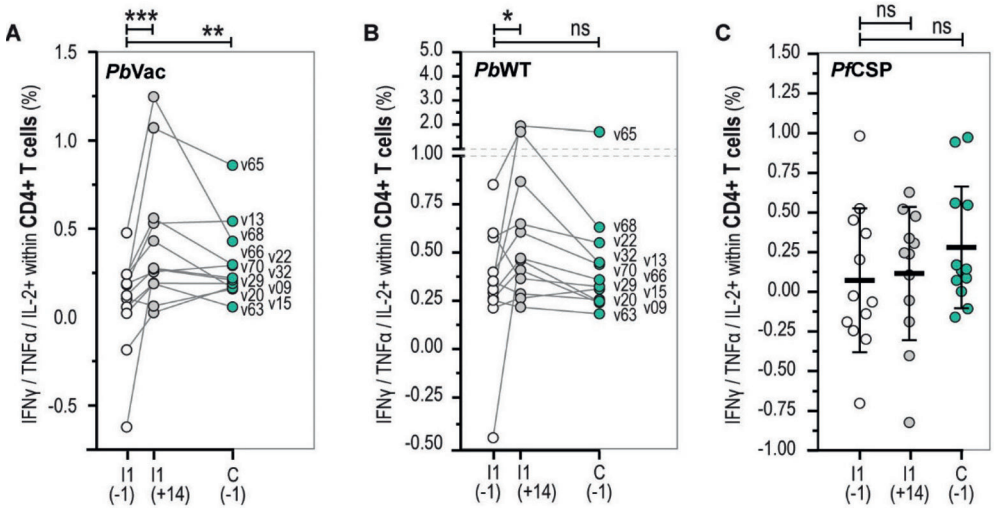
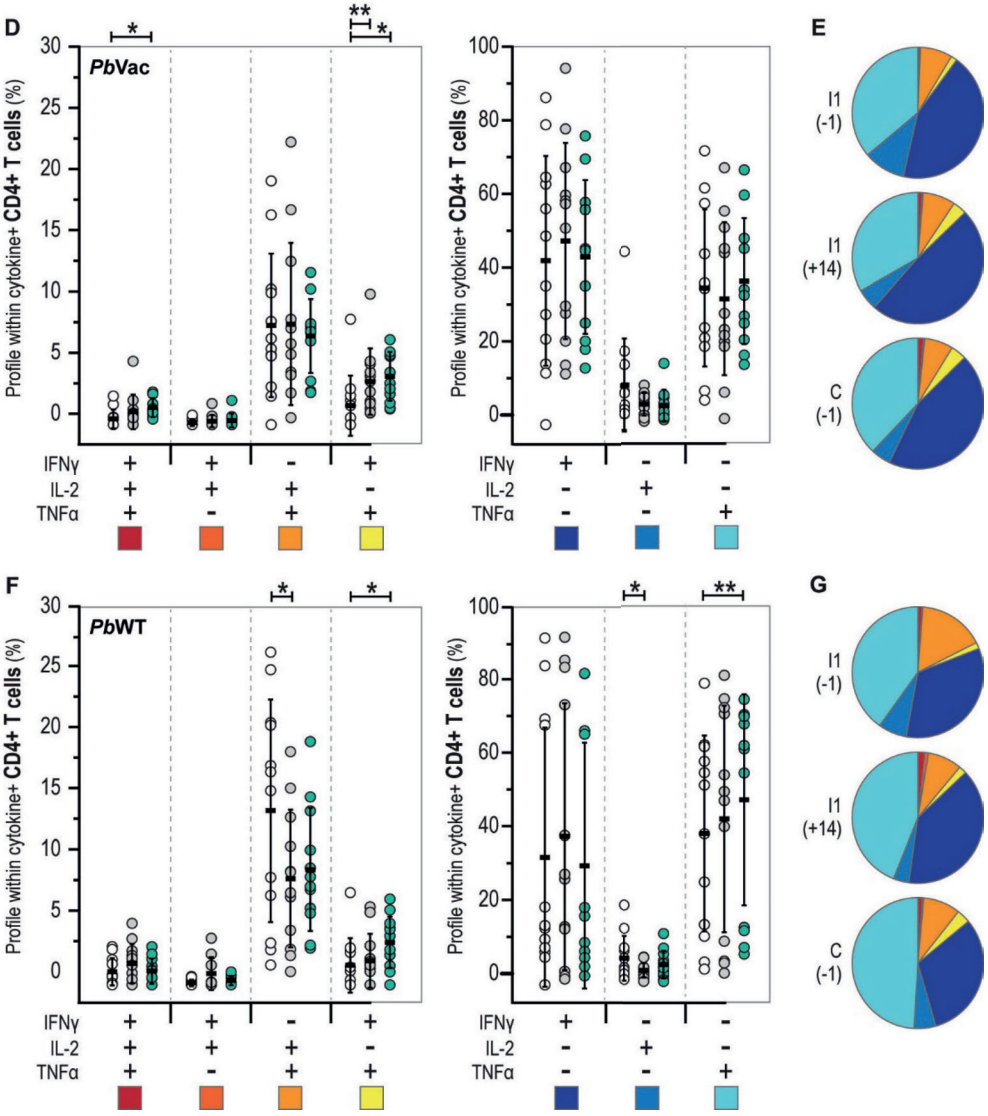


Figure S4 – Polyfunctionality of $CD4^+$ T cell-produced cytokines

(A-C) Percentage of $CD4^+$ T cells producing any combination of IFNγ, IL-2 and TNFα cytokines in peripheral blood samples collected pre-immunization (I1(-1) – white circles), 14 days after the first immunization (I1(+14d) – grey circles) and before CHMI (C(-1) – green circles) following stimulation with *Pb*Vac spz (A) or surrogates for the *Pb*Vac components, *Pb*WT spz (B) and *Pf*CSP (C).



Continued Figure S4 – Polyfunctionality of $CD4^+$ T cell-produced cytokines

(D-H) Comparative changes (D and F) and overall relative cytokine proportions within $CD4^+$ T cells producing IFN-γ, IL-2 and/or TNF-α (E and G) for each combination of cytokines upon stimulation with *Pb*Vac spz (D and E) or *Pb*WT spz (F and G). In (D and F), left panels show the percentage of polyfunctional $CD4^+$ T cells in the various peripheral blood samples collected and the right panels show the percentage of mono-functional $CD4^+$ T cells. Pie charts in (E and G) are colored for each cytokine combination as indicated below (D and F), respectively, and represent the total populations of cells producing any combination of IFN-γ, IL-2 and TNF-α. Differences between groups were assessed by applying the Wilcoxon matched-pairs signed rank test (*≤0.05; **≤0.01; ***≤0.001). In (C, D and F), bars represent the mean ± standard deviation. Vxx denotes volunteer number.

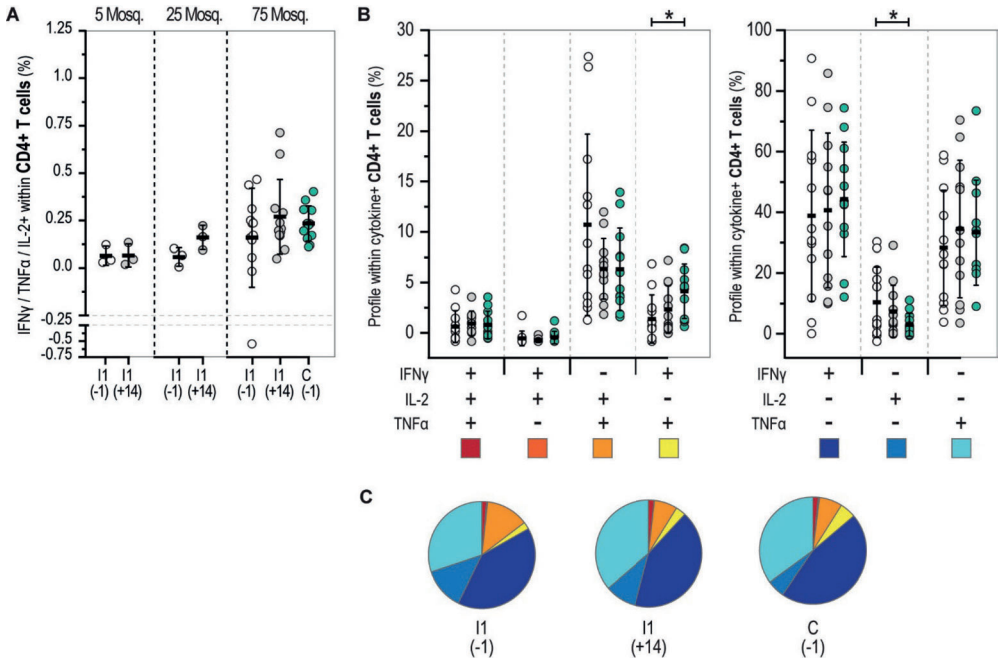


Figure S5 – Polyfunctionality of cross-species cytokine responses

(A) Percentage of IFNγ-producing $CD4^+$ T cells following stimulation with *Pf* spz in peripheral blood samples collected pre-immunization (I1(-1)) and 14 days after immunization (I1(+14)) with 5 and 25 mosquito bites, or collected pre-immunization (I1(-1)), 14 days after the first immunization (I1(+14d)) with 75 mosquito bites and before CHMI (C(-1))**; (B and C)** Comparative changes (B) and overall relative proportion within $CD4^+$ T cells producing IFNγ, IL-2 and/or TNFα (C) for each combination of cytokines produced upon stimulation with *Pf* spz. In (B), left panels show the percentage of polyfunctional $CD4^+$ T cells in the various peripheral blood samples collected and the right panels show the percentage of mono-functional $CD4^+$ T cells. Pie charts in (C) are colored for each cytokine combination as indicated below (B) and represent the total population of cells producing any combination of IFN-γ, IL-2 and TNF-α. Differences between groups were assessed by applying the Wilcoxon matched-pairs signed rank test (*≤0.05; **≤0.01; ***0.001). Bars represent the mean ± standard deviation.

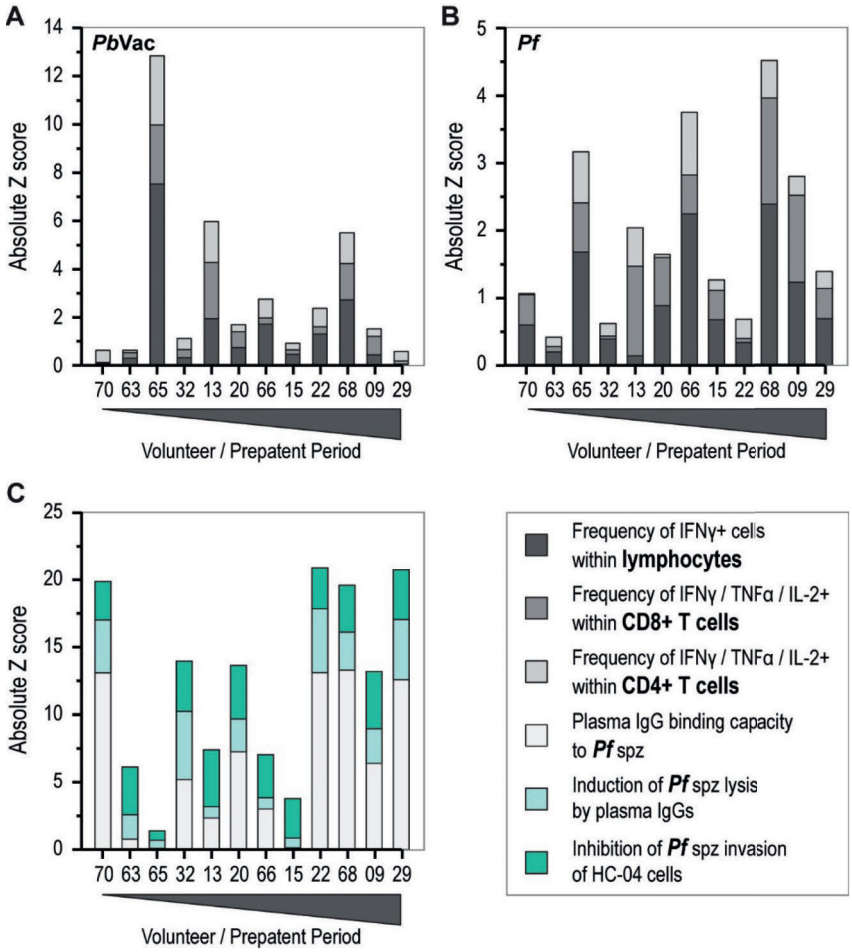


Figure S6 – Analysis of heterogeneity of individual responses to immunization
Individual cellular immune responses to *Pb*Vac **(A)** or to *Pf* **(B)**, and humoral immune responses against *Pf* **(C)** were globally scored for each volunteer by comparing their immune responses with the entire group's mean values prior to immunization.

		Group 1	Group 2	Group 3	Group 4
No. of participants		3	3	12	6
Gender (no. of participants, %)	Female	3 (100)	1 (33)	7 (58)	4 (67)
	Male	-	2 (67)	5 (42)	2 (33)
Race	Caucasian	2	3	10	6
	Asian	0	0	1	0
	Other	1	0	1	0
Age (median (range))		21.1 (18-23)	26.1 (21-30)	21.5 (19-26)	22.5 (21-26)

Table S1 – Baseline characteristics

		Group 1 (n=3) 5 mosq. bites		Group 2 (n=3) 25 mosq. bites		Group 3 (n=12)										Group 4 (n=6)	
						~75 mosq. bites (immunization 1)		~75 mosq. bites (immunization 2)		~75 mosq. bites (immunization 3)		~75 mosq. bites (immunization 4)		P. falciparum challenge		P. falciparum challenge	
		Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2
Local	Pruritis	-	-	3 (100%)	-	9 (75%)	-	9 (75%)	-	-	-	10 (83%)	-	1 (8%)	-	1 (17%)	-
	Edema arms	-	-	-	-	-	-	-	-	-	-	1 (8%)	-	-	-	-	-
	Painful skin arms	-	-	-	-	1 (8%)	-	-	-	-	-	-	-	-	-	-	-
	Hematoma/pain after venapuncture	-	-	1 (33%)	-	-	-	-	-	-	-	-	-	-	-	1 (17%)	-
Systemic	Headache	1 (33%)	1 (33%)	2 (67%)	-	3 (25%)	1 (8%)	1 (8%)	-	-	1 (8%)	-	-	6 (50%)	-	6 (100%)	-
	Abdominal pain	1 (33%)	-	-	-	1 (8%)	-	-	-	-	-	-	-	-	-	-	-
	Nausea	2 (67%)	-	-	-	1 (8%)	1 (8%)	-	-	-	-	-	1 (17%)	1 (8%)	-	3 (50%)	1 (17%)
	Diarrhea	1 (33%)	-	1 (33%)	-	-	-	-	-	-	-	-	-	1 (8%)	-	-	-
	Stomach pain	-	-	1 (33%)	-	-	-	-	-	-	-	-	-	-	-	-	-
	Malaise	-	-	-	-	1 (8%)	-	1 (8%)	-	-	-	1 (8%)	-	1 (8%)	-	2 (33%)	-
	Fatigue	-	-	-	1 (33%)	-	-	-	-	-	-	1 (8%)	-	3 (25%)	-	-	-
	Angio-edema	-	-	-	-	1 (8%)	-	-	-	-	-	-	-	-	-	-	-
	Dizziness	-	1 (33%)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Night sweating	-	-	-	-	-	-	-	-	-	-	1 (8%)	-	-	-	-	-
	Chills	-	-	-	-	-	-	-	-	-	-	-	-	1 (8%)	-	-	-
	Myalgia	-	-	-	-	-	-	-	-	-	-	-	-	1 (8%)	-	-	1 (17%)

Table S2 – Adverse Events

	Group 1 (n=3)		Group 2 (n=3)		Group 3 (n=12)										Group 4 (n=6)	
	5 mosq. bites		25 mosq. bites		~75 mosq. bites (immunization 1)		~75 mosq. bites (immunization 2)		~75 mosq. bites (immunization 3)		~75 mosq. bites (immunization 4)		P. falciparum challenge		P. falciparum challenge	
	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2	Grade 1	Grade 2
Leukocytes decreases (<3.5*10^9/ml)	-	-	-	-	1 (8%)	-	-	-	-	-	-	-	3 (25%)	-	1 (17%)	-
Lymphocytes decreases (<0.5*10^9/ml)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1 (17%)	-
elet decreases (<150*10^9/ml)	-	-	1 (33%)	-	1 (8%)	-	-	-	-	-	-	-	1 (8%)	-	1 (17%)	-
AST/ALT elevations (female >31/34U/L; male >35/45U/L)	-	-	-	-	1 (8%)	1 (8%)	-	-	-	-	1 (8%)	-	2 (17%)	1 (8%)	1 (17%)	-
Bilirubin increases (> 17µmol/L)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2 (33%)
Creatinine increases (female >90 µmol/L; male >115 µmol/L)	-	-	1 (33%)	-	-	-	-	-	1 (8%)	-	-	-	-	-	2 (33%)	-

Table S3 – laboratory abnormalities

		Number of mosquito bites		Days to parasitemia		1st generation parasites	
		r	P value	r	P value	r	P value
Plasma IgG binding to spz	PbVac	0.333	0.288	0.384	0.217	-0.333	0.283
	PbWT	0.018	0.960	0.313	0.320	-0.245	0.433
	Pf	0.572	0.055	0.277	0.382	-0.371	0.229
Plasma IgM binding to spz	PbVac	-0.081	0.795	-0.503	0.078	0.462	0.131
	PbWT	-0.165	0.599	-0.280	0.326	0.340	0.278
	Pf	0.175	0.583	-0.334	0.245	0.259	0.413
Plasma IgG reactivity to PfCSP	Full length	0.821	0.002	0.162	0.613	-0.312	0.316
	Repeat sequence	0.709	0.012	0.122	0.705	-0.273	0.381
Purified plasma IgG binding to Pf spz		0.219	0.033	0.219	0.492	-0.269	0.387
C3b deposition of Pf spz		0.119	0.778	0.119	0.712	-0.144	0.646
Pf spz lysis		-0.176	0.005	-0.176	0.520	0.189	0.553
Inhibition of Pf spz invasion		0.187	0.670	0.187	0.559	-0.116	0.711

Table S4 – Correlation between humoral responses elicited by *Pb*Vac, total number of mosquito bites after 4 immunizations, and CHMI outcome

The non-parametric Spearman correlation test was employed to identify potential correlations between the humoral responses assessed in various experimental assays and the total number of mosquito bites administered or the outcome of CHMI, as represented by the increase in pre-patency and the decrease in the number of 1st generation parasites. Significant correlations are highlighted in grey ($p \leq 0.05$).

	Number of mosquito bites				Days to parasitemia				1st generation parasites			
	I1 (+14)		C (-1)		I1 (+14)		C (-1)		I1 (+14)		C (-1)	
	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value
CD4 T cells (%)	-0.409	0.179	-0.225	0.473	-0.112	0.652	-0.424	0.141	0.411	0.184	0.669	0.020
CD8 T cells (%)	-0.453	0.137	-0.463	0.128	0.194	0.543	-0.119	0.647	0.158	0.623	0.480	0.116
TCRγδ cells (%)	-0.032	0.905	0.148	0.643	0.387	0.212	0.360	0.248	-0.235	0.451	-0.349	0.258
TCRγδ-Vδ2 cells (%)	0.093	0.771	0.389	0.210	0.353	0.259	0.384	0.217	-0.193	0.537	-0.417	0.174
NKT cells (%)	-0.119	0.703	-0.105	0.736	0.277	0.382	0.729	0.009	-0.098	0.754	-0.617	0.035
NK cells (%)	0.274	0.387	0.389	0.210	0.075	0.816	0.395	0.203	-0.182	0.561	-0.518	0.085
Monocytes (%)	-0.409	0.179	-0.225	0.473	-0.244	0.388	0.115	0.722	0.039	0.908	-0.245	0.433

Table S5 – Correlation between changes in frequency of circulating sub-families of immune cells, total number of mosquito bites after 4 immunizations, and CHMI outcome

The non-parametric Spearman correlation test was employed to identify potential correlation between the changes in circulating immune cells and outcome of *Pb*Vac immunization, as represented by the increase in pre-patency and the decrease in the number of 1st generation parasites. Significant correlations are presented in grey ($p \leq 0.05$).

	Number of mosquito bites						Days to parasitemia						1st generation parasites					
	I1 (+5)		I1 (+7)		I1 (+10)		I1 (+5)		I1 (+7)		I1 (+10)		I1 (+5)		I1 (+7)		I1 (+10)	
	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value
Interferon α	-0.059	0.854	0.081	0.804	-0.077	0.811	0.155	0.646	0.474	0.121	-0.301	0.305	-0.301	0.359	-0.560	0.060	0.237	0.480
Interferon γ	-0.018	0.952	0.056	0.864	0.055	0.892	-0.510	0.073	-0.661	0.016	-0.492	0.124	0.504	0.097	0.623	0.034	0.333	0.349
TNF α	0.219	0.515	0.030	0.939	0.184	0.634	-0.074	0.777	-0.367	0.266	-0.672	0.042	0.077	0.823	0.401	0.250	0.667	0.059
IL18	0.119	0.728	0.110	0.749	-0.137	0.677	-0.353	0.235	-0.306	0.300	-0.400	0.179	0.219	0.515	0.169	0.619	0.319	0.336
IL12	-0.426	0.213	-0.207	0.552	0.030	0.946	-0.367	0.238	-0.120	0.649	-0.642	0.038	0.261	0.462	-0.055	0.872	0.479	0.166
MCP1 / CCL2	-0.158	0.614	-0.116	0.711	-0.077	0.804	-0.639	0.020	-0.639	0.020	-0.277	0.333	0.585	0.049	0.525	0.082	0.312	0.322
IL8 / CXCL8	0.055	0.875	0.150	0.658	0.114	0.739	0.369	0.263	-0.220	0.460	0.196	0.561	-0.292	0.374	0.109	0.749	-0.214	0.515
IL6	0.264	0.429	0.269	0.422	-0.128	0.713	-0.038	0.839	-0.597	0.042	-0.640	0.034	0.068	0.844	0.533	0.095	0.620	0.061
IL10	0.188	0.607	0.030	0.946	0.119	0.793	-0.063	0.784	-0.107	0.691	-0.406	0.246	-0.109	0.751	-0.079	0.818	0.527	0.186

Table S6 – Correlation between changes in serum cytokine levels after the first *Pb*Vac administration, total number of mosquito bites after 4 immunizations, and CHMI outcome

The non-parametric Spearman correlation test was employed to identify potential correlations between changes in serum cytokine levels after the first *Pb*Vac administration and the outcome of *Pb*Vac immunization, as represented by the increase in pre-patency and the decrease in the number of 1st generation parasites. Significant correlations are presented in grey ($p \leq 0.05$).

	CD4 T cells		CD8 T cells		TCRγδ cells		TCRγδ (Vδ2) cells		NKT cells		NK cells		Monocytes	
	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value	r	P value
Interferon α	0.373	0.261	0.091	0.796	-0.278	0.397	-0.191	0.577	-0.145	0.673	-0.300	0.371	0.645	0.037
Interferon γ	0.236	0.513	-0.139	0.707	-0.851	0.003	-0.770	0.013	-0.576	0.088	-0.042	0.918	0.297	0.407
TNF α	0.850	0.006	0.300	0.437	-0.695	0.040	-0.533	0.148	-0.783	0.017	-0.450	0.230	0.117	0.776
IL18	0.155	0.654	-0.236	0.485	-0.246	0.455	-0.109	0.755	-0.082	0.818	0.073	0.838	0.645	0.037
IL12	0.224	0.537	-0.176	0.632	-0.347	0.316	-0.164	0.657	-0.515	0.133	-0.273	0.448	0.527	0.123
MCP1 / CCL2	0.350	0.266	-0.007	0.991	-0.102	0.744	0.021	0.956	-0.021	0.956	0.077	0.817	0.587	0.049
IL8 / CXCL8	-0.291	0.096	-0.282	0.279	0.633	0.431	0.600	0.313	0.455	0.349	0.218	0.039	0.545	0.838
IL6	0.564	0.386	0.382	0.402	-0.274	0.041	-0.358	0.056	-0.333	0.163	-0.673	0.521	0.079	0.088
IL10	0.310	0.462	0.190	0.665	0.527	0.185	0.548	0.171	0.048	0.935	-0.429	0.299	0.810	0.022

Table S7 – Correlation between changes in serum cytokine levels 10 days after the first *Pb*Vac administration and changes in frequency of circulating sub-families of immune cells before CHMI

The non-parametric Spearman correlation test was employed to identify potential correlation between changes in serum cytokine levels after the first *Pb*Vac administration and changes in frequency of circulating sub-families of immune cells before CHMI. Significant correlations are presented in grey ($p \leq 0.05$).

Chapter 3

Induction of transmissible *Plasmodium falciparum* gametocyte densities by controlled human malaria infection with blood-stage or mosquito bite inoculation

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Submitted

Abstract

Background
For malaria elimination efforts it is important to better understand parasite transmission to mosquitoes and to develop models for early-clinical evaluation of transmission-blocking interventions. We previously described a controlled human malaria infection protocol for induction of gametocytemia in malaria-naïve volunteers by mosquito bite (CHMI-trans) (1).

Methods
Here, we compared gametocyte production and infectivity in healthy adults following CHMI initiated by either *Plasmodium falciparum (Pf)*- infected mosquito bite or intravenous blood-stage inoculation of *Pf*-infected-erythrocytes. Participants received (sub) curative treatments with gametocyte-permissive piperaquine or sulfadoxine-pyrimethamine.

Results
Blood-stage inoculation induced higher gametocyte densities than mosquito bite inoculation, which was predicted by *PfAP2-G* transcript levels – indicative of gametocyte commitment – and resulted in *Pf*-positive mosquito infections in 9/12 participants compared to 0/12 participants after mosquito bite inoculation.

Conclusion
This direct comparison establishes the CHMI-transmission model with intravenous administration of asexual parasites as a tool for early-clinical evaluation of interventions that aim to interrupt *Pf*-transmission.

Introduction

Plasmodium transmission to mosquitoes depends on the presence of mature male and female gametocytes in the peripheral blood. Gametocyte formation is triggered by activation and expression of *PfAP2-G* (2, 3). Following maturation in the bone marrow and spleen (4), mature gametocytes are released into the circulation where they can be ingested by blood-feeding mosquitoes, thus propagating infection. Gametocyte density is a key determinant of *Plasmodium* transmission to mosquitoes (5).

Malaria elimination strategies depend on a clear understanding of human to mosquito transmission and would benefit from interventions that specifically aim to reduce transmission (6, 7). Effective models for the early-clinical evaluation of candidate transmission-blocking interventions (TBIs) are required. Recently, we developed a controlled human malaria infection model (CHMI-trans) that permitted safe induction of mature gametocytes in malaria-naïve volunteers by *Pf*-infected mosquito bite, but failed to induce sufficiently high gametocyte densities to allow mosquito infection prevalence to be used as an outcome measure (1). Here, we have demonstrated in a direct comparison, that the mosquito-bite (MB) CHMI-trans model can be improved by instead initiating infections with intravenous inoculation of *Pf*-infected erythrocytes, so called induced blood stage malaria (IBSM) (8). We evaluated the induction of infectious gametocytes following different inoculation and treatment regimens, demonstrated the predictive value of *PfAP2-G* transcripts for gametocyte development and determined the sample size requirements for evaluation of TBIs using the improved CHMI-trans study design.

Methods

Study design, ethics approval and registration

This randomized, open-label, single center trial was conducted at the Radboud university medical center (Nijmegen, the Netherlands) between May and November 2018. Screening procedures and eligibility criteria were described earlier (1). All participants were between 18 and 35 years and provided written informed consent. The trial protocol (research file number NL63552.000.17) was approved by the central committee for research involving human subjects (CCMO), and the Western Institutional Review Board (WIRB), and registered at ClinicalTrials.gov, identifier NCT03454048 and EudraCT, identifier 2017-00040005-40.

Study procedures

24 participants were enrolled in two time-separated cohorts/groups. Within each group, participants were randomly assigned to one of two treatment arms **(Table 1).** In group 1, n=12 were infected by bites from five *Pf* 3D7-infected mosquitoes (MB) (1); In group 2 (IBSM), n=12 were infected by intravenous injection with ~2800 *Pf* 3D7-infected erythrocytes (8). Once parasitemia was detected by 18S qPCR (9) at a density of 5,000 parasites/mL in the MB group, or on day 8 post-inoculation in the IBSM group, treatment was initiated with low-dose piperaquine (LD-PQP 480 mg; T1). If asexual parasitemia exceeded 1,500 parasites/mL by qPCR after T1 and before day 21 after inoculation, participants received a second subcurative treatment with LD-PQP 480 mg (T2) to extend asexual parasitemia and allow induction of gametocytes. On day 21 post-inoculation or upon recrudescence after T2, participants were curatively treated with either 960mg PQP or 1000mg/50mg SP (T3) (**Table 1**). All study participants received a final treatment with a three-day regimen of 1000mg/400mg atovaquone/proguanil (Malarone) per day starting on day 36 post-inoculation or upon recrudescence after curative treatment (T3).

Female and male gametocytes were assessed by quantitative reverse-transcriptase PCR (qRT-PCR) for *CCp4* (female) and *PfMGET* (male) mRNA (10) with a threshold of 5 gametocytes/mL for positivity (1). *PfAP2-G* (2) and *SBP-1* (8, 11) qRT-PCR was performed

	MB		**IBSM**	
	Arm 1	**Arm 2**	**Arm 3**	**Arm 4**
Subjects (n)	6	6	6	6
Infection method	Mosquito	Mosquito	Blood-stage	Blood-stage
Treatment 1	LD-PIP	LD-PIP	LD-PIP	LD-PIP
Treatment 2	LD-PIP	LD-PIP	LD-PIP	LD-PIP
Treatment 3	PIP	SP	PIP	SP
Sex				
Female	3/6 (50%)	4/6 (66.7%)	4/6 (66.7%)	4/6 (66.7%)
Age				
Median	24.5 (18-30)	22.5 (19-26)	25.5 (20-29)	20.0 (19-26)
BMI (kg/m2)				
Median	22.2 (20.8-29.3)	24.2 (22.7-26.9)	20.4 (18.1-22.8)	24.7 (20.8-27.7)
Hemoglobin (mmol/L)	8.8 (8.0-9.7)	8.2 (7.6-9.9)	8.8 (7.6-10.0)	8.7 (7.7-9.4)

Table 1 – Baseline characteristics
Values between brackets represent range (min-max). MB; mosquito bite inoculation, IBSM; induced blood stage malaria. LD-PIP; piperaquine 480mg, PIP; piperaquine 960mg, SP; sulfadoxine-pyrimethamine 1000mg/50mg.

on day of first treatment and the ratio of *PfAP2-G*:*SBP-1* expressed as an indication of the proportion of sexually committed ring-stage parasites (12). Infectivity of gametocytes to mosquitoes was assessed on days 21, 24 and 29 post-inoculation for all gametocyte positive individuals by direct skin feeding assays (DFA), direct membrane feeding assay with whole blood (DMFA), following serum replacement (MFA-SR), or following enrichment for gametocytes by magnetic-activated cell sorting (MFA-MACS) (13). Mosquitoes were dissected on day 7-9 post-feeding and microscopically examined for oocysts following staining with mercurochrome.

Study outcomes

Primary outcomes were the prevalence of gametocytes and the frequency and severity of adverse events. The prevalence of gametocytes was defined as the presence of female gametocytes by *CCp4* qRT-PCR at any of the daily measurements from day 15 after MB inoculation or day 10 after IBSM inoculation. Secondary outcomes were gametocyte dynamics, commitment and maturation; and infectiousness to *Anopheles stephensi* mosquitoes.

Statistical analysis

Based on preliminary data, we anticipated that >95% of individuals would develop gametocytes (1). The CHMI-trans approach was considered unsuitable if <50% of individuals would develop mature gametocytes. Therefore, we powered the trial to meet a lower limit of the 99% confidence interval of >50%. The enrolment of 12 individuals per inoculation group of whom 11 would become gametocytemic, would mean we can estimate the proportion of gametocytemic individuals with a lower limit of the 99% confidence interval of 52%. We anticipated that ~73% of the IBSM inoculated individuals would infect at least one mosquito (8). Within each inoculation group of 12 individuals, we thus expected 8 or 9 transmitting individuals, resulting in a lower limit of the 95% confidence interval of 34%.

Statistical analyses were performed using GraphPad Prism 5 and SPSS version 25.0 (SPSS Inc, Chicago, IL, USA). The non-parametric Mann-Whitney U test was used for comparison between differences in continuous variables between study arms, for dichotomous variables the Fisher's exact test was used.

AUC was computed using GraphPad Prism 5, with the $(\Delta X)*(Y1 +Y2)/2$ formula. Correlations were assessed using non-parametric Spearman's rho. To obtain an estimate of the proportion of male gametocytes weighted for gametocyte density, for each individual

the sum of observed male gametocytes across time-points was divided by the sum of all gametocytes. Each individual thus provided one weighted proportion; the median of these proportions was calculated per inoculation group. The proportion of infected mosquitoes prior to treatment and after treatment was compared between treatment groups using Generalized Estimating Equations (GEE), adjusting for correlations between mosquitoes feeding on the same donor and using robust standard errors; outcomes were presented as odds ratios with 95% confidence intervals (95% CI). For sample size estimates for future TBI studies, mosquito infection data from DFAs in the current study and a previous study using IBSM and treatment with LD-PQP on day 8 post-challenge (8), were used, assuming three DFAs per individual and 30 examined mosquitoes per assay. The Bayesian statistical model takes into account variation between individuals, within individuals and between days of sampling. Power estimates were based on 4000 simulations per trial design, accounting for uncertainty estimated transmission probability.

Results

From a total of 41 screened volunteers, 24 healthy adults (**Table 1; Figure 1**) were enrolled and randomly assigned to experimental malaria infection with *P. falciparum* 3D7 parasites by MB or IBSM. To treat asexual parasite infection, participants received subcurative treatment with piperaquine (LD-PQP, 480 mg) that was administered a second time upon parasite recrudescence (n=6 from MB; n=5 from IBSM). Within each of the two inoculation groups (MB or IBSM), participants were randomized to receive curative treatment with either PQP (960mg) or sulfadoxine-pyrimethamine (SP, 1000mg/50mg), resulting in four study arms.

All participants completed the study and developed qPCR-detected asexual parasitemia 6.5 to 16.5 days after the MB inoculation or 4 to 5 days after IBSM. Peak parasite densities ranged from 2,428-53,203 parasites/mL after MB infection compared to 3,262-271,790 parasites/mL after IBSM (p=0.478) (**Table 2**, **Figure 2A** and **2B**). Female gametocytes were detected in 23/24 participants and male gametocytes in 18/24 participants by qRT-PCR targeting *CCp4* and *PfMGET*, respectively (10). All other participants had detectable gametocyte mRNA transcripts that were below the pre-defined limit of detection of 5 gametocytes/mL. Mean time of first gametocyte detection relative to first asexual parasite appearance was 13.9 days (range: 10.0-20.0 days) for MB and 9.2 days (range 8.0-11.0 days) for IBSM for female gametocytes (p<0.001), and 17.3 days (range 10.5-22.0 days) for MB and 9.3 days (range 8.0-11.0 days) for IBSM for male gametocytes (**Table 2, Figure 2**; p<0.001). Gametocyte sex-ratio was female-biased; the median proportion of male gametocytes was 0.20 (IQR 0.14-0.50) for the MB group and 0.31 (IQR 0.22 – 0.51) for the IBSM group (p=0.142) (**Figure 2C** and **2D**).

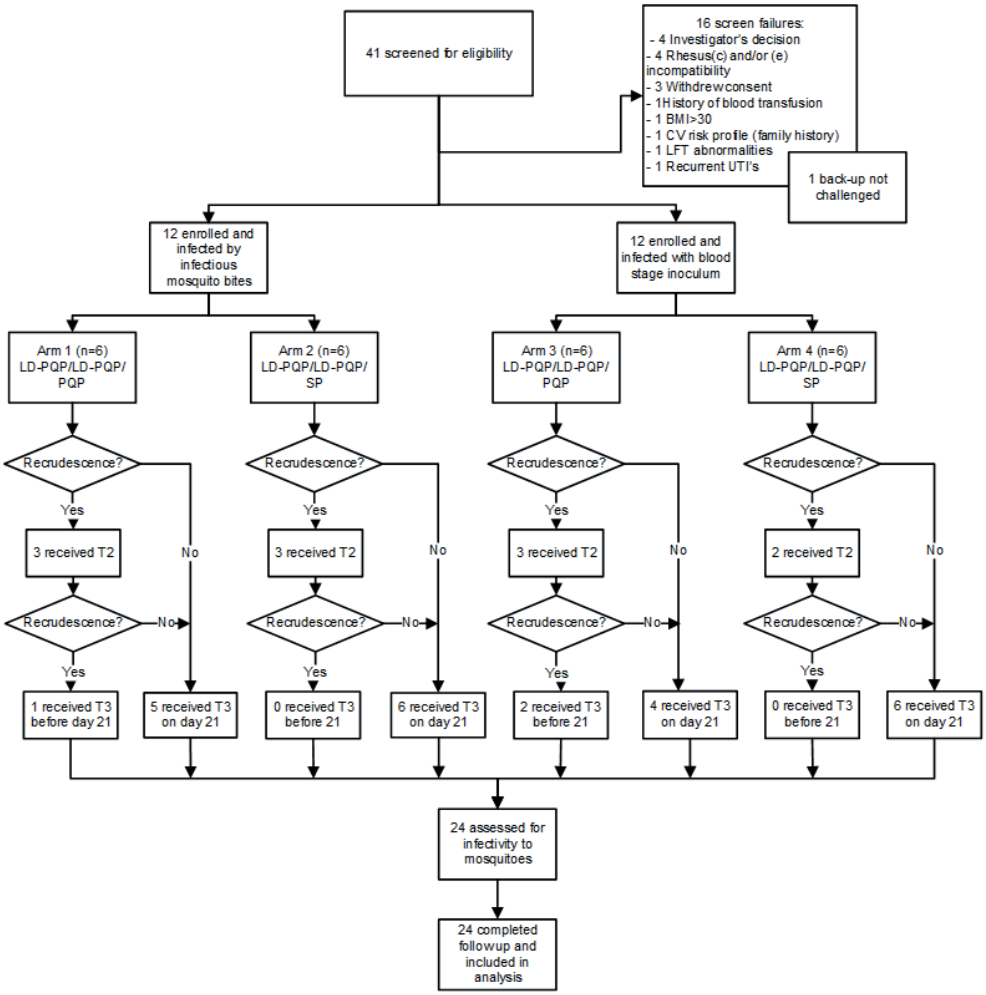


Figure 1 – Trial flow chart

Forty-one individuals were screened for eligibility, of whom 24 were included and divided over 4 study arms. All participants received LD-PQP on day 8 (IBSM group) or when treatment criteria were met (MB group). Participants received a second treatment with LD-PQP (T2) if recrudescence occurred. Due to thrombocytopenia 1 participant received T1 7.5 days post-inoculation and a curative treatment (T3) on day 12.5. As recrudescence occurred 15 days later, the final treatment with atovaquone/proguanil was initiated on day 27. Two IBSM participants developed asexual recrudescence after T1 and both received T3 directly (20.5 days post inoculation). All remaining participants of both cohorts received a curative dose PQP or SP (T3) on day 21 and final treatment with atovaquone/proguanil 36 days post inoculation. LD-PQP; low dose piperaquine 480mg. PQP; piperaquine 960mg. MB; mosquito bite, IBSM; induced blood stage malaria, T1; first subcurative treatment, T2; second subcurative treatment, T3; curative treatment.

	MB	IBSM	p-value
Time to T1 (days)	12.25 (7.5-19.50)	8	N.A.
Number of participants receiving T2 (% (n))	50 (6)	42 (5)	NS
Time between T1-T2 (days)	2.00 (1.75-6.50)	6.25 (4.25-12.50)	0.024
Peak parasite density (pf/mL)	32,807 (2,428-53,203)	27,700 (3,262-271,790)	0.478
Peak gametocyte density (gct/mL)	11.8 (2.5-727.9)	1,304 (179-3,826)	<0.001
Area under the curve (AUC)			
Total parasitaemia	37,654 (4,260-103,994)	38,639 (3,067-303,669)	0.977
Gametocytaemia	99 (16.6-5,330)	11,043 (1,643-37,326)	<0.001
Day of gametocyte detection after infection (days)	21.0 (20.0-27.0)	14.0 (13.0-16.0)	<0.001
Time to gametocyte detection* (days)	14.0 (10.0-20.0)	9.0 (8.0-11.0)	<0.001

**relative to first detection of asexual parasites.*

Table 2 – Course of treatments and parasitemia in different inoculation groups
Peak parasitemia and AUC total parasitemia were defined by 18S qPCR, peak gametocyte density and AUC gametocytemia were defined by the cumulative of *CCp4* female specific and *PfMGET* male specific qRT-PCR. MB; mosquito bite inoculation, IBSM; induced blood stage malaria.T1; first subcurative treatment, T2; second subcurative treatment. Presented data are median values with range between brackets.

The median peak total gametocyte density was 1304 gametocytes/mL (IQR: 308-1607) in the IBSM group compared to 12 gametocytes/mL (IQR 7-29) in the MB group (p<0.001) (**Figure 2A, B**). Peak gametocyte density strongly correlated with peak asexual parasitemia within both the MB (ρ= 0.64; p=0.024) and IBSM groups (ρ= 0.77; p=0.003) (**Figure 3A**); in a linear regression model, peak asexual parasite density for MB (ß=0.800; 95% CI 0.458-1.142, p<0.001) and IBSM (ß=1.472; 1.125-1.819, p<0.001) groups were both strongly predictive of gametocyte density without evidence of interaction. The ratio of *PfAP2-G* transcripts to ring-stage *SBP-1* transcripts, as an indicator of sexually committed parasites (12), strongly correlated with peak gametocyte density across groups (ρ=0.62; p=0.002) **(Figure 3B).**

In direct feeding assays (DFA), where mosquitoes directly fed on the skin of participants, 75% (9/12) of the IBSM group infected at least one mosquito compared to 0% (0/12) of the MB group (p<0.001) (**Figure 4A**). The median percentage of infected mosquitoes was 5% (range 3-20%) with 1-2 oocysts (**Figure 4B**); mosquito infection prevalence was strongly associated with venous blood gametocyte density (ρ = 0.556; p=0.017) (**Supplementary Figure 1**). Direct membrane feeding assays (DMFA) on venous blood and membrane feeding assays where participants plasma was replaced with malaria-naïve serum (MFA-SR) resulted in lower mosquito infection rates (**Supplemental Information**). In MFA's that

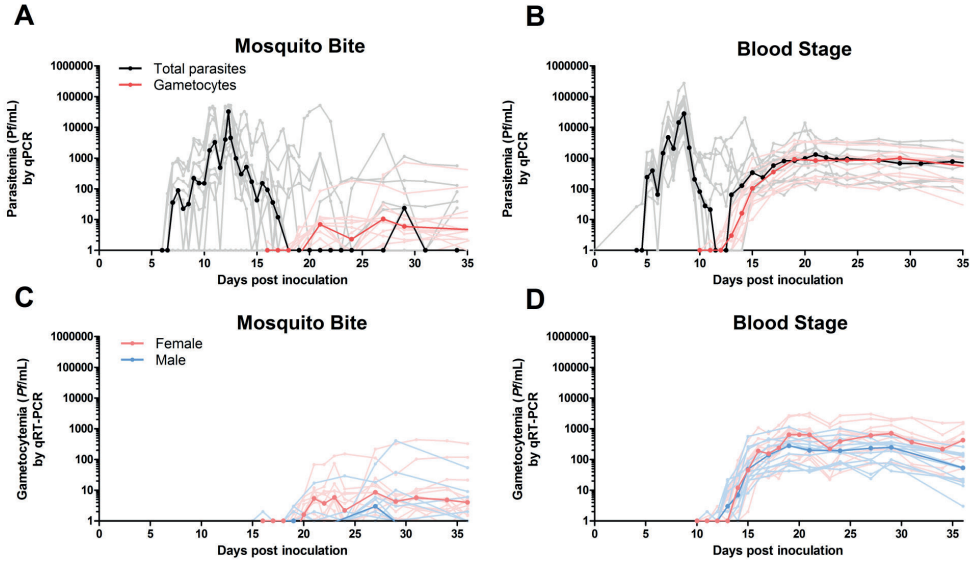


Figure 2 – Asexual parasitemia and gametocytemia following mosquito bite or blood stage inoculation

(A/B) Black lines represent median of 18S qPCR asexual parasitemia, grey lines represent individual participant data. Red lines represent median gametocytemia, pink lines represent individual participant data (sum of *CCp4* and *PfMGET* qRT-PCR). **(C/D)** Dark pink lines represent median female gametocytemia, light pink lines represent individual participant data (*CCp4* qRT-PCR) female specific gametocytes. Dark blue line represents median male gametocytemia, light blue lines represent individual participant data (*PfMGET* qRT-PCR).

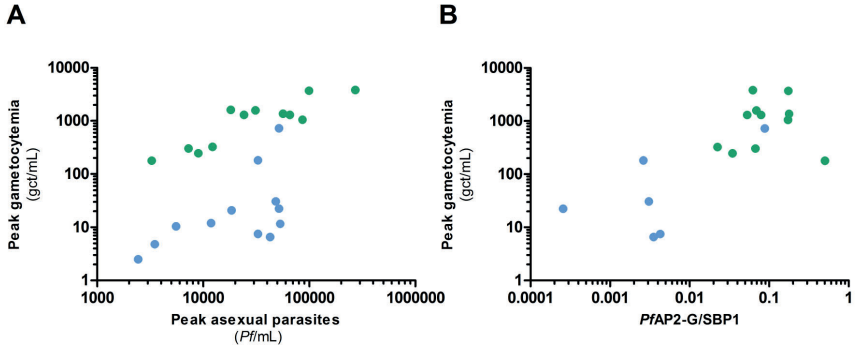


Figure 3 – The association between asexual parasite density, transcripts indicative of sexual commitment and subsequent gametocyte density

Blue dots represent data from mosquito bite inoculation; green dots represent data from blood-stage inoculation. **(A)** Correlation between peak asexual parasitemia and peak gametocytemia after mosquito bite and blood-stage inoculation. Mosquito bite inoculation (Spearman's ρ; P-value 0.64; 0.024), blood-stage inoculation 0.77; 0.003. **(B)** Correlation between peak gametocytemia and the ratio of *PfAP2-G* transcripts over ring-stage asexual transcripts *SBP-1* (Spearman's ρ; P-value 0.66; 0.001).

used gametocytes enriched by magnetic-activated cell sorting (MFA-MACS), samples from 100% (12/12) of participants in the IBSM group, and 8% (1/12) of the MB group infected mosquitoes (p<0.001) **(Figure 4A; 4B)** with a strong correlation between the original gametocyte density and the proportion of infected mosquitoes (ρ=0.558; p=0.016).

Prior to curative treatment (T3), the proportion of infected mosquitoes by DFA was 2.99% (11/368) and was not different between those who subsequently received PQP or SP (OR 0.743; 95% CI 0.113-4.868; p=0.756 after adjustment for correlation between mosquitoes feeding on the same participant). However, following curative treatment, the proportion of infected mosquitoes was considerably lower after SP treatment (0.28%; 1/363) compared to PQP treatment (4.46%; 16/359) (**Figure 4C,** and **4D**; OR 0.059; 95% CI 0.0074-0.478). When considering IBSM participants treated with PQP only, 83% (5/6) of participants were infectious to mosquitoes by DFA. Using this optimum procedure – IBSM inoculation followed by PQP treatment – we explored the suitability of the CHMI-transmission model with its current performance for future early-clinical testing of TBIs. Sample size estimates indicated that 7, 10 or 15 participants would be required per study arm to detect a statistically significant difference in the likelihood of mosquito infection between participants receiving a TBI and controls, when assuming TBI efficacy of 95%, 90% or 80%, respectively (**Figure 5**).

Study procedures were well tolerated in all participants; possible and probable related adverse events are listed in **Supplementary Table 1**. No serious adverse events occurred. Laboratory abnormalities are shown in **Supplementary Table 2** and returned to baseline before the end of study.

Discussion

Gametocytes develop from their asexual progenitors, and asexual parasite density is a strong determinant of subsequent gametocyte density in malaria-naïve volunteers (1, 8). However, in this study, we found considerably higher gametocyte densities following IBSM compared to MB inoculation despite a similar asexual parasite burden. A higher ratio of *PfAP2-G*:*SPB-1* transcripts – indicative of gametocyte commitment (2, 14) – was strongly predictive of subsequent gametocyte density, suggesting differences in sexual commitment between inoculation routes. Our data provide the first prospective *in vivo* evidence that *PfAP2-G* transcripts may serve as a correlate of gametocyte formation (2, 12, 15). The reasons behind this apparent higher sexual commitment after IBSM remain elusive. We observed a strong association between duration of asexual parasitemia prior to first treatment and estimated sexual commitment rates, even when restricting our

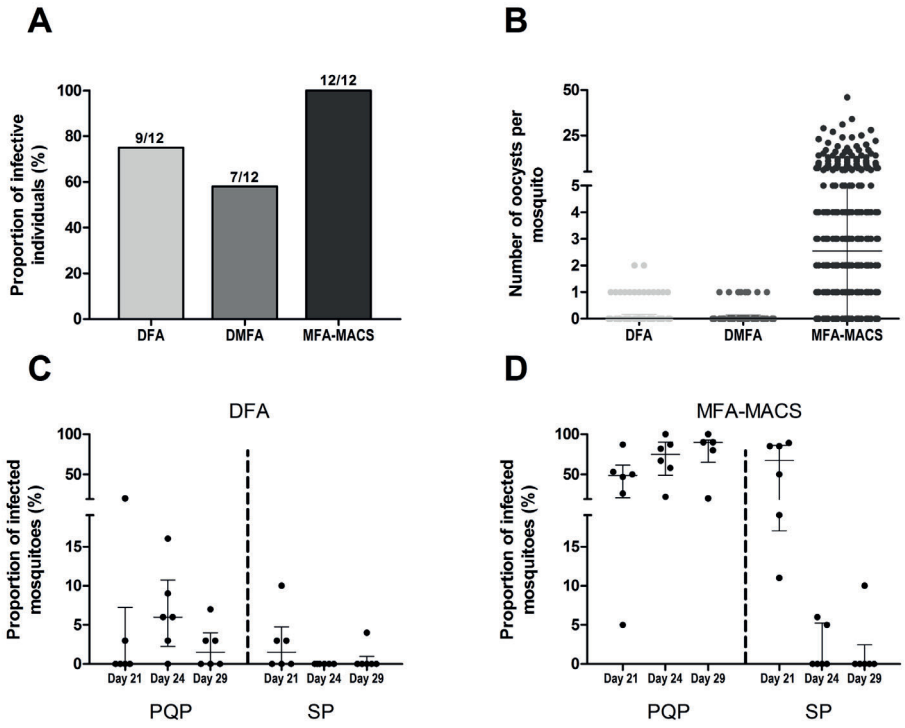


Figure 4 – Transmission to Anopheles mosquitoes by Direct Skin Feeding or by Membrane Feeding of venous blood samples

A total of 71 direct feeding assays (DFA) were conducted (median: 31 examined mosquitoes per experiment; IQR: 28 - 32); alongside 71 direct membrane feeding assays (DMFA) (exactly 25 examined mosquitoes per experiment), and 71 DMFA experiments following gametocyte concentration by magnetic-activated cell sorting (MFA-MACS; median: 18 mosquitoes examined per experiment; IQR: 16-19). **(A)** Total of proportion of individuals infectious to mosquitoes by DFA, DMFA, and MFA-MACS, cumulative for different feeding assay time points. **(B)** Number of oocysts per mosquito in DFA, DMFA, and MFA-MACS feeding experiments. **(C/D)** Proportion of infected mosquitoes per individual per time-point by curative treatment group (PQP or SP) in DFA and MFA-MACS. Day 21 is pre-treatment, day 24 and day 29 are post treatment. PQP = Individuals that received piperaquine as a curative treatment, SP = Individuals that received sulfadoxine-pyrimethamine as a curative treatment.

analysis to MB infections (**Supplementary Figure 3**). Differences in inflammation may also have contributed to differences in gametocyte formation (16).

We aimed to maximize asexual parasite burden (i.e. the area under the curve of asexual parasite density versus time) by adding a second subcurative treatment with piperaquine. This indeed resulted in a significantly higher asexual parasite burden compared to the

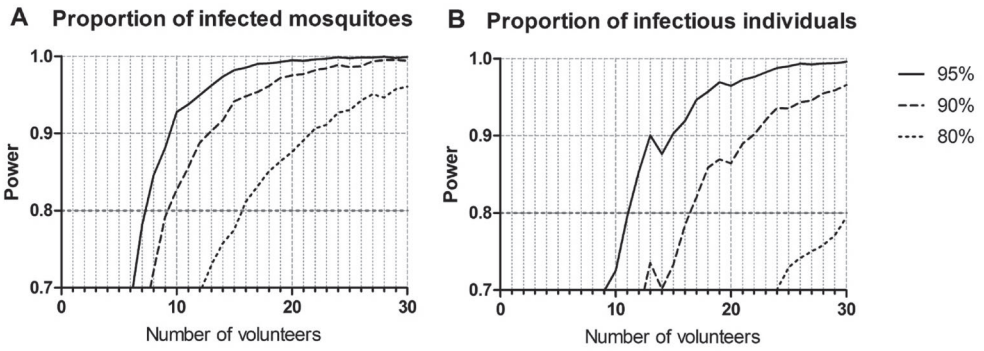


Figure 5 – Sample size requirements to use the current model to examine the efficacy of transmission blocking interventions
Sample size calculations were performed using all available data from the current study (n=6) and the CHMI transmission trial conducted in Brisbane (n=12) (8). Three mosquito feeding time-points per person were assumed. Power estimates are based on 4000 simulations per trial design, accounting for uncertainty estimated transmission probability. Line-types indicate vaccine efficacy of 80%, 90% or 95% and power was calculated for comparisons of whether people were infectious (B; infecting ≥1 mosquito) and the proportion of mosquitoes they infected (A). Detecting a statistically significant difference in the proportion of infected mosquitoes between vaccinated and non-vaccinated participants with vaccine efficacy of 95%, 90% or 80% required 7, 10 or 15 volunteers per arm, respectively (80% power; p-value <0.05).

previous study using a single subcurative treatment (p=0.02) (1) (**Supplementary Figure 2A**). However, the group size was too small to determine if this resulted in a meaningful increase in gametocyte density or infectivity to mosquitoes.

Our work supports the use of PQP over SP for CHMI-trans studies. Whilst treatment with SP previously showed promise in our model (1), current data strongly suggest SP may compromise infectivity of mature gametocytes (17, 18). This is contrary to our field study data that indicate high and transmissible gametocytemia after SP treatment (19, 20). This difference could plausibly be due to parasite genetic differences (17, 18).

Further improvements of CHMI-trans may be conceivable, for example by using clinical isolates or parasite lines with intrinsically or inducible higher gametocyte commitment (12, 21). However, in its current form, our protocol involving IBSM with piperaquine treatment allows early-clinical testing of highly potent transmission blocking interventions achieving ≥80% transmission reduction (6, 22), with approximately 15 participants per study arm – similar to CHMI models for pre-erythrocytic and erythrocytic malaria vaccines (23).

In conclusion, we report major improvements over our earlier mosquito bite CHMI-trans model (1) by demonstrating in a direct comparison, a striking effect of CHMI inoculation route on gametocyte development and infectivity. We define the optimal design of a CHMI-transmission study that allows the safe induction of transmissible gametocyte densities. The presented model thus paves the way for early-clinical evaluation of transmission-blocking interventions that are deemed crucial for successful malaria elimination.

Acknowledgements

We would like to thank the staff from the Radboudumc Technology Center Clinical Studies. We also thank Daphne Smit and Annemieke Jansens for their assistance and project support during the trial. We thank the following individuals for their assistance during the trial: Wouter Graumans, Roel Heutink, Rianne Stoter, Laura Pelser, Jolanda Klaassen, Astrid Pouwelsen, and Jacqueline Kuhnen for the mosquito infection and dissection work, Foekje Stelma, and all the thick smear microscopists for reading many smears. This trial was supported mainly with funds from PATH's Malaria Vaccine Initiative. KL and KAC and TB are supported by a fellowship from the European Research Council (ERC-2014-StG 639776 to TB). Finally and foremost, we would like to thank the study volunteers who participated in this trial. The funder, PATH's Malaria Vaccine Initiative, was involved in the study design, analysis and interpretation of the data, the preparation of the report, but not data collection. IJR, MA, GdJ, KC, RWS and TB had full access to all study data with final responsibility for the decision to submit the report for publication.

Supplemental Information

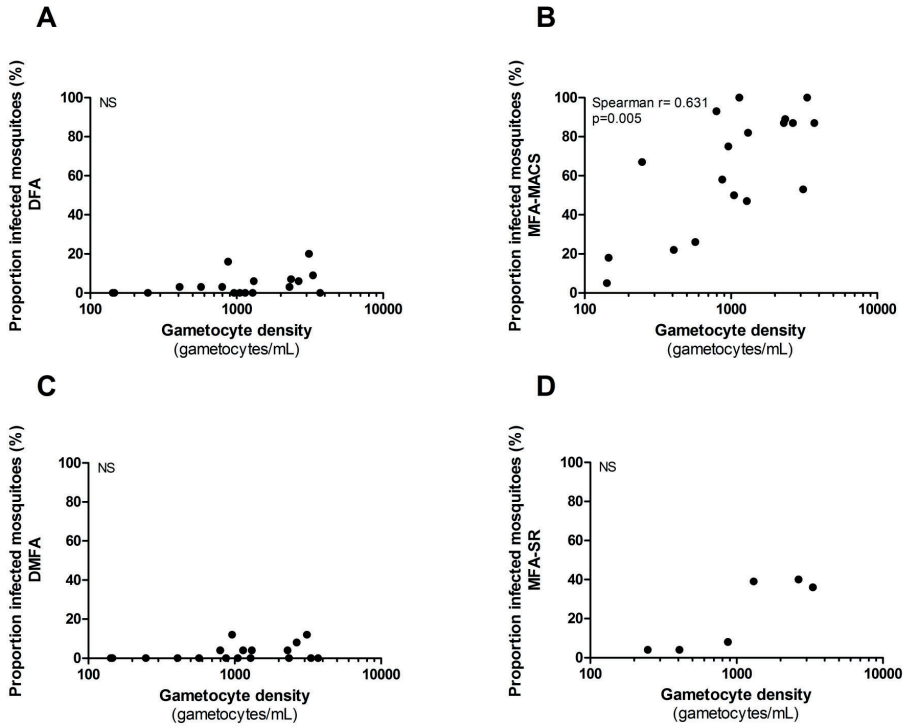
AP2-G PCR

Total nucleic acid was RQ1 DNaseI digested (Promega) and cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturers protocol. 2 ul of cDNA was run in a total reaction volume of 20 ul of GoTaq qPCR mixture (Promega) containing 900 nM primers. Standard cycling conditions were used.
Primers:
FW: 5'- tggtggtaataagaacaacagaggt-3'
Rev: 5'- ccatcataatcttcttcttcgtcg-3'

Supplemental Results

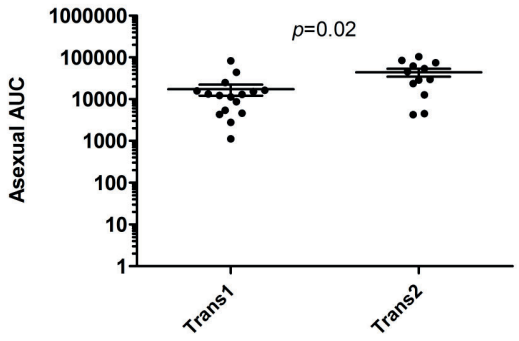
Membrane feeding assays after serum replacement

As an exploratory objective, a total of 24 DMFA experiments were conducted where volunteer plasma was replaced with malaria-naïve serum (MFA-SR; exactly 25 mosquitoes examined per experiment). MFA-SR experiments were conducted only on day 24 post inoculation for both inoculation routes. MFA-SR resulted in 67% (8/12) infectious individuals and 12% (35/298) infected mosquitoes in the IBSM group. These results were considerably lower than the infection rates after MFA-MACS where 100% (12/12), 67% (8/12) and 58% (7/12) of individuals were infectious, with 51% (109/215), 34% (75/218) and 41% (74/182) infected mosquitoes on day 21, day 24 and day 29 post inoculation respectively. No measurable transmission was observed after MFA-SR in the MB group.

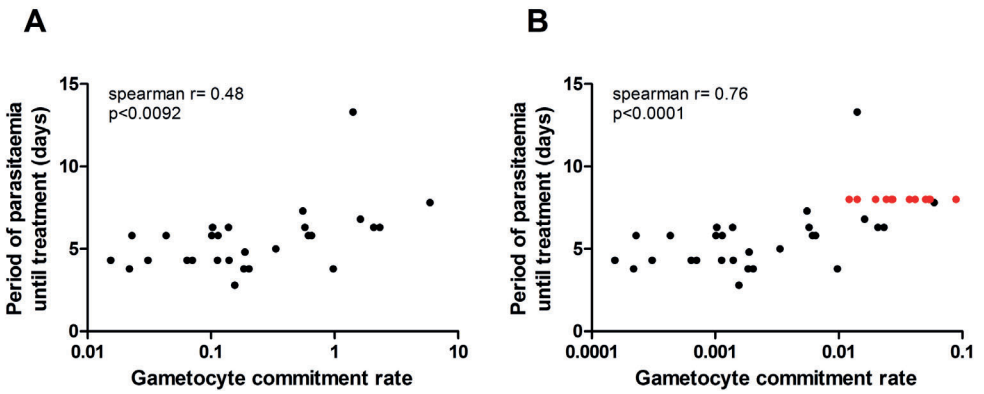


Supplementary Figure 1 – Correlation between proportion of infected mosquitoes and gametocyte density

Only data from study arm 4 (N=6) that IBSM inoculated and received piperaquine as a curative treatment was included. **(A)** DFA; direct skin feeding assays. **(B)** MFA-MACS; membrane feeding assay following enrichment for gametocytes by magnetic-activated cell sorting. **(C)** DMFA; direct membrane feeding assay. **(D)** MFA-SR; membrane feeding assay following serum replacement.



Supplementary Figure 2 – Difference in parasite burden between CHMI-transmission studies
Trans1; participants of our previous trial (1) and Trans 2; this current studies.



Supplementary Figure 3 – Association between the period of asexual parasitemia and estimation of commitment ratio
(A) Data from 2 CHMI studies using mosquito bite inoculation only (black dots) (1). **(B)** All data of A, including data from the blood-stage inoculation (red dots). The period of asexual parasitemia was calculated from the moment of inoculation (day 0) until day of treatment for participants of the blood-stage inoculation, and day 6.5 (the estimated moment of parasites entering the bloodstream) after mosquito bite inoculation until day of treatment. The gametocyte commitment rate is estimated by dividing the peak gametocyte by the peak of asexual parasites.

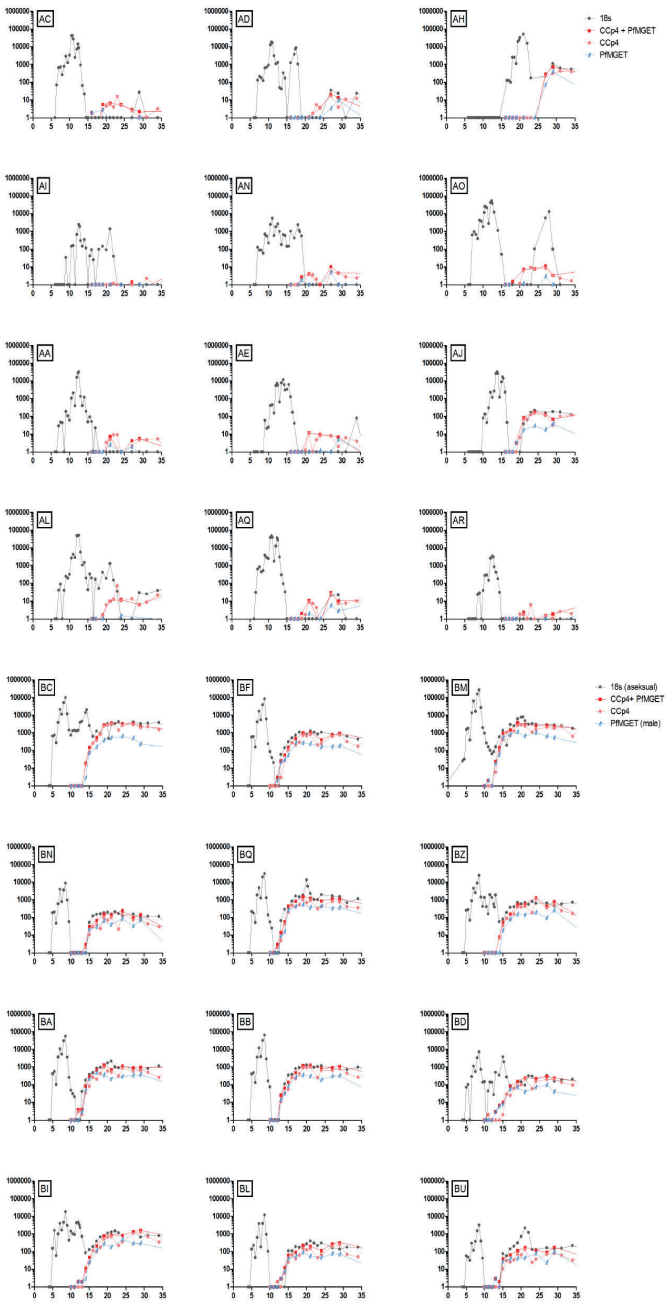
	MB						IBSM					
	Arm 1			Arm 2			Arm 3			Arm 4		
	No of subjects	No of episodes	Median duration in days (Range)	No of subjects	No of episodes	Median duration in days (Range)	No of subjects	No of episodes	Median duration in days (Range)	No of subjects	No of episodes	Median duration in days (Range)
Fever	6	16	0.5 (0.0-1.3)	6	11	0.4 (0.0-1.0)	6	13	0.3 (0.1-1.1)	3	5	0.3 (0.0-0.6)
Chills	6	13	0.4 (0.0-3.4)	1	2	0.3 (0.0-0.7)	4	7	0.2 (0.0-4.6)	1	2	0.3 (0.1-0.5)
Syncope	0	N.A.	N.A.	1	1	0.0	0	N.A.	N.A.	0	N.A.	N.A.
Abdominal pain	0	N.A.	N.A.	2	2	0.5 (0.0-0.9)	5	6	0.3 (0.0-2.0)	2	2	0.3 (0.4-0.5)
Fatigue	4	7	1.0 (0.1-3.4)	5	13	1.0 (0.5-3.9)	5	12	1.1 (0.0-8.0)	3	6	0.4 (0.0-1.9)
Headache	6	27	0.5 (0.0-4.0)	6	26	0.9 (0.0-3.6)	6	33	0.7 (0.0-4.0)	6	20	0.8 (0.1-3.5)
Malaise	4	8	1.8 (0.0-3.9)	4	15	1.2 (0.3-3.3)	1	3	3.1 (0.9-6.5)	2	2	0.5 (0.1-0.8)
Myalgia	1	1	2.1	5	6	1.8 (0.9-3.2)	4	7	0.7 (0.1-5.1)	4	5	0.3 (0.1-2.2)
Nausea	5	13	0.5 (0.0-1.8)	4	12	0.9 (0.1-2.3)	6	9	0.5 (0.0-2.0)	3	6	0.1 (0.0-0.6)
Decreased appetite	3	3	4.6 (3.5-6.9)	2	2	1.8 (0.6-3.0)	2	3	1.3 (0.9-3.0)	0	N.A.	N.A.
Dizziness	2	5	0.5 (0.0-1.5)	1	3	1.4 (1.2-1.5)	2	8	1.0 (0.0-4.0)	2	4	0.6 (0.3-1.0)
Diarrhea	1	1	0.3	1	2	0.8 (0.0-1.7)	1	1	2.0	0	N.A.	N.A.
Palpitations	1	1	5,3	0	N.A.	N.A.	0	N.A.	N.A.	0	N.A.	N.A.
Back pain	0	N.A.	N.A.	0	N.A.	N.A.	2	3	0.9 (0.1-2.0)	0	N.A.	N.A.
Arthralgia	0	N.A.	N.A.	0	N.A.	N.A.	0	N.A.	N.A.	1	1	0.3
Nonspecific thoracic pain	0	N.A.	N.A.	0	N.A.	N.A.	2	2	0.1 (0.0-0.2)	0	N.A.	N.A.
Total	**6**	**95**		**6**	95		6	107		6	53	

Supplementary Table 1 – Adverse Events

	MB						IBSM					
	Arm 1			Arm 2			Arm 3			Arm 4		
	N (% of total) Grade 1	N (% of total) Grade 2	N (% of total) Grade 3	N (% of total) Grade 1	N (% of total) Grade 2	N (% of total) Grade 3	N (% of total) Grade 1	N (% of total) Grade 2	N (% of total) Grade 3	N (% of total) Grade 1	N (% of total) Grade 2	N (% of total) Grade 3
Decreased hemoglobin	3 (50)	3 (50)	0 (0)	3 (50)	2 (33)	0 (0)	3 (50)	1 (17)	0 (0)	3 (50)	1 (17)	0 (0)
Decreased leukocytes	4 (67)	1 (17)	0 (0)	2 (50)	1 (17)	1 (17)	2 (33)	2 (33)	0 (0)	1 (17)	1 (17)	1 (17)
Decreased platelets	4 (67)	0 (0)	1 (17)	3 (50)	1 (17)	0 (0)	3 (50)	0 (0)	0 (0)	2 (33)	0 (0)	0 (0)
Increased AST and/or ALT	2 (33)	3 (50)	1 (17)	4 (67)	0 (0)	2 (33)	2 (33)	3 (50)	0 (0)	0 (00	2 (33)	0 (0)
Elevated Creatinine	0 (0)	0 (0)	0 (0)	1 (17)	1 (17)	0 (0)	0 (0)	0 (0)	0 (0)	1 (17)	2 (33)	0 (0)

Grading was based on the Food and Drug Administration's Toxicity Grading Scale. No grade four abnormalities were reported.

Supplementary Table 2 – Laboratory abnormalities



Supplementary Figure 4 – Individual parasite curves

Grey lines represent individual 18S qPCR asexual parasitemia. Red lines represent total gametocytemia (sum of *CCp4* and *PfMGET* qRT-PCR). Pink lines represent female gametocytemia (*CCp4* qRT-PCR). Blue line represents male gametocytemia (*PfMGET* qRT-PCR).

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Part II

Heterogeneity in host response after *Plasmodium* spp. infection

Chapter 4

Systematic review of the role of Angiopoietin-1 and Angiopoietin-2 in *Plasmodium* species infections: Biomarkers or therapeutic targets?

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Jaap J. van Hellemond, Perry J.J. van Genderen

Malaria Journal. 2016 Dec 1;15(1):581

Abstract

Background

Levels of both Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) correlate with malaria disease severity and are proposed as biomarkers and possible therapeutic targets. To establish their role in malaria, a systematic review was performed of the literature on Ang-1 and Ang-2 with regard to their potential as biomarkers in malaria and discuss their possible place in adjuvant treatment regimens.

Methods

Ten electronic databases were systematically searched to identify studies investigating Ang-1 and Ang-2 in human and murine malaria in both clinical and experimental settings. Information about the predictive value of Ang-1 and Ang-2 for disease severity and their regulatory changes in interventional studies were extracted.

Results

579 studies were screened; 26 were included for analysis. In all 5 studies that determined Ang-1 levels and in all 11 studies that determined Ang-2 in different disease severity states in *P. falciparum* malaria, a decline in Ang-1 and an increase of Ang-2 levels was associated with increasing disease severity. All 9 studies that determined angiopoietin levels in *P. falciparum* patients to study their ability as biomarkers, could distinguish between multiple disease severity states; the more the disease severity states differed, the better they could be distinguished. 5 studies differentiating malaria survivors from non-survivors with Ang-2 as marker, found an AUROC in a range of 0.71-0.83, which performed as well or better than lactate. Prophylactic administration of FTY720, rosiglitazone or inhalation of nitric oxide (NO) during malaria disease in mice resulted in an increase in Ang-1, a decrease in Ang-2 and an increased survival. For rosiglitazone a decrease in Ang-2/Ang-1 ratio was observed after post-infection treatment in mice and humans with malaria, but for inhalation of NO an effect on Ang-1 and survival was only observed in mice.

Conclusion

Both Ang-1 and Ang-2 levels correlate with and can distinguish between malaria disease severity states within the group of malaria infected patients. However, distinct comparisons of disease severity states were made in distinct studies and not all distinctions made, had clinical relevance. Changes in levels of Ang-1 and Ang-2 might also reflect treatment effectiveness and are promising therapeutic targets as part of multi-targeted therapy.

Background: Possible role of Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) in malaria

General aspects of malaria and endothelial cell activation

Even though many disease control interventions have been implemented, malaria remains a major health problem with an estimated 214 million cases and 438.000 deaths worldwide in 2015 (1). Most of these malaria deaths were caused by *P. falciparum*, one of the six *Plasmodium* species (*P. cynomolgi, P. falciparum, P. knowlesi, P. malariae, P. ovale, P. vivax*) that can infect humans. The high pathogenicity of *P. falciparum* is in part related to the expression of *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP-1) on the membranes of infected erythrocytes. The infected erythrocytes with *Pf*EMP-1 on the membrane adhere to the vascular endothelium of vital organs. This process is called sequestration and will cause a partial obstruction of the blood flow. Together with the increased deformability of uninfected erythrocytes and adherence of uninfected erythrocytes to infected erythrocytes, which is called rosetting (2), extensive sequestration will lead to a decline in oxygen delivery to the organs. Without treatment, the lack of oxygen will result in acidosis and multi-organ failure; along with endothelial barrier dysfunction and inflammation. This may eventually lead to death in untreated cases, especially in non-immune individuals. Of the non-falciparum species, *P. vivax,* which may run a relatively benign course compared to *P. falciparum*, is most important and can also cause severe malaria and result in death. The pathogenesis of *P. vivax* infection differs from *P. falciparum* infection and is still poorly understood. *P. vivax* infects only young erythrocytes – reticulocytes – which can also adhere to the vascular endothelium, but showed 10 times less cytoadhesion compared to *P. falciparum* infected erythrocytes *in vitro* (3). Also the cytokine response differs between the species: *P. vivax* showed a stronger response than *P. falciparum* did (4). Whether cytoadherence in *P. vivax* leads to disproportional organ-specific parasitaemia and what role the cytokine response plays in the pathogenesis remains unclear.

Endothelial cell activation

Endothelial cell activation is crucial in the pathogenesis of *P. falciparum* infection as *Pf*EMP-1 on the erythrocyte membranes interacts with several cytoadherence receptors that show a low, basal expression pattern in a non-inflammatory environment, but are upregulated during malaria infection (5). Activation of endothelial cells is complex and interconnected with other processes, such as coagulation and inflammation. Quiescent endothelial cells have anticoagulant properties due to expression of several proteins, such as protein C and thrombomodulin. On the other hand, after activation by e.g. thrombin (6), fibrin (7, 8), complement factors C5-C9 (9) or platelets (10), endothelial cells express

or release procoagulant and inflammatory proteins, such as Tissue Factor, von Willebrand Factor (vWF), Ang-2, I-CAM, V-CAM and E-selectin (5, 11). Endothelial cell activation occurs not only during malaria but also during many other infectious diseases, such as bacterial sepsis (12) and dengue haemorrhagic fever (13, 14). To study endothelial cell activation during infectious diseases, expression levels of multiple endothelial-cell activation markers have been determined and were found to be increased, including the vascular growth factors Ang-1 and Ang-2, which were extensively studied during systemic inflammation (reviewed in (15-20)). Their role in endothelial cell activation during malaria has also been investigated.

Ang-1, Ang-2 and endothelial cell activation

Ang-1 and Ang-2 are ligands of the Tie-2 receptor, which is expressed on endothelial cells and regulates endothelial quiescence during normal physiological conditions. Ang-1 is constitutively produced and excreted into the blood by pericytes and smooth muscle cells and it is also stored in platelets. Ang-1 binds to the Tie-2 receptor, thereby acting as an agonist. Upon Ang-1/Tie-2 interaction, the most important downstream signalling event is protein kinase B phosphorylation by phosphoinositide-3-kinase resulting in an anti-apoptotic and anti-inflammatory status of the endothelial cell.

Ang-2 is produced in endothelial cells and pre-stored in the Weibel-Palade bodies (WPB) together with vWF. Upon activation of endothelial cells, exocytosis of the WPB is induced and their content is released into the bloodstream (21). The increased Ang-2/Ang-1 ratio causes a replacement of the Ang-1-Tie-2 interaction by an Ang-2-Tie-2 interaction. In the presence of Ang-1, Ang-2 acts as a functional antagonist and the Ang-2-Tie-2 interaction results in the blocking of the protective, anti-inflammatory and anti-apoptotic effect of Ang-1 (see **Figure 1**). Therefore, Ang-2 release and binding to Tie-2 receptors further stimulates the inflammatory response of endothelial cells. However, an agonistic function of Ang-2 has also been described and is influenced by cell type and experimental conditions (22). A decrease in the Ang-1 concentration during pro-inflammatory conditions, also contributes to the increased Ang-2/Ang-1 ratio. The underlying mechanisms of the Ang-1 decrease have not been defined yet. From these findings, it may be hypothesized that the role of Ang-1 is protective during malaria and the role of Ang-2 may be harmful.

Ang-1 and Ang-2 are likely to play a role early in malaria pathogenesis

During malaria a decrease in Ang-1 and increase in Ang-2 concentration have been observed (15). It is not known whether the decrease of Ang-1 and increase of Ang-2 are the result of the endothelium activation status or that the altered levels also contribute

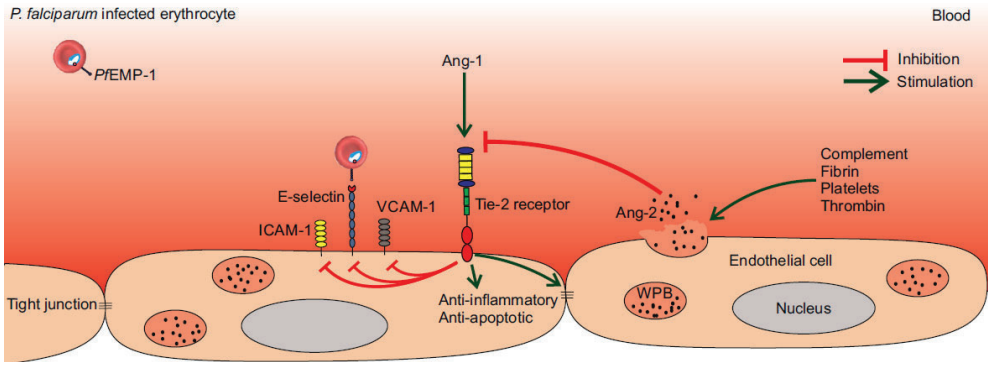

Figure 1 – Schematic overview of function and localisation of Ang-1, Ang-2 and the Tie-2 receptor

Ang-2 is pre-stored in the Weibel-Palade bodies in endothelial cells and is released upon endothelial cell activation. Ang-2 replaces Ang-1 by binding the Tie-2 receptor, preventing its activation and thereby blocking the anti-inflammatory, anti-apoptotic and tight-junction supporting effects of Ang-1. *Abbreviations*: **Ang-1,** angiopoietin-1; **Ang-2**, angiopoietin-2**; ICAM-1, E-selectin, VCAM-1**, adhesion molecules; ***Pf*EMP-1**, *Plasmodium falciparum* erythrocyte membrane protein 1; **WPB**, Weibel-Palade body.

to endothelium activation. However, during the initial processes that lead to pathology in malaria, Ang-1 and Ang-2 might play a role as i) Ang-1 and Ang-2 levels strongly correlate with disease severity and mortality (15), ii) in sepsis, which shares many similarities with severe malaria (15, 18, 23), interfering in the angiopoietin-Tie-2 system, had a huge effect on the inflammatory response, vascular leakage and survival (24) and iii) Ang-2 can be released rapidly upon activation of endothelial cells as it is pre-stored in WPBs (21). After exocytosis of WPBs, released Ang-2 may then have extended effects in further activation of endothelial cells by activation of a pro-inflammatory amplification loop.

Because of the correlation of Ang-1 and Ang-2 levels with disease severity and mortality, Ang-1 and Ang-2 have been evaluated as biomarkers for malaria disease severity in multiple studies. However, each individual study compared different disease severity states and the studies were performed in distinct populations at different geographical locations around the world. To gain more insight in their role during malaria, the current literature concerning the use of Ang-1 and Ang-2 as biomarker in a clinical setting is here reviewed systematically. Furthermore, the role of Ang-1 and Ang-2 as potential therapeutic targets in malaria is evaluated as they might play a causative role in the pathogenesis.

Methods

Literature search and inclusion criteria

A broad systematic literature-search strategy was created combining synonyms for angiopoietin with synonyms for malaria or *Plasmodium*. In Embase, Medline and CInahl thesaurus terms were used together with words in title and/or abstract. In the other databases only titles and/or abstracts were searched. The databases Embase.com, Medline (Ovid), Cochrane Central registry of trials, Web of Science, Scopus, cinahl (EBSCOhost), Pubmed as supplied by publisher (for the most recent not yet indexed articles), lilacs, scielo, ProQuest and Google scholar (an additional search), were searched without data limit, from inception lastly on 15 June 2016 (**Additional file 1**). Based on the titles and abstracts, potentially relevant articles were selected by two independent reviewers (GMdJ, JJS) for full text screening. Articles in English meeting the inclusion criteria were included (**Additional file 2**). In case of disagreement between the reviewers, a third independent reviewer was consulted (JJvH).

Reported studies were included if Ang-1 and/or Ang-2 protein or mRNA levels were determined in any specimen in humans and/or mice with a confirmed *Plasmodium* infection. Through tracking the citations in selected articles, no additional articles were found.

Data extraction

The selected articles were grouped, depending on the study aim, into: *i)* studies performed in non-pregnant humans and *ii)* interventional studies. The aim of studies in group *i* was to correlate Ang-1 and Ang-2 levels with disease severity and in group *ii* to find an adjuvant therapy next to antimalarial treatment.

For the articles of group *i*, information was extracted on the *Plasmodium* species, the study population, the Ang-1 and/or Ang-2 protein and/or mRNA levels or the Ang-2/Ang-1 ratio in various disease states, and information about the associations of Ang-1 and Ang-2 levels with other variables. In the studies included in group *ii*, data was extracted regarding study subject, mice species or population characteristics, details of the studied intervention, Ang-1, Ang-2 protein or mRNA levels and the Ang-2/Ang-1 ratio.

Results

Characteristics of included studies

26 studies assessing Ang-1 and/or Ang-2 protein and/or mRNA levels in human and/or mice with a confirmed *Plasmodium* infection were identified and included for analysis. 22 studies were performed in humans only, of which 5 included children and adults in Asia and Canada as a single group (**Additional file 3**), 6 studies included adults only and were performed in Asia and Brazil (**Additional file 4**), 8 studies included children only and were all performed in only African countries (**Additional file 5**) and 1 study included a separate group of children and adult (25) (**Additional file 4 and 5**). 3 studies provided data on mice only (**Additional file 6**). 1 interventional study combined results of studies in mice and humans (26) (**Additional file 6**).

In 9 studies a discriminative value of Ang-1, Ang-2 levels or the Ang-2/Ang-1 ratio for disease severity was reported (25, 27-35) (**Additional file 7**). 4 studies determined the Ang-1 and/or the Ang-2 level in humans after start of antimalarial treatment (29, 34, 36, 37). 3 studies investigated the effect of adjuvant therapy next to standard treatment on survival and on Ang-1 and/or Ang-2 levels in mice (38-40), 2 in humans (41, 42) and 1 in both humans and mice (43) during *Plasmodium* species infection (**Additional file 6**). 1 study used a mouse model with one deleted Ang-1 allele (43) (**Additional file 6**).

Aim i: Ang-1 and Ang-2 levels as biomarkers for malaria disease severity

Ang-1 is decreased and Ang-2 is increased during Plasmodium species infection in non-pregnant humans

In 10 out of 11 studies comparing healthy controls with either uncomplicated (25, 32, 34, 35, 37, 44-46), severe (34, 35, 44, 46, 47) or cerebral *P. falciparum* malaria (25, 32, 37, 45, 46) or uncomplicated (44, 48) or severe (44) *P. vivax* malaria, a similar pattern in changes was observed for Ang-1 and Ang-2 levels: a decrease of Ang-1 (25, 32), an increase of Ang-2 (25, 32, 34, 35, 37, 44-48) and consequently an increase of the Ang-2/Ang-1 ratio (25, 32) (**Additional files 3, 4, 5**). Adults and children, living either in endemic areas or travellers in non-endemic areas all showed these similar changes. However, 1 study (36) out of 3 (25, 32, 36) that determined the Ang-1 levels in *P. falciparum* malaria found an increase in Ang-1 compared with the levels in healthy controls opposed to a decline in the other studies. The absolute concentration of Ang-2 was significantly higher in *P. vivax* infected patients compared to *P. falciparum* infected patients in Indonesia, independent of the observed peripheral parasitaemia (48). Contrastingly, a significant lower Ang-2 concentration was observed in *P. vivax* compared to *P. falciparum* infected travellers in

Canada (49). Barber et al (44) determined also the Ang-2 levels in both uncomplicated *P. vivax* malaria (median pg/ml (IQR)) (4557 (3463-6197)) and *P. falciparum* malaria (3230 (2123-5243)) and in severe *P. vivax* malaria (8857 (6547-9734)) and *P. falciparum* malaria (8371 (3963-13463). However, this study did not statistically compare the levels between the *P. vivax* and *P. falciparum* malaria, although Ang-2 seems to be higher in *P. vivax* infected patients especially when the higher parasitaemia of *P. falciparum* infected patients is taken into account.

Differences in the absolute Ang-1 and Ang-2 concentrations were also found between disease severity states: lower Ang-1 and higher Ang-2 concentrations were observed in severe disease compared to uncomplicated disease in both *P. falciparum* (25, 28-30, 32, 34, 44-46) and *P. vivax* malaria (31, 44) and in non-survivors compared to survivors infected with *P. falciparum* (25, 27, 30, 32-35, 41, 47) (**Additional files 3-6**).

Furthermore, an elevated Ang-2 concentration was associated with respiratory distress (50), impaired consciousness (50), acute kidney injury (33, 47), multi-organ failure (47), anaemia (33), jaundice (33), hypoglycaemia (33), pure cerebral malaria (cerebral malaria patients without the other complications of severe malaria according to WHO 2000 criteria) (33) and higher Ang-1 levels were associated with a higher platelet count in *P. falciparum* and *P. vivax* infections. (31, 33, 51).

Ang-1 increases and Ang-2 decreases after start antimalarial treatment in survivors

An increase in Ang-1 and a decrease in Ang-2 levels in survivors compared to non-survivors was observed after the start of treatment in both children (29) and adults (34, 36) (**Additional file 3,4,5**) and one study found an average Ang-2 decrease of 2.7 ng/ml over a 24 hour period in survivors (34). Moxon et al (37) found a nearly two-fold decline in Ang-2 level in children with uncomplicated malaria and a five-fold decline in children with severe malaria 28 days after the start of treatment. However, 28 days after treatment the Ang-2 levels in uncomplicated malaria were still significantly higher than Ang-2 levels in healthy controls. This lack of decrease to baseline levels might be caused by a 50% loss to follow-up, which may have led to a biased difference in Ang-2 value between healthy control and patients after treatment.

Ang-1 and Ang-2 levels have a varying discriminative power for distinguishing disease severity states in humans

Uncomplicated *P. falciparum* malaria can rapidly progress into life-threatening disease due to the exponential increase in parasite biomass and sequestration of infected erythrocytes, which may lead to organ failure. The currently used biomarkers for disease severity, have a limited predictive value to identify those patients that will rapidly progress towards

complicated disease (52). Several new markers have been proposed and investigated (53) as prognostic parameters for severe malaria, including Ang-1 and Ang-2. In distinct studies Ang-1, Ang-2 and the Ang-2/Ang-1 ratio were used to investigate whether they could distinguish different disease severity states and survivors from non-survivors (25, 27-35) (**Additional file 7**).

The Ang-1 concentration was found to be the best discriminator between uncomplicated malaria and severe malaria (28) with a sensitivity of 86% (95% CI 71-94) and a specificity of 85% (95% CI 76-92) (**Additional file 7**). As expected the more the disease severity states reflected both ends of the spectrum (e.g. uncomplicated malaria versus cerebral malaria) the better they could be distinguished (25, 28, 29, 32) (**Additional file 7**).

Since cerebral malaria is a complication of *Plasmodium falciparum* malaria and therefore a manifestation of severe malaria, distinguishing cerebral malaria from other forms of severe malaria was difficult as shown by the observation that only the Ang-1 concentration had a predictive value significantly higher than chance (28) with a sensitivity of 72% (95% CI 56-84) and specificity of 66% (95% CI 55-75). The predictive value of the Ang-2 concentration for fatal outcome was found to be as well as plasma lactate in Vietnamese adults with severe malaria (33) and in Malawian children with cerebral malaria (27), and even better in Indonesian adults with severe malaria (34) (**Additional file 7**). In order to increase the discriminative power, two studies used a combination of markers (27, 30). Incorporating the *i)* Ang-2 concentration or *ii*) the Ang-1, Ang-2 and Tie-2 concentrations in a clinical model, comprising age, body condition score, respiratory distress and severe anaemia as other parameters, significantly improved the ability to accurately predict survival or fatal outcome compared to the clinical model alone (c-index (95% CI), p-value) (*i*: 0.78 (0.68-0.82), 0.02; *ii*: 0.79 (0.72-0.84), 0.03) (27). The combination of Ang-2 with CXCL10 and sICAM-1 had a sensitivity of 100% and a specificity of 93% to distinguish survivors from non-survivors) (30).

In addition to the use as biomarker for disease severity in humans infected with *Plasmodium* species, the Ang-1 and Ang-2 concentration may also be used for distinguishing cerebral malaria from other diseases involving the central nervous system (CNS) in critically ill patients. Distinguishing cerebral malaria from other febrile diseases affecting the CNS is challenging, but can have major treatment implications. Ang-1 was found to differentiate between cerebral malaria, diagnosed by malaria specific retinopathy, and other febrile CNS affecting diseases with a sensitivity and specificity of 88% (95% CI 69-96) and 87% (95% CI 73-94), respectively (**Additional file 7**) (29).

Ang-1 and Ang-2 levels do not correlate with parasitaemia, sequestration and erythrocyte rosetting

Assuming that Ang-1 and Ang-2 concentrations and parasitaemia, parasite biomass, sequestration and erythrocyte rosetting all reflect disease severity during malaria, a tight correlation between them may be anticipated. However, no correlation was found between concentrations of Ang-1 and Ang-2 or Ang-2/Ang-1 ratio and sequestration in cerebral malaria in brain autopsies (33), microvascular obstruction measured with an Orthogonal Polarization Spectral device in the rectal mucosa (47) or rosetting as determined by IgG antibody levels to the infected erythrocyte surface of *ex vivo* isolates and corrected for disease severity (50). Parasite biomass as measured by *Pf*HRP2 was correlated with Ang-2 in *P. falciparum* (34, 44, 47), but not as measured by PvLDH and pLDH in *P. vivax*. (44). 3 studies could not find a correlation between parasitaemia and Ang-1 and Ang-2 in *P. falciparum* (25, 33, 34) infected children and adults. However, 3 studies did observe a correlation between Ang-2 and the parasitaemia in *P. falciparum* and *P. vivax* infected children and adults (28, 44) or the Ang-2/Ang-1 ratio (33). Taken together, the relation between Ang-1 and Ang-2 and parasite abundance related markers varies between the studies.

Aim ii: Adjuvant therapy next to antimalarial treatment

Ang-1 and Ang-2 levels are influenced by anti-inflammatory therapies

Ang-1 and Ang-2 concentrations have both been suggested as adjuvant treatment targets in malaria (15, 54), but so far interventional studies in mice models or in humans have not been performed. Several studies used Ang-1 and Ang-2 as biomarkers for endothelial cell activation after an intervention aiming at another target.

Adjuvant treatment strategies

In distinct included studies *P. berghei-Anka* infected mice and/or *P. falciparum* infected humans received next to artesunate either a functional S1P receptor antagonist: FTY720 or LX2031 (38), a PPAR-γ agonist: rosiglitazone (43) or inhaled nitric oxide (40-42). FTY720 and LX2931 influence the homeostasis of inflammation and of vascular endothelium activity (55-57). Rosiglitazone increases CD36-mediated phagocytosis of infected erythrocytes, has anti-inflammatory properties and improved survival in a cerebral malaria model previously (58, 59). NO inhibits the exocytosis of Weibel-Palade bodies (60), organelles in endothelial cells that store both vWF and Ang-2. NO levels and cofactors required for NO synthesis are known to be reduced during *P. falciparum* infection (34, 35, 45) and higher NO levels are associated with lower Ang-2 levels (34), whereas NO inhibitors positively correlate with Ang-2 (46). Several processes contributing to the decreased NO levels during malaria have been studied, such as substrate limitation and increase in NO inhibitors (35), but these are beyond the scope of this review.

Adjuvant treatment in mice

No change in Ang-1 levels have been observed in mice treated with FTY720, 1, 3 or 5 days after infection nor an increase in survival in treated mice 3 or 5 days after infection (38). The survival rates did also not increase in both prophylactic and post-infection LX2931-treated mice. However, a higher Ang-1 concentration and survival rate were observed in mice receiving prophylactic treatment with either FTY720 or iNO compared to placebo treated mice (38) (**Additional file 6**). Survival rates increased also in mice receiving adjuvant treatment with rosiglitazone or iNO 3 or 5 days after infection compared to artesunate only treated mice (40). Rosiglitazone prevented the decrease in the Ang-1 concentration that was seen in mice treated with artesunate only (43). The Ang-2/Ang-1 mRNA levels, measured in homogenized brain tissue of mice, were significantly lower in mice prophylactically treated with iNO (40) or post-infectiously with rosiglitazone compared to mice treated with artesunate only (43). These results demonstrated an association between prophylactic therapy, decreased endothelium activation and increased survival rates.

When rosiglitazone was given to *P. berghei-Anka* infected mice with one Ang-1 allele deleted (Ang-1^{del}), production of the Ang-1 levels was 30%-50% compared to wildtype mice 5-6 days post-infection (43). An increase in survival was only seen in wildtype mice treated with rosiglitazone and not in the Ang-1^{del} mice treated with rosiglitazone (43). This emphasizes that proper Ang-1 production apparently increases survival in a direct or indirect way, and therefore, a role of Ang-1 in the pathogenesis during malaria is likely.

Adjuvant treatment in humans

iNO as adjuvant therapy for severe malaria and rosiglitazone for uncomplicated malaria was also studied in humans. In a total of 272 Ugandan children adjuvant iNO was given in 2 studies with either nitrogen (42) or air (41) as placebo treatment. No significant differences in Ang-1, Ang-2 concentrations nor survival between the iNO treated and placebo groups was found in either study. In rosiglitazone treated patients the Ang-2/Ang-1 ratio was significantly lower compared to patients receiving placebo as measured 3 days after treatment was started and parasite clearance was significantly enhanced (43). None of the patients died or was admitted to intensive care in both the treatment and control group.

Discussion

The role of Ang-1 and Ang-2 as biomarkers and possible therapeutic targets in severe malaria are evaluated in this systematic review. Decreased Ang-1 and increased Ang-2

concentrations seem to be valuable as biomarker for disease severity and survival. The role of Ang-1 and Ang-2 in follow-up of treatment effectiveness as well as their role as future therapeutic targets are promising, but further studies are warranted. Nonetheless, some discussion points before use as biomarkers or therapeutic targets remain.

Ang-1 and Ang-2 as biomarkers for disease severity

Uncomplicated malaria vs. severe malaria and cerebral malaria

The results indicate that the decreased Ang-1 and the increased Ang-2/Ang-1 ratio are robust biomarkers to distinguish uncomplicated malaria from cerebral malaria (25, 28, 29, 32). However, it is easier to distinguishing uncomplicated malaria from cerebral malaria by a rapid clinical examination procedure as defined by the WHO (cerebral malaria: severe *P. falciparum* with coma (Glasgow coma scale <11, Blantyre coma score <3), or malaria with coma persisting >30 min after a seizure). Nevertheless, Ang-1, Ang-2 and the Ang-2/Ang-1 ratio could be valuable markers if they can identify patients with uncomplicated malaria at risk for progressing to severe disease. The AUROC (area under the receiver operating characteristic) for distinguishing uncomplicated malaria from severe malaria is 0.88 (p=0.001) and the sensitivity and specificity are 86% (95% CI 71-94) and 85% (95% CI 77-92), respectively (28) (**Additional file 7**). This AUROC is higher than for plasma lactate (0.67 (95 CI% 0.57-0.75)) or parasitaemia (0.53 (95% CI 0.45-0.62) as found in an endemic area (61) and the sensitivity higher than in nonimmune travellers (67%) (62). Although the discriminating power of Ang-1 levels is promising, conclusion should be drawn with caution, as only one study in children (N=193) evaluated the AUROC to distinguish uncomplicated malaria from severe malaria (28).

Survivors vs. non-survivors

Ang-1 and Ang-2 levels can be used to distinguish malaria disease severity states, but prediction of outcome might be of more clinical value. Several studies that focussed on clinical outcome demonstrated that the Ang-2 level predicted mortality as well as or better than lactate blood levels in adults and children in Asia and Africa with severe and cerebral malaria (27, 33, 34). Additional prospective studies that evaluate Ang-1 and/or Ang-2 as markers of disease progression and clinical outcome should be performed to confirm these results.

Cerebral malaria vs. other febrile CNS affecting diseases

A marker other than parasitaemia to distinguish cerebral malaria from 'other diseases with involvement of the CNS' might be useful in patients with parasitaemia, as asymptomatic mild parasitaemia is not uncommon in malaria endemic areas (63, 64). Direct and indirect ophthalmoscopy can be used for this aim if retinopathy is observed with certain malaria-

specific characteristics, such as retinal whitening, vessel changes, retinal haemorrhages, and papilledema. This clinical procedure has a sensitivity of 95% and a specificity of 90% for diagnosing cerebral malaria (65). The Ang-1 concentration and the Ang-2/Ang-1 ratio were found to be good discriminators between 'cerebral malaria with retinopathy' and 'other diseases with involvement of the CNS' (**Additional file 7**). However, 'cerebral malaria with retinopathy' is already a selected group within the total group of patients with cerebral malaria, and therefore, it might overestimate the sensitivity and specificity to diagnose cerebral malaria. In addition, the group of patients that will benefit from a marker for cerebral malaria - patients with mild parasitaemia and a non-malaria disease that involves the CNS -, were not represented in both studies that investigated the capacity of angiopoetin levels to distinguish cerebral malaria from 'other diseases with involvement of the CNS' as parasitaemia in the latter group was absent. Nevertheless, to evaluate whether Ang-1 and/or Ang-2 levels can identify these patients might be of clinical value.

Treatment follow-up

Early parameters that indicate whether treatment is effective in malaria patients are valuable as treatment regimens may be reconsidered when patients do not properly recover. In both humans (29, 34, 36, 37, 42, 43) and mice (38-40, 43) recovery from malaria and survival was associated with an increase in Ang-1 and decrease in Ang-2 level (**Additional file 3, 4, 5 and 7**). However, the threshold for an Ang-1 increase or Ang-2 decrease that reflects proper recovery was not investigated. Neither were Ang-1 and Ang-2 levels compared with other clinical or laboratory indices, e.g. fever or parasite clearance, which would be of interest.

Variation between studies

Even though Ang-1 and Ang-2 concentrations could discriminate between disease severity states during malaria, distinct studies found varying levels of sensitivity and specificity (**Additional file 7**). This variation may be due to: *a)* host-related factors, such as genetic differences between study populations and differences in acquired immunity towards *Plasmodium* species infection between study populations, *b)* pathogen-related factors, such as infections with different strains with inherently differences in pathogenicity, and *c)* environmental-related factors, such as differences in access to healthcare that may result in delayed diagnosis or delayed start of proper treatment. In addition, the methods by which Ang-1 and Ang-2 levels are determined are still further developed and currently lack proper standardization. Additional investigations are required before conclusions on the varying levels can be drawn.

Ang-1 and platelet activation

A lower Ang-1 level was found in healthy controls compared to *P. falciparum* infected patients in 1 study which collected blood in CTAD tubes (36). These tubes contain the platelet stabilizing agents citrate, theophylline, adenosine and dipyridamole, which together result in a nearly complete block of *in vitro* platelet activation (66, 67). The studies determining Ang-1 in serum (32) or in venous blood (not further specified) (25) found a higher level Ang-1 in healthy controls compared to *P. falciparum* infected patients. In addition, other studies that determined Ang-1 in malaria patients found a negative correlation between Ang-1 and disease severity. It is remarkable that the only study using tubes that avoid *in vitro* platelet activation found a higher level of Ang-1, which is stored in platelets and released upon activation, in patients than in healthy controls. Platelet activation may occur *in vivo* during malaria as induction of coagulation is proven even in early malaria (68). To exclude altered Ang-1 levels due to *in vitro* platelet activation after blood collection, platelet-poor specimens are preferred to determine platelet activation *in vivo*. 11-dehydrothromboxane B2 is a breakdown product of thromboxane A2 that is produced *in vivo* by activated platelets. Since 11-dehydrothromboxane B2 is excreted in urine, a specimen that is free of platelets, determination of 11-dehydrothromboxane B2 in urine samples may estimate *in vivo* platelet activation most accurately (69).

Lack of consistent associations between Ang-1, Ang-2 and the parasitaemia in P. falciparum

Even though Ang-1 and Ang-2 concentrations correlate with disease severity, the results of the correlation between Ang-1 or Ang-2 concentrations in *P. falciparum* infection and parasitaemia, sequestration or rosetting vary between the studies (25, 28, 33, 34, 44, 47, 50). The lack of a consistent correlation of the Ang-1 concentration, Ang-2 concentration and Ang-2/Ang-1 ratio with parasitaemia may be due to the fact that this parameter is determined in peripheral blood and does not reflect the true parasite load as sequestered parasites are not adequately taken into account. A correlation between *Pf*HRP2, which reflects total parasite load during *P. falciparum* infection and Ang-2 was found in 3 studies (34, 44, 47), but sequestration (47) and rosetting corrected for disease severity (50) did not correlate with Ang-1 and/or Ang-2 concentrations. The correlation between Ang-2 with parasite load but not with sequestration and rosetting might be due to *a)* the fact that *Pf*HRP2 levels (34, 44, 47), measuring sequestration with a OPD device (47) and IgG antibody levels (50) are all markers that may not estimate their biological phenomena similarly, *b)* a temporal dissociation of the determinations (47), *c)* loss of correlation due to the fact that locally observed phenomena –sequestration and rosetting– were compared to a systemic phenomenon –Ang-2 release– (47), or *d)* the infected erythrocytes may activate endothelial cells not by direct, physical contact, but indirectly by inducing other endothelial cell activators, such as cytokines (70, 71). Next to the quantity of parasite biomass, also the *Plasmodium* strain and the host response might contribute to the

variations in Ang-2 increase. Although it should be noted that an included study (50) did not show a relation between Ang-2 and group A-like *Pf*EMP expression if corrected for disease severity. However, the possible differences in other genes were not examined in that study. Taken together, the host-response related marker Ang-2 might be a good or better biomarker for disease severity and treatment follow-up than pathogen-related markers, such as parasite load or parasitaemia.

Ang-2 in P. vivax vs. P. falciparum malaria

Data on Ang-2 levels in *P. vivax* and *P. falciparum* differed between distinct studies: 2 studies found a higher Ang-2 level in *P. falciparum* compared to *P. vivax* (44, 48) and 1 study found a higher Ang-2 level in *P. vivax* compared to *P. falciparum* (49). However, comparison should be done with caution as these studies were performed in different populations: patients in endemic areas (44, 48) versus travellers in non-endemic regions (49) and the parasitaemia was not reported in all studies (49). In addition, the blood samples of *P. vivax* and *P. falciparum* of infected Canadian travellers were processed differently (49), which may have influenced the Ang-2 levels results. Higher Ang-2 levels in *P. vivax* compared to *P. falciparum* are plausible as *P. vivax* is known to induce a stronger cytokine response (4), which was also shown in an included study in adult in an endemic area (48). These cytokines might contribute to endothelial cell activation and therefore to Ang-2 release from Weibel-Palade bodies. Interestingly, in *P. vivax* infected patients Ang-2 levels did not correlate with pLDH and PvLDH, which were proposed as markers that take both peripheral and hidden parasite load into account. Instead, a correlation was found between Ang-2 levels and parasitaemia. This might suggest a cytokine-independent endothelial cell activation, as both hidden and peripheral infected erythrocytes should contribute to the induction of the cytokine response. On the other hand, pLDH and PvLDH are not as well established markers for the parasite biomass in *P. vivax* infected patients (72) as *Pf*HRP2 is in *P. falciparum* infected patients, and therefore, no firm conclusions can be drawn yet. Additional research is necessary to elucidate the differences in pathogenesis between *P. vivax* and *P. falciparum* malaria and the role of endothelium activation during both *Plasmodium* infections.

Clinical implementation

Although Ang-1 and Ang-2 levels can be used to differentiate between disease severity states in malaria disease and might predict disease progression, their use in routine clinical practice is not possible yet. Malaria disease mainly occurs in resource-poor settings where it is not possible to determine Ang-1 or Ang-2 with complex laboratories techniques. If Ang-1 and Ang-2 are shown to be robust biomarkers for disease progression, new simple rapid-point-of-care tests need to be developed for clinical use, as was done before for CRP (73) and for multiple haematological, biochemical and coagulation parameters (74).

Furthermore, the clinical consequences of a marker for disease progression are currently limited as no adjuvant treatment is available yet next to medication to eradicate the parasite. However, development of adjuvant treatment and biomarkers to indicate which patients should receive adjuvant treatment go hand in hand and multiple adjuvant treatment strategies are currently investigated (as reviewed by (75, 76)).

The possible role of Ang-1 and Ang-2 in adjuvant treatment regimens for *P. falciparum* malaria

Studies using Ang-1 or Ang-2 as therapeutic target were only performed in sepsis, which shares many similarities with severe malaria caused by a *P. falciparum* infection. Both sepsis and malaria are characterized by cytokines release, coagulation alterations and endothelial cell activation (15, 18, 23). In sepsis Ang-1 was the mostly studied angiopoietin in interventional murine models (as reviewed by (24)). Ang-1 was found to be protective in endotoxin-induced shock or hyperoxia-induced lung injury models. However, most studies used Ang-1 treatment prior to infection which is not useful in a clinical setting for sepsis nor for malaria. Some of the studies giving Ang-1 after development of sepsis or acute lung injury found no reduction in mortality while others reported a positive effect (24). In addition, it should be noted that *P. berghei* ANKA infected CB57BL/6 mice, which are widely used as a model for human cerebral malaria, do show histopathological features that differ substantially from cerebral malaria in humans. Human cerebral malaria is characterized by sequestration, whereas inflammation is central in murine cerebral malaria and the amount of sequestration limited (77). These differences may contribute to the contrasting results in outcome between iNO treated *P. berghei* ANKA infected mice and P. falciparum infected humans. This is supported by the observation that most successful interventions in murine cerebral malaria show no significant effect in human cerebral malaria (as reviewed by (78)). More investigations on the therapeutic effect of post infection use of Ang-1 in different disease models are needed before solid conclusions about the therapeutic properties of Ang-1 and/or Ang-2 can be drawn.

The complexity of the host response to the *Plasmodium* species infections possibly plays a role in the lack of effect of adjuvant treatment when given post-infection. During malaria disease in humans and mice the immune system, coagulation system and the activity of endothelial cell are all systematically altered. These processes are complex and interweaved which each other (see **Figure 2**). Providing even multi-targeted adjuvant treatment with FTY720 or iNO at the moment that these regulatory systems are already systemically activated and upregulating each other, may have too limited effect to halt these feed-forward stimulated processes. Multi-targeted adjuvant treatment such as rosiglitazone, that - unlike FTY720 or iNO - also was found to have an effect on Ang-1 and

Ang-2 levels and survival when administered post-infection, may however interfere with all the distinct regulatory systems in a way that reduces morbidity and mortality (43) (see **Figure 2**), although further studies are definitely needed.

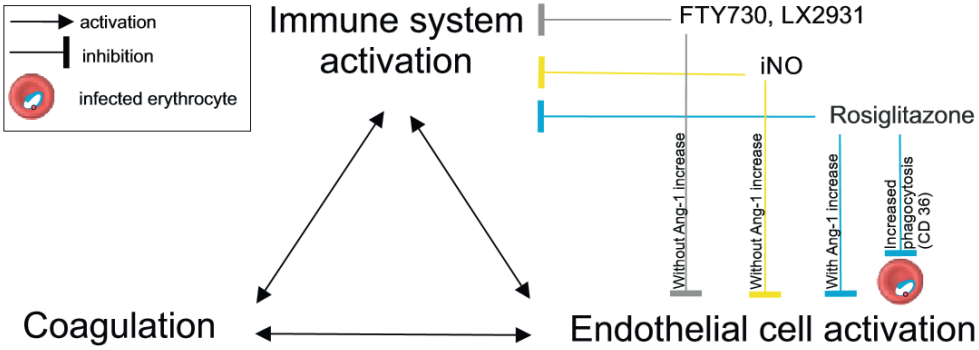

Figure 2 – Interactions between different regulatory systems and the effects of therapeutics on the determinants

Coagulation, endothelial cell activation and immune system activation are processes that stimulate each other. FTY730, LX2931 and NO inhibit the immune system activation and endothelial cell activation. Rosiglitazone inhibits immune system activation, endothelial cell activation and upregulates CD36 which result in increased phagocytosis of the infected erythrocytes.

Conclusion

Ang-1 and Ang-2 concentrations correlate with disease severity and survival in malaria and showed a good discriminative power to distinguish disease severity states with predictive values equal to, or higher than, the currently used biomarkers plasma lactate and parasitaemia. However, different disease severity states were compared in the distinct studies and not every distinction has clinical impact. The possible ability of Ang-1 and Ang-2 concentrations to distinguish cerebral malaria from other febrile CNS affecting disease and to predict those patients progressing from uncomplicated malaria towards severe malaria will have clinical value and should be studied in more detail before it can be used in routine practice.

For further optimization of treatment of patients with severe disease, multi-targeted adjuvant treatment regimens are needed. Agents interfering with Ang-1 and or Ang-2 may be included in such adjuvant therapy as their role as therapeutic target is promising. However, it is conceivable that agents interfering with multiple pathways are necessary, since multiple regulatory systems are consecutively activated during malaria infection.

Declarations

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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Authors' contributions
GMdJ, JJvH and PJvG conceived of the study. GMdJ and JJS performed the literature search and selected the relevant articles, with JJvH acting as third reviewer in case of disagreement. JJvH, PJvG and AV participated in revising the manuscript. All authors read and approved the final manuscript.

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Additional data

Additional file 1 – Search strategy
Search performed on 15 June 2016

Database	Number of publications	Number of publications after deduplication
Embase.com	170	166
Medline (ovid)	45	9
Web-of-science	112	32
Scopus	128	2
Cochrane	5	0
cinahl (ebsco)	5	1
lilacs	3	2
scielo	3	0
ProQuest	8	8
Google scholar	100	58
Total	**579**	**278**

Table 1: Number of publications extracted from the indicated databases

Syntaxis of search strategies

Embase.com
(angiopoietin/exp OR 'angiopoietin 1'/exp OR 'angiopoietin 2'/exp OR 'angiopoietin receptor'/exp OR 'angiogenic protein'/exp OR 'angiogenic factor'/exp OR (angiopoietin* OR angiogenic* OR angpt* OR 'ang 1' OR 'ang 2' OR ang1 OR ang2 OR (tie NEAR/3 (receptor* OR protein*)) OR tek OR tie1 OR tie2 OR (Tyrosine* NEAR/3 kinase* NEAR/3 immunoglobulin*))) AND (malaria/exp OR Plasmodium/exp OR 'antimalarial agent'/de OR (malaria OR Plasmodium OR antimalari*))

Medline (ovid)
(exp angiopoietins/ OR exp "Receptors, TIE"/ OR "Angiogenic Proteins"/ OR (angiopoietin* OR angpt* OR "ang 1" OR "ang 2" OR ang1 OR ang2 OR (tie ADJ3 (receptor* OR protein*)) OR tek OR tie1 OR tie2 OR (Tyrosine* ADJ3 kinase* ADJ3 immunoglobulin*))) AND (exp malaria/ OR exp Plasmodium/ OR "Antimalarials"/ OR (malaria OR Plasmodium OR antimalari*))

Cochrane
((angiopoietin* OR angiogenic* OR angpt* OR 'ang 1' OR 'ang 2' OR ang1 OR ang2 OR (tie NEAR/3 (receptor* OR protein*)) OR tek OR tie1 OR tie2 OR (Tyrosine* NEAR/3 kinase* NEAR/3 immunoglobulin*))) AND ((malaria OR Plasmodium OR antimalari*))
Web-of-science
TS=(((angiopoietin* OR angiogenic* OR angpt* OR "ang 1" OR "ang 2" OR ang1 OR ang2 OR (tie NEAR/3 (receptor* OR protein*)) OR tek OR tie1 OR tie2 OR (Tyrosine* NEAR/3 kinase* NEAR/3 immunoglobulin*))) AND ((malaria OR Plasmodium OR antimalari*)))

Scopus
TITLE-ABS-KEY(((angiopoietin* OR angiogenic* OR angpt* OR "ang 1" OR "ang 2" OR ang1 OR ang2 OR (tie W/3 (receptor* OR protein*)) OR tek OR tie1 OR tie2 OR (Tyrosine* W/3 kinase* W/3 immunoglobulin*))) AND ((malaria OR Plasmodium OR antimalari*)))

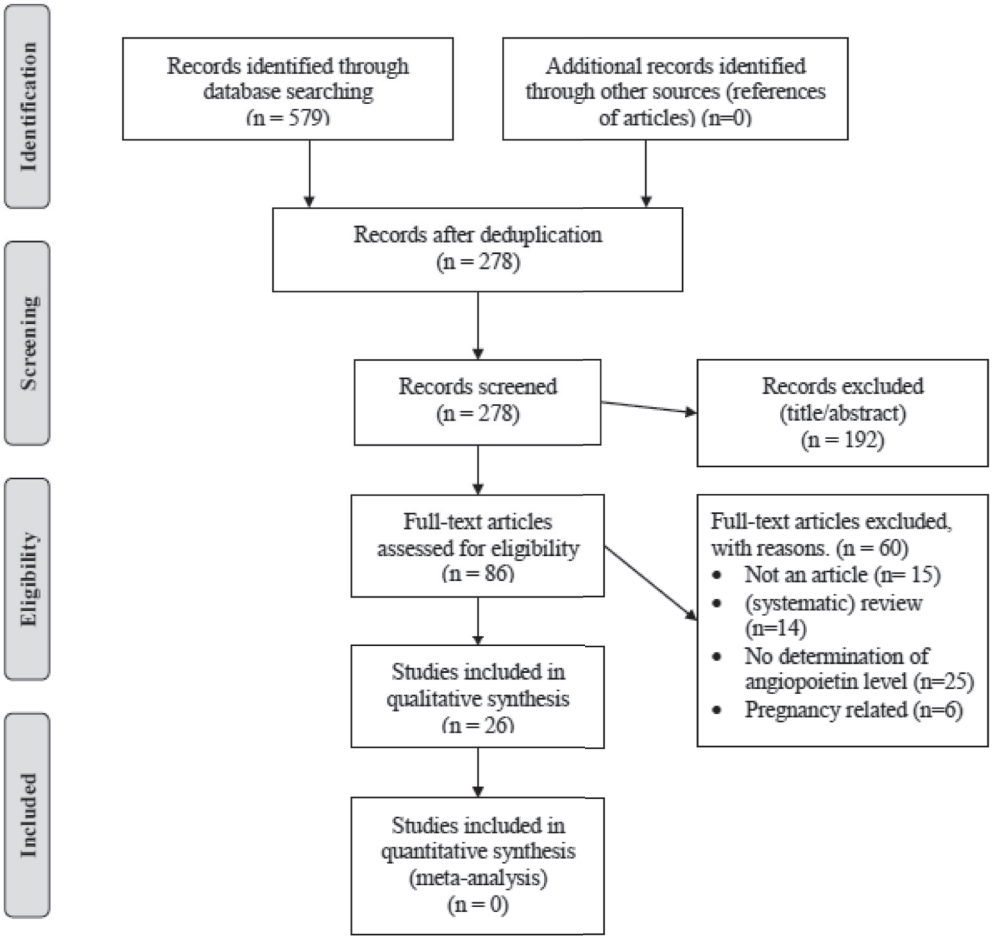
cinahl (ebsco)
(MH "Angiogenic Proteins" OR (angiopoietin* OR angpt* OR "ang 1" OR "ang 2" OR ang1 OR ang2 OR (tie N3 (receptor* OR protein*)) OR tek OR tie1 OR tie2 OR (Tyrosine* N3 kinase* N3 immunoglobulin*))) AND (MH malaria+ OR MH "Antimalarials" OR (malaria OR Plasmodium OR antimalari*))

Google scholar
angiopoietin|angiopoietins|angiogenic|angpt|angpt1|angpt2|angpt3|angpt4|"ang|tie 1|2|3|4"|ang1|ang2|ang3|ang4|"tek|tie1|tie2|tie receptor|protein"|"Tyrosine kinase immunoglobulin" malaria|Plasmodium|antimalaria

lilacs

scielo
(angiopoietin* OR angiogenic OR angpt* OR ang1 OR ang2 OR ang3 OR ang4 OR tie1 OR tie2 OR "tie receptor" OR "tie protein" OR "Tyrosine kinase immunoglobulin") AND (malaria OR Plasmodium OR antimalaria*)

ProQuest
(ti(angiopoietin* OR angiogenic OR angpt* OR ang1 OR ang2 OR ang3 OR ang4 OR tie1 OR tie2 OR "tie receptor" OR "tie protein" OR "Tyrosine kinase immunoglobulin") OR ab(angiopoietin* OR angiogenic OR angpt* OR ang OR tie OR ang1 OR ang2 OR ang3 OR ang4 OR tie1 OR tie2 OR "tie receptor" OR "tie protein" OR "Tyrosine kinase immunoglobulin")) AND (ti(malaria OR Plasmodium OR antimalaria*) OR ab(malaria OR Plasmodium OR antimalaria*))



From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *Preferred Reporting Items for Systematic Reviews and Meta-Analyses:* The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

Additional file 2 – Flow diagram: selection of relevant artides

	References	Population, N Study type Age in years	Ang-1 (ng/ml)	Ang-2 (ng/ml)	Ang2/Ang1 ratio
Pf.	Conroy et al. (2009)	Thailand, N=193 Prospective cohort study UM: 26 (13-50) SM: 24 (14-59) CM: 26 (14-61)	UM > CM > SM	UM < SM and CM	UM < SM and CM
	Jain et al. (2011)	India, N=183 Retrospective cohort study HC: 25 (14-32) MM: 19 (12-36) CM-S: 25 (12-40) CM-NS: 25 (13.5-37.5)	HC (12.4) > MM (4.6) > CM S (2.8) and CM NS (2.4)	HC (0.34) < MM (0.97) < CM S (3.6) < CM NS (7.8)	HC (0.02) < MM (0.16) < CM S (1.42) < CM NS (2.69)
	Brouwers et al. (2015)	Indonesia, N=52 Prospective case-control study HC: 24 (22-29) Malaria:13 (6-26)	HC < malaria Further increase after start treatment	HC < malaria Decline after start treatment	HC = malaria
	MacMullin et al. (2012)	Canada, N=186 Retrospective cohort study 0-55+	FB = CB	FB = CB; *Pv.* (0.34) < *Pf.* (1.1)* FB: *Pv.* (0.31) < *Pf.* (1.0)* CB: *Pv=Pf*	
Pv./Pf	Barber et al. (2015)	Malaysia, N=266 Prospective cohort study Controls: 35 (23-44) Non-severe *Pv.*:24 (18-29) Severe *Pv.*: 39 (30-52) Non-severe *Pf.*: 25 (17-39) Severe *Pf.*: 33 (19-45)	Not determined	HC (1.2) < non-severe *Pv.* (4.6) < severe *Pv.* (8.9) non severe *Pf.* (3.2) < severe *Pf.* (8.4)	

Additional file 3 – Studies on children and adults showing significant differences in Ang-1 and Ang-2 levels in *Plasmodium falciparum* and *Plasmodium vivax* infection

Variables were presented as median; inter quartile range (IQR) unless otherwise specified; **CB**, Canadian born; **CM,** cerebral malaria (WHO definition); **FB**, foreign born; **HC**, healthy control; **MM**, Mild malaria, defined as parasitemia of < 25,000 parasites/µl and no evidence of severe malaria and no past history of mental/metabolic illness, tuberculosis, meningitis, or accidental head injury; ***Pf.***, *Plasmodium falciparum;* ***Pv.***, *Plasmodium vivax*; **S**, survivors, **SM**, severe malaria; **UM**, uncomplicated malaria. * Mean

	References	Population, N Study type Age in years	Ang-1 (ng/ml)	Ang-2 (ng/ml)	Ang2/Ang1 ratio
Pf.	Yeo et al. (2008, 2010 (ref. 35))	Indonesia, N=146 Prospective cohort study HC: 25 (18–44)** MSM: 28 (18–56)** SM: 29 (18–56)**	Not determined	HC (2.3) < MSM (5.3) < SM (15.0)* SM S < SM NS (admission levels) Decrease in SM S (2.7 ng/ml/24h) during treatment but not in SM NS	
	Lovegrove et al. (2009)	Thailand, N=60 Prospective cohort study HC: 32 (25-48)* UM: 22 (14–63* CM: 25 (17–50)*	HC (378) > UM (82.25) > CM (3.51)*	HC (0.0089) < UM (1.84) < CM (6.19)*	HC ($3*10^{-5}$) < UM (0.017) < CM (3.47)*
	Prapansilp et al. (2013)	Vietnam, N=63 SM, CM patients, 23 were used for autopsia UK, N=18 autopsy controls Retrospective case-control study Controls: 58.5 (36-74.5)* Cases: 32 (26-43)*	Lower incidence of high expression in neurons/astroglial cells in non-malaria vs SM and CM, not in vessels.	Lower incidence of high expression in neurons and lower expression in astroglial cells in non-malaria vs SM and CM. S (15) < NS (28)*	S (30.6) < NS (42.5)*
	Hanson et al. (2015)	Bangladesh, India, N=142 Prospective observational study 35 (25–45)*	Not determined	HC (1.6) < SM (19.1)* S (14.9) < NS (21.9)* comatose patients < not comatose patients	
Pv./Pf	Yeo et al. (2010) (ref. 48)	Indonesia, N=143 Prospective observational study HC: 25 (18–44)** *Pf.*28 (18–56)** *Pv.* 23 (18–35)**	Not determined	HC (2.3) <uncomplicated *Pf.* (5.3) < uncomplicated *Pv.* (7)*	
Pv.	Gomes et al. (2015)	Brazil, N=80 Retrospective case-control study Case: 38.5 (25-55)* Control 39 (28-49)*	Non-severe (19.9) > severe (12)* Normal platelet count > thrombocytopenia	Non-severe (5.9) < severe (8.2)* Normal platelet count < thrombocytopenia	Non-severe (0.3) < severe (0.8)* Normal platelet count < thrombocytopenia

Additional file 4 – Studies on adults showing significant differences in Ang-1 and Ang-2 levels in *Plasmodium falciparum* and *Plasmodium vivax* infection

CM, cerebral malaria (WHO definition); **HC**, healthy control; **MSM**, moderate severe malaria, defined as fever within the preceding 48 h, with >1,000 asexual *P. falciparum* parasites/L, with no WHO warning signs or criteria for SM; **NS**, non-survivors; ***Pf.***, *Plasmodium falciparum;* ***Pv.***, *Plasmodium vivax*; **S**, survivors, **SM**, severe malaria; **UM**, uncomplicated malaria. *Median (IQR), **Mean (range).

References	Population; N Study Age in years	Ang-1 (ng/ml)	Ang-2 (ng/ml)	Ang2/Ang1 ratio
Lovegrove et al. (2009)	Uganda, N=164 Prospective cohort study HC: 7 (3.2–12)* UM: 7 (3.0–12)* CM: 5.4 (3.2–12)*	HC (64.4) > UM (25.0) > CM (9.0)* CM S (9.1) > CM NS (0.39)	HC (0.068) < UM (0.28) < CM (0.83) *	HC (0.0015) < UM (0.013) < CM (0.14)* CM S (0.13) < CM NS (2.6)
Conroy et al. (2010)	Malawi, N=123 Retrospective case-control study UM: 3.3 (0.7-8.0)* NMC: 2.2 (0.1-9.1)* CM-N: 4.3 (1.2-13.3)* CM-R: 2.8 (1.1-6.8)*	UM and NMC > CM-R CM-N > CM-R Admission level CM-R survivors < 28 days post-treatment	UM and NMC < CM-R CM-N < CM-R Admission level CM-R > 28 days post-treatment	UM and NMC < CM-R CM-N < CM-R Admission level CM-R > 28 days post-treatment
Erdman et al. (2011)	Uganda, N=156 Retrospective case-control study UM: 4.4 (2.1-8.1)* CM: 3.0 (1.5-4.3)* SMA; 1.3 (0.9-2.0)*	Not determined	UM < CM, SMA S < NS: CM S < CM NS SMA S < SMA NS	
Conroy et al. (2012)	Malawi, N=155 Retrospective case-control studyCM-N: 3.6 (2.6-6.6)* CM-R: 2.7 (2.1-3.7)*	CM-N > CM-R	CM-N < CM-R CM-R S < CM-R NS	

Additional file 5 – Studies on children showing significant differences in Ang-1 and Ang-2 levels in *Plasmodium falciparum* infection

References	Population; N Study Age in years	Ang-1 (ng/ml)	Ang-2 (ng/ml)	Ang2/Ang1 ratio
Moxon et al. (2014)	Malawi, N=226 Prospective case-control study HC: 4.6 (3.7–5.7)** UM: 4.7 (4.1–5.3)** CM: 4.1(3.3–5.2)**	Not determined	HC (0.23) < UM (0.58), CM (1.54)*** (enrolment); Still increased after 4 weeks in UM (0.32) compared to HC (0.23) 50% patients lost-to-follow-up	
Weinberg et al. (2014)	Tanzania, N=211 Prospective cohort study HC: 8 (7 –8)* MSM: 5 (4–6)* SM: 5 (4–8)* CM: 4 (4–5)*	Not determined	HC (1.1) < MSM (2.0) and SM (2.3) < CM (3.7)	
Abdi et al. (2014)	Kenya, N=213 Retrospective cohort study UM: 2.7 (1.8-3.3)* SM: 3.9 (3-6.2)*	Not determined	UM < SM	
Rubach et al. (2015)	Tanzania, N=259 Prospective observational study HC:12.8 (2.6–3.2)** UM: 3.7 (3.3-2.1)** CM: 4.3 (4.6)** NMC: 2.4 (1.9-2.8)**	Not determined	HC (0.815), UM (1.88), NMC (1.15) < CM (3.45)*	

Continued additional file 5

CM, cerebral malaria (WH's definition); **CM-N,** cerebral malaria without retinopathy; **CM-R**, cerebral malaria with retinopathy; **HC**, healthy control; **MSM,** moderate severe malaria: fever within the preceding 48 h, with >1,000 asexual *P. falciparum* parasites/L, with no WHO warning signs or criteria for SM and a requirement for inpatient parenteral therapy because of an inability to tolerate oral treatment; **NMC**, non-malaria central nervous system conditions*;* **NS**, non survivors; **S**, survivors; **SM,** severe malaria; **SMA**, severe malarial anaemia; **UM,** uncomplicated malaria.

*Median, (IQR), ** Mean (95% CI), *** Geometric mean

	References	Population, N	Intervention	Ang-1 or Ang-2	Ang2/Ang1	Survival rate
Mice, PbA infected	Finney et al. (2011)	C57BL/6, 6-8 weeks, female	FTY720 or LX2931administration i.p. 1 day prior to infection or 1, 3 or 5 days after infection. All mice received artesunate once 5 days after infection.	FTY720: Ang-1, higher in prophylactic treated mice vs untreated mice. No effect in mice treated after infection.		FTY720: increased in mice treated prophylactic or treated 1 day after infection. Not increased when treatment was given 3 or 5 days post-infection. LX2931: no increase in survival.
	Serghides et al. (2011)	C57BL/6, 7-9 weeks, male	Inhalation of NO or placebo (air) 1 day prior to infection or 3 or 5.5 days post infection Nitrite in drinking water day of infection compared with drinking water without additions. All mice treated 3 or 5 days after infection received also artesunate.	Ang-1: higher in mice receiving prophylactic iNO compared to air treated mice.	Lower in mice receiving prophylactic iNO compared to air treated mice.	iNO: increased in both prior to and post infection treated mice compared to control mice. Nitrite: increased in nitrite treated mice compared to control mice.
	Kim et al. (2014)	C57BL/6, C5aR-/-, C5L2-/-, 7-11 weeks, male, female	Infection of all groups of mice.	Ang-1 protein level: Higher in C5aR-/- compared to WT; No difference between C5L2-/- and WT Ang-2 mRNA: Lower in C5aR-/- compared to WT; No difference between C5L2-/- and WT		Increased in C5aR-/- mice compared to WT. No increase in C5L2-/- mice
	Serghides et al. (2014)	C57BL/6, 7-8 weeks, female	Treatment with artesunate and adjuvant rosiglitazone or placebo 3 days post-infection or on onset CM signs (5-6 post infection).	Ang-1: uninfected mice = rosiglitazone+artesunate > artesunate (in blood)	Rosiglitazone < artesunate (in brain homogenates)	Increased in mice receiving rosiglitazone+artesunate compared to artesunate alone
		BALB/C Ang-1del, BALB/C WT (Ang-1 sufficient)	Treatment with artesunate and rosiglitazone or artesunate alone, start day 5.5 post infection.	Ang-1: Ang-1^{del} mice produce 30-50% of the Ang-1 levels of Ang-1 sufficient mice.		Increased in rosiglitazone treated Ang-1 sufficient mice but not in Ang-1del mice.

Additional file 6 – Studies on the effect of anti-inflammatory interventions on Ang-1 and Ang-2 levels during *Plasmodium spp* infections in humans and mice

	References	Population, N	Intervention	Ang-1 or Ang-2	Ang2/Ang1	Survival rate
Humans, *Pf*. infected	Serghides et al. (2014)	Thailand, N=140 Randomized double-blind placebo controlled trial Age in years (mean, (SD)) Rosiglitazone: 26,5 (10.3) Placebo: 26.1 (9,5)	Atovaquone-proguanil+rosiglitazone vs atovaquone-proguanil+placebo		Decreased 3 days after start treatment for rosiglitazone treated humans vs placebo treated humans.	
	Mwanga-Amumpair et al (2015)	Uganda, N=92 Randomized open label clinical trial, Phase II Age in years (mean (SD)): $N_{2:}$ 2.8 (1.9) NO:3.5 (1.9)	Adjuvant iNO or iN_2 treatment (for 48-120h)	Ang-1: Increased in NO group over 48h, not in N_2 group. No intergroup differences Ang-2: Decreased in NO and N_2 group over 48h. No intergroup differences.	Decreased in NO and N_2 group over 48h. No intergroup differences	No intergroup differences.
	Hawkes et al (2015)	Uganda, N=180 Randomized placebo-controlled, blinded trial Age in years (median (IQR)): iNO:2.0 (1.0–3.0); Placebo: 2.0 (1.0–3.0)	Adjuvant iNO or air placebo treatment. (72h)	Ang-2: no significant differences between groups during 72h of hospitalization. Ang-2 at admission S < NS		

Continued additional file 6

Ang-1, angiopoietin-1; **Ang-2**, angiopoietin-2; **Ang-1^{del} mice**, mice with one Ang-1 allele; **CM**, cerebral malaria; **FTY720**: antagonist of Sphingosine-1-phosphate receptor; **iN2**, inhaled nitrogen; **iNO**, inhaled nitric oxide; **i.p.**, intraperitoneal; **NS**, non survivors; ***PbA***, *Plasmodium Berghei* ANKA; ***Pf***. *Plasmodium falciparum*; r**osiglitazone**, PPARγ agonist, antidiabetic drugs in thiazolidinedione class, anti-inflammatory features; **S**, survivors; **WT**, wildtype; **-/-**, knock-out.

Study species	Distinguishing	AUC Ang-1 (95% CI; p-value) Sensitivity (95% CI); specificity (95% CI)	AUC Ang-2 (95% CI; p-value) Sensitivity (95% CI); specificity (95% CI)	AUC Ang-2/Ang-1 (95% CI; p-value) Sensitivity (95% CI); specificity (95% CI)
Yeo et al. (2008, 2010 (ref. 35)) *P. falciparum*	SM S - SM NS		0.84 (0.71-0.96) Higher AUROC than lactate 0.63 (0.41-0.83), comparable AUROC with HRP2 0.86 (0.73-0.94)	
Lovegrove et al. (2009) Thailand – adults *P. falciparum*	UM - CM	1 (1-1; p<0.001) 100 (87-100); 100 (87-100)	0.84 (0.72-0.95; p<0.001) 72 (52-86); 84 (65-94)	1 (1-1; p<0.001) 100 (87-100); 100 (87-100)
Uganda – children P. falciparum	UM - CM	0.79 (0.71-0.86; p<0.001) 70 (58-79); 75(63-83)	0.69 (0.60-0.78; p<0.001) 83 (72-90); 60 (48-71)	0.78 (0.70-0.86; p<0.001) 73 (61-82); 70 (58-79)
Conroy et al. (2009) *P. falciparum*	UM – SM	0.88 (p<0.001) 86 (71-94); 85 (76-92)	0.76; (p<0.001) 69 (53-82); 79 (68-87)	0.86 (p<0.001) 81 (65-90); 83 (72-90)
	UM – CM	0.78 (p<0.001) 71 (61-80); 74(63-83)	0.77 (p<0.001) 76 (66-84); 77 (66-85)	0.82 (p<0.001) 78 (68-86); 83(72-90)
	SM – CM	0.74 (p<0.001) 72 (56-84); 66 (55-75)	0.53 (p=0.663) 47 (32-63); 64 (54-74)	0.60 (p=0.084) 44 (30-60); 83 (74-89)
	UM – SM and CM	- 71 (62-78); 77 (66-85)	- 74 (66-81); 77 (66-85)	- 79 (71-85); 83 (74-89)
Conroy et al. (2010) *P. falciparum*	CM-N - CM-R	0.64 (0.51-0.78; p=0.046) 68 (53-81); 44 (28-62)	0.77 (0.65-0.89; p≤0.05) 71 (55-83); 72 (54-85)	0.74 (0.60-0.87; p=<0.05) 74 (58-85); 66 (47-80)
	UM - CM-R	0.96 (0.93-1; p≤0.05) 94 (80-98); 87 (73-94)	0.65 (0.51-0.79; p=0.036) 63 (45-77); 71 (55-83)	0.96 (0.93-1.0; p=<0.05) 94 (80-98); 95 (83-99)
	CNS - CM-R	0.93 (0.88-0.99; p≤0.05) 88 (69-96); 87 (73-94)	0.71 (0.55-0.84; p≤0.05) 67 (47-82); 71 (55-83)	0.93 (0.87-0.99; p=<0.05) 71 (51-85); 95 (83-99)

Additional file 7 – Ang-1 and Ang-2 as biomarkers for disease severity during human *Plasmodium spp.* infection

Study species	Distinguishing	AUC Ang-1 (95% CI; p-value) Sensitivity (95% CI); specificity (95% CI)	AUC Ang-2 (95% CI; p-value) Sensitivity (95% CI); specificity (95% CI)	AUC Ang-2/Ang-1 (95% CI; p-value) Sensitivity (95% CI); specificity (95% CI)
Erdman et al (2011) *P. falciparum*	S – NS		0.83 (0.75-0.90; p<0.01) 78 (56-93); 79 (68-87)	
Jain et al. (2011) *P. falciparum*	MM – CM NS	0.35	0.95 90; 90	0.90 86; 83
	CM S – CM NS	0.50	0.76 83;64	0.69 66;60
Conroy et al. (2012) *P. falciparum*	CM S - CM NS	0.56 (0.47-0.65; p=0.20)	0.71 (0.63-0.78; p≤0.0001) AUROC not different from lactate 0.67 (0.59-0.78; p=0.002)	
Prapansilp et al. (2013) *P. falciparum*	S – NS		0.73 (0.60-0.85) AUROC not different from lactate 0.67 (0.53-0.81)	0,66 (0.52-0.80) AUROC not different from lactate (0.67 (0.53-0.81)
Gomes et al. (2015) *P. vivax*	All patients		0.67 78; 50	0.74 61; 84
	Platelet count <75000 µL		0.83 88; 67	0.88 75; 91
	Platelet count >75000 µL		0.53 80; 29	0.65 60; 68

Continued additional file 7

CM, cerebral malaria; **CM-N**, cerebral malaria without retinopathy; **CM-R**, cerebral malaria with retinopathy; **CNS**, fever and decreased consciousness, not malaria **MM**, mild malaria; defined as parasitemia of < 25,000 parasites/µl and no evidence of severe malaria and no past history of mental/ metabolic illness, tuberculosis, meningitis, or accidental head injury; **NS**, non-survivors; **S**, survivors; **SM**, severe malaria; **UM**, uncomplicated malaria

Chapter 5

Liver injury in uncomplicated malaria is an overlooked phenomenon: An observational study

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Abstract

Background

Liver injury is a known feature of severe malaria, but is only incidentally investigated in uncomplicated disease. In such cases, drug-induced hepatotoxicity is often thought to be the primary cause of the observed liver injury, and this can be a major concern in antimalaria drug development. We investigated liver function test (LFT) abnormalities in patients with imported uncomplicated malaria, and in Controlled Human Malaria Infection (CHMI) studies.

Methods

Clinical and laboratory data from 484 imported malaria cases and 254 CHMI participants were obtained from the Rotterdam Malaria Cohort database, and the Radboud University Medical Center database (between 2001-2017), respectively. Routine clinical LFTs, clinical profiles, parasite densities, hematological, and inflammation parameters were assessed in 217 patients with imported falciparum malaria upon admission, and from longitudinal data of 187 CHMI participants.

Results

Upon admission, the proportion of patients with imported uncomplicated malaria and elevated liver enzymes was 128/186 (69%). In CHMI, 97/187 (52%) participants showed LFT abnormalities, including mild (64%, >1.0 ≤2.5x upper limit of normal (ULN)), moderate (20%, >2.5 ≤5.0xULN) or severe (16%, >5.0xULN). LFT abnormalities were primarily ALT/AST elevations and to a lesser extent γGT and ALP. LFT abnormalities peaked shortly after initiation of treatment, regardless of drug regimen, and returned to normal within three to six weeks. Positive associations were found with parasite burden and inflammatory parameters, including cumulative inflammatory cytokine responses and oxidative stress markers (r=0.65, p=0.008, and r=-0.63, p=0.001, respectively).

Conclusion

This study shows that reversible liver injury is a common feature of uncomplicated falciparum malaria, most likely caused by an enduring pro-inflammatory response post treatment. The recognition of this phenomenon is of clinical relevance for individual patient care as well as clinical development of (new) antimalarial drugs.

Introduction

Malaria continues to play a critical role in the global infectious disease burden with significant morbidity and mortality, especially in sub-Saharan Africa. International travelers are also at risk in more than 90 countries worldwide, mainly in Africa, Asia, and the Americas (1). Clinical malaria is characterized by a systemic inflammatory response induced by asexual *Plasmodium* parasites. *Plasmodium falciparum* is responsible for most malaria-related deaths globally (1). Severity of disease is determined by multiple factors, including parasite species and timing of antimalarial treatment (2). The majority of severe falciparum malaria manifestations are most likely related to cytoadherence of parasitized red blood cells (RBCs) to the vascular endothelium, to each other (platelet-mediated agglutination) (3), or to uninfected erythrocytes (rosetting) (4, 5). The subsequent sequestration in the microvasculature results in decreased local oxygen supply and a disturbed metabolism, eventually leading to vital organ failure.

Hepatic dysfunction and jaundice are common features of severe malaria (6-9). Histopathological changes in the liver range from hepatocyte necrosis, granulomatous lesions, Kupffer cell hyperplasia, malarial pigmentation, cholestasis, monocyte infiltrations to malarial nodules (7, 10, 11). These complications can contribute significantly to liver failure and other systemic complications (9, 12, 13). The underlying pathogenesis of liver damage is largely unknown. Furthermore, liver involvement has only been incidentally investigated in uncomplicated malaria (14-16).

Previously, we regularly observed pronounced elevations in liver function tests (LFTs) often shortly upon initiation of curative treatment in patients with imported uncomplicated malaria. We hypothesized that liver injury might arise through a mechanism other than drug-induced hepatotoxicity, or the substantial sequestration observed in severe disease (4, 17). Understanding this clinical feature of uncomplicated malaria could support clinicians in drug-related decision making, and in particular drug development, where frequency of adverse hepatic reactions is a leading concern. Here, we performed a retrospective analysis to determine the frequency of LFT abnormalities in patients with imported uncomplicated malaria, and used longitudinal prospective data of controlled human malaria infection (CHMI) studies to further investigate the significance, dynamics and pathophysiological basis of liver injury.

Methods

Study design and population

Clinical and laboratory data from 484 imported malaria cases and 254 individuals participating in 17 CHMI studies were obtained from the Rotterdam Malaria Cohort database, and the Radboud University Medical Center database, respectively. The Rotterdam Malaria Cohort consists of patients diagnosed with imported malaria who presented at the Rotterdam Harbour Hospital or the Erasmus University Medical Center between 2002 and 2017. For this study, clinical and laboratory data from patients with imported *P. falciparum* malaria were selected (**S1 figure**). Patients with a known history of liver disease, or concomitant infections at admission were excluded (e.g. hepatitis B, HIV, schistosomiasis, urinary tract infections, and respiratory tract infections). Furthermore, patients that received antimalarial treatment prior admission were also excluded. Data on concomitant medication use other than antimalarial treatment, antipyretic therapy (e.g. acetaminophen) or information on (chronic) alcohol intake were not available. Because of the retrospective nature of the analysis and use of anonymized data, ethical approval or informed consent was not necessary, as stated in the Medical Research involving Human Subject Act (WMO).

Healthy malaria-naïve male and female participants aged 18-35 years were recruited for the CHMI studies between 2001 and 2016. Extended screening included hematology and biochemistry parameters, serology for asexual stages of *P. falciparum,* and hepatitis B, C and HIV, as previously described (16, 18). All study participants provided written informed consent at screening visit. All clinical trials were approved by the Radboudumc Committee on Research Involving Human Subjects (CMO), the Central Committee on Research Involving Human Subjects (CCMO) of the Netherlands, or the Western Institutional Review Board (WIRB). The studies were conducted according to the principles outlined in the Declaration of Helsinki and Good Clinical Practice standards, and registered at ClinicalTrials.gov. Study EHMI-3, conducted in 2001, was not registered in an online database such as ClinicalTrials.gov.

Controlled Human Malaria Infection

CHMI participants were all challenged by bites of malaria-infected mosquitoes or direct venous inoculation of sporozoites/blood stage parasites. The challenges were conducted with different parasite clones: NF54 (airport malaria) (19), 3D7 (clone of NF54) (20), NF135 (Cambodia) (21), and NF166 (Guinea, West Africa) (22). Daily blood sampling included parasitological assessment (thick blood smears and qPCR) and/or safety laboratory parameters.

Laboratory investigations

All clinical laboratory data were assessed by standard hematological and biochemical tests on peripheral blood specimens. Biochemical liver tests included: aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (γGT), alkaline phosphatase (ALP), lactate dehydrogenase (LD), and total bilirubin. Reference laboratory values for AST were <30 or <35 U/L (female or male); ALT <35 or <45 U/L (female or male); γGT <40 or <55 U/L (female or male); ALP <100 or <115 U/L (female or male); total bilirubin <17 μmol/L; LD <250 U/L (**S2 table**).

Inflammatory cytokines and oxidative stress analysis

Inflammatory cytokines (IL-1β, IFN-α, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33) were determined in study CHMI-trans1 using flow-based multiplex (Legendplex). For the oxidative stress analysis, TaqMan gene expression Assays (Applied Biosystem) were used to measure antioxidant gene expression levels for Superoxide dismutases (SOD1 and SOD2), Nitric oxide synthase (NOS3), Catalase (CAT), Peroxiredoxin (PRDX1) and Glutathione S-transferase Kappa (GSTK1) from whole blood by using qPCR (**S1 Material**). The cumulative anti-oxidant and cytokine responses were determined through transformation of data into a Z-score (each value subtracted by the mean of the group and divided by the SD of the group).

Definitions

Liver function test (LFT) abnormalities were graded using an adaptation of the World Health Organization (WHO) Adverse Event Grading System 2003: **mild** LFT elevations (>1.0 ≤ 2.5x upper limit of normal (ULN)), **moderate** (>2.5 ≤5.0xULN), and **severe** (>5.0XULN) elevations. Imported malaria patients were classified as having uncomplicated malaria by absence of WHO criteria 2014(23) for severe *P. falciparum* malaria on admission or during hospitalization. Abnormal bilirubin levels were not included in the incidence or grading of LFT abnormalities, since the bilirubin increases found in the imported malaria cases most likely reflect haemolysis and could possibly bias the elevated liver enzyme prevalence in imported malaria. Similarly, lactate dehydrogenase (LD) was not included in the incidence or grading of LFT abnormalities, due to its strong relation to haemolysis and low liver specificity. Patients with imported malaria were considered partially-, semi-, or non-immune. In short, patients born in a malaria-endemic country living in the Netherlands were considered partially immune. Patients that were born and still residing in a malaria-endemic area were presumed semi-immune. All other patients were considered non-immune (24). Data on previous episodes of malaria were not available.

The parasite load was estimated by measuring the area under the curve of parasitaemia over time, and computed by GraphPad Prism 5 (2009, San Diego California USA).

Statistical analysis

All data were analyzed with GraphPad Prism 5 or SPSS software version 24 (IBM Inc., Chicago, IL, USA). Descriptive analyses are presented as percentages, mean with standard deviation, or median with inter-quartile range. Mann Whitney U test was used for comparison of non-normal continuous data or independent groups/samples. Chi square test or Fisher's exact test was used for comparison of qualitative variables and for determining association between various variables. Correlation between different parameters was assessed by Spearman's rho. A P-value of < 0.05 was considered as statistically significant.

Results

The general characteristics on admission of the 217 included patients with imported *P. falciparum* malaria are shown in **table 1** and **S1 table**. On admission day, LFTs were increased in 128/186 (69%) of patients with uncomplicated falciparum malaria (**table 1**) and 27/31 (87%) in severe disease (**S1 table**). In uncomplicated disease, abnormalities appeared to be mild in 93/186 (50%), moderate in 26/186 (14%), and severe in 9/186 (4.8%) of the cases. The median ALT of all uncomplicated malaria patients with LFT abnormalities was 48 U/l (range 15-242 U/l, IQR 34-68 U/l), and the median AST was 41 U/l (range 9-326 U/l, IQR 31-63 U/l). The median γGT was 71 U/l (range 20-447 U/l, IQR 49-113 U/l), the median ALP was 83 U/l (range 36-265 U/l, IQR 64-101 U/l), the median total bilirubin was 22 μmol/l (3-115 μmol/l, IQR 17-43 μmol/l), and the LD was 286 μmol/l (range 52-656 μmol/l, IQR 113-346 μmol/l). No statistically significant differences were found between ages, gender, or immune status in patients with- or without LFT abnormalities. Furthermore, LFT abnormalities appeared in all treatment groups (**table 1**). In a sub-group analysis of the malaria cohort, increases in LFT abnormalities were also found in 23/50 (46%) of the patients with a *Plasmodium vivax* infection (**S1 table**). No significant differences in other parameters than in LFTs between cases with and without LFT abnormalities were observed (**S1 table**).

To better define the dynamics and basis of liver injury, we used data of CHMI studies, which entails extensive screening to minimise confounding effects. Longitudinal data of 17 CHMI studies with a total of 254 participants with patent parasitaemia, showed that LFTs were elevated in 97/187 (52%) of the participants with LFTs measured during infection (**table**

	No LFT# (n=58)	LFT# (n=128)	p-value
Age	37 (29-50)	39 (30-47)	0.960
Gender			0.390 [a]
female	18 (31%)	32 (25%)	
male	40 (69%)	96 (75%)	
Immunity			0.270 [a]
Unknown	3 (5%)	1 (1%)	
Non-immune	27 (47%)	57 (45%)	
Partially	26 (45%)	65 (51%)	
Semi	2 (3%)	5 (4%)	
Parasitaemia/µL	7.611 (1.582-44.550)	5.466 (1.146-47.100	0.811
AST (U/L)	21 (17-26)	41 (31-63)	<0.001
ALT (U/L)	25 (19-30)	48 (34-68)	<0.001
γGT (U/L)	30 (20-37)	71 (49-113)	<0.001
Alkaline phosphatase (U/L)	65 (57-79)	83 (64-101)	<0.001
Bilirubin (total) (µmol/L)	21 (10-28)	22 (15-31)	0.039
LD (U/L)	275 (196-304)	286 (113-346)	0.181
Haemoglobin (mmol/L)	8.1 (7.4-8.9)	8.7 (7.7-9.4)	0.006
Leukocytes (x10^9/L)	4.9 (4.0-5.8)	4.9 (3.6-6.4)	0.863
Lymphocytes (x10^9/L)	0.9 (0.6-1.4)	0.9 (0.6-1.4)	0.998
Thrombocytes (x10^9/L)	125 (69-174)	87 (56-131)	0.008
CRP (mg/L)	64 (30-116)	106 (56-158)	0.001
Creatinine (µmol/L)	95 (81-108)	95 (81-108)	0.400
Urea (mmol/L)	4.9 (3.9-6.2)	5.2 (4.0-6.4)	0.498
Sodium (mmol/L)	136 (133-138)	135 (133-138)	0.578
Potassium (mmol/L)	3.8 (3.5-4.2)	3.7 (3.5-4.0)	0.299
Lactate (mmol/L)	1.3 (1.0-1.5)	1.4 (1.1-1.9)	0.017
Temperature (°C)	37.6 (36.9-38.8)	38.9 (37.5-39.5)	0.001
Treatment			0.532 [a]
atovaquone/proguanil	50 (86%)	108 (84%)	
quinine	4 (7%)	4 (3%)	
artemether/lumefantrine	4 (7%)	2 (2%)	
artesunate		13 (10%)	
chloroquine		1 (1%)	
Severity LFT#			n/a
Mild LFT#	n/a	93 (73%)	
Moderate LFT#	n/a	26 (20%)	
Severe LFT#	n/a	9 (7%)	

All data are presented as median (IQR) or N (%). LFT#= liver function test abnormalities. Statistical differences are tested using Mann-Whitney or
[a]Chi-square. *Clinical laboratory reference ranges can be found in S2 table.

Table 1 – General characteristics of patients with imported uncomplicated falciparum malaria on admission

2). In most cases standard LFT measurement time-points were obtained at baseline, 2 days post-treatment, and at the end of study. Elevations were primarily limited to ALT/AST elevations, and to a lesser extent, to γGT and ALP. Increased transaminases were found in 83/129 (64%) of the CHMI participants when treated at positive thick smear (treatment threshold of ~>5 parasites/μL), including mild (50/83 (60%), >1.0 ≤ 2.5xULN), moderate (18/83 (22%), >2.5 ≤5.0xULN) and severe (15/83 (18%), >5.0xULN) elevations (**table 2**). The incidence of LFT elevations was 14/58 (24%) in volunteers treated at positive qPCR (treatment threshold of >0.1 parasites/μL), including mild (12/14 (86%)), moderate (1/14 (7%)), and severe (1/14 (7%)) elevations (**table 2**). Among all CHMI participants the median peak ALT was 69 U/l (range 13-870 U/l, IQR 46-98 U/l), and median peak AST was 52 U/l (range 22-723 U/l, IQR 43-85 U/l) from all sample collection time-points during infection. Median peak γGT was 40 U/l (range 11-184 U/l, IQR 26-68 U/l), median peak ALP was 79 U/l (range 17-218 U/l, IQR 67-94 U/l), median peak total bilirubin was 11 μmol/l (range 3-31 μmol/l, IQR 9-15 μmol/l), and median peak LD was 422 μmol/l (range 170-981 μmol/l, IQR 198-396 μmol/l). Only 9/187 (5%) showed mild elevations (>1.0 ≤ 2.5xULN) of total bilirubin levels.

When more frequently assessed over the course of the study (2 CHMI studies, n=34), mild LFT elevations became apparent just prior to initiation of treatment (**figure 1**). The majority of LFT abnormalities exceeded the upper limit of normal on the day of treatment, and peaked around 2-6 days after initiation of antimalarials. This corroborates with data from EHMI-3 (n=5) where treatment is initiated 48 hours after a positive thick smear. The observed abnormalities were transient, normalizing at study end (around day 35–42 after challenge infection). Furthermore, a distinct pattern of timing of peak of transaminase elevations is found between individuals. Some participants show peak elevations early after treatment and others more delayed. These specific patterns, as well as the severity of the observed abnormalities, seem regardless of different drug regimens used.

To compare the dynamics of LFT abnormalities between CHMI and imported cases, data of 51 patients with imported uncomplicated malaria with multiple LFTs measurements were also obtained to analyze the course of LFT elevations (**S2 figure**). A similar LFT pattern was found in both naturally-acquired infections and CHMI participants, where LFT elevations peaked after treatment and normalized during follow-up. In contrast to CHMI-participants, total bilirubin levels in patients with imported *P. falciparum* malaria were increased on admission day, suggesting a concomitant haemolysis most likely due to 2-3 log higher parasite densities.

Study	Year	Challenge	Treatment drug	Subjects with parasitaemia (n)	Mean peak parasiteamia (Pf/ µL)	Subjects with LFT#[a] (n(%))	Severity			Treatment criterion	LFT timepoints	Registered
							mild	moderate	severe			
EHMI-3	2001	3D7- mosquito bite	chloroquine	5	21.6	4 (80)	2	2	0	48h after positive TS[b]	multiple timepoints[e]	-
EHMI-8A	2007	NF54- mosquito bite	artemether-lumefantrine	5	15.1	*LFTs not measured during infection[d]*				positive TS[b]	-	NCT00442377
LSA-3	2008	NF54- mosquito bite	artemether-lumefantrine	18	26.7	18 (100)	10	4	4	positive TS[b]	multiple timepoints[e]	NCT00509158
EHMI-8B	2009	NF54- mosquito bite	atovaquone-proguanil	7	3.7	4 (57)	4	0	0	positive TS[b]	Standard[c]	NCT00757887
TIP1	2010	NF54-NF135 mosquito bite	atovaquone-proguanil	10	17.6	*LFTs not measured during infection[d]*				positive TS[b]	-	NCT01002833
TIP2	2010/2011	NF54- PfSPZ I.M.*	atovaquone-proguanil	15	43.6	5 (33)	4	0	1	positive TS[b]	Standard[c]	NCT01086917
ZonMw1	2011	NF54- mosquito bite	atovaquone-proguanil	12	28.5	*LFTs not measured during infection[d]*				positive TS[b]	-	NCT01218893
EHMI-9	2011	3D7- mosquito bite or BS challenge**	atovaquone-proguanil	19	9.5	11 (58)	9	1	1	positive TS[b]	standard[c]	NCT01236612
ZonMw2	2012	NF54- mosquito bite	atovaquone-proguanil	9	27.8	6 (67)	6	0	0	positive TS[b]	standard[c]	NCT01422954
TIP4	2012	NF135- mosquito bite	atovaquone-proguanil	19	81.1	8 (42)	6	2	0	positive TS[b]	standard[c]	NCT01660854
TIP3	2012	NF54-NF135-NF166 mosquito bite	atovaquone-proguanil	15	37.4	*LFTs not measured during infection[d]*				positive TS[b]	-	NCT01627951
TIP5	2012/2013	NF54- mosquito bite	atovaquone-proguanil	21	15.5	11 (52)	4	6	1	positive TS[b]	standard[c]	NCT01728701
BMGF2a	2014	NF54-NF135-NF166 mosquito bite	atovaquone-proguanil	20	20.1	*LFTs not measured during infection[d]*				2 pos. qPCR (≥500Pf/ml)	-	NCT02149550
BMGF1	2014	NF54- mosquito bite	atovaquone-proguanil	8	0.1	0 (0)	0	0	0	pos. qPCR (>100Pf/ml)	standard[c]	NCT02080026
BMGF2b	2015	NF54-NF135-NF166 mosquito bite	atovaquone-proguanil	31	1.5	7 (23)	6	0	1	pos. qPCR (>100Pf/ml)	standard[c]	NCT02098590
BCG-EHMI	2016	NF54- mosquito bite	atovaquone-proguanil	19	1.0	7 (37)	6	1	0	pos. qPCR (>100Pf/ml)	standard[c]	NCT02692963
CHMI-trans1	2016	3D7- mosquito bite	sulfadoxine-pyrimethamine/ piperaquine	16	20.0	16 (100)	5	3	8	positive TS[b]	multiple timepoints[e]	NCT02836002
	Total subjects with LFTs measured			187		97 (52)	62 (33)	19 (10)	16 (9)			
	Total subjects with LFTs measured (thick smear studies)			129		83 (64)	50 (39)	18 (14)	15 (12)			
	Total subjects with LFTs measured (qPCR studies)			58		14 (24)	12 (21)	1 (2)	1 (2)			

[a]liver function test abnormalities; [b]TS = thick smear; [c]standard LFT measurements at 3 time-points: baseline, 2 days after treatment, and at the end of study; [d]LFTs are only measured on baseline and at the end of study. [e]>3 LFT measurement time-points (range of 4-13). *intra-muscular injection; **blood stage challenge

Table 2 – Overview of CHMI studies

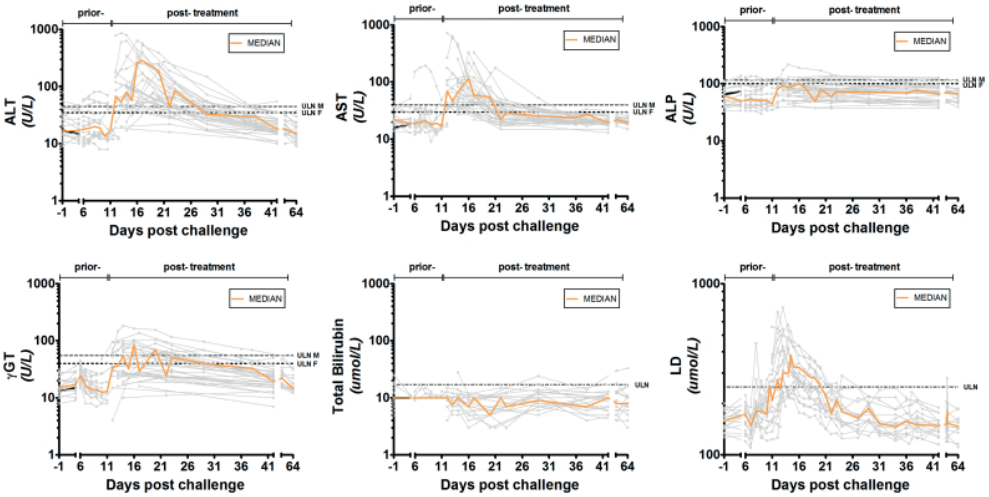

Figure 1 – Course of liver function test (LFT) levels during CHMI
Multiple LFT measurements of the LSA-3 and CHMI-trans1 study (n=34). LFT sampling time-points differ between studies and individual participants. The line interruption indicates sampling time-points prior- and post-treatment. ALT= alanine aminotransferase, AST= aspartate aminotransferase, γGT= gamma glutamyl transferase, ALP= alkaline phosphatase. Orange curves are the median of all grey individual participants curves per LFT.

Association between parasite load & liver function tests

CHMI participants with LFT abnormalities presented with a higher parasite load than those without LFT abnormalities ($p<0.001$), although the range was relatively large (f**igure 2A**). There was a positive association between LFT values and parasite load in the CHMI studies (r=0.33, $p<0.001$) (**figure 2B**). No significant differences were found between the different groups of LFT severity grades. In addition, in clinical cases with imported uncomplicated *P. falciparum* infections, a positive association was found between parasite density on admission day, and LFT abnormalities (r=0.25, $p=0.005$).

Clinical laboratory parameters and liver function tests

Analysis of clinical laboratory parameters in CHMI participants showed that lowest platelet, leukocyte, and lymphocyte counts, as well as maximum LD levels, independently associated with LFT abnormalities ($p<0.0001$, $p=0.01$, $p<0.0001$, $p<0.0001$, respectively) (f**igure 3**). However, prior to treatment these parameters poorly predicted LFT abnormalities.

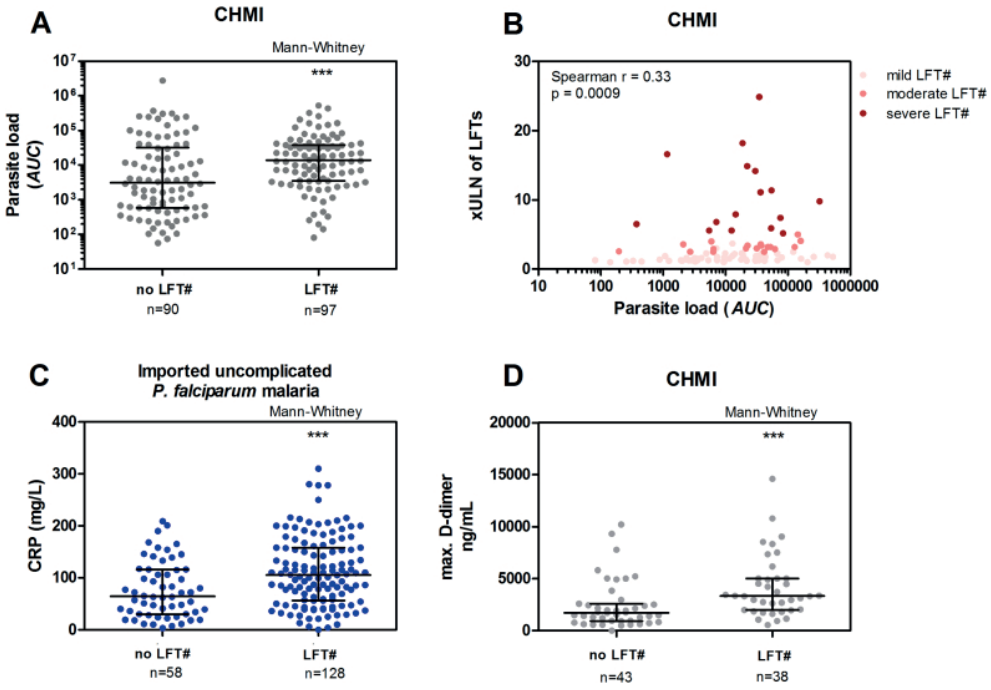

Figure 2 – Parasite load, inflammation markers, and liver function test (LFT) abnormalities
A) Parasite load in CHMI without- (no LFT#), and with LFT abnormalities (LFT#). Error bars represent median with inter-quartile range. Parasite load as the area under the curve (AUC) represents the total parasite exposure over time. **B**) Association of parasite load and severity of liver function abnormalities in x the upper limit of normal of LFTs (xULN of LFTs) in CHMI subjects. **C)** C-reactive protein (CRP) levels of patients with imported uncomplicated *P. falciparum* malaria on admission day. **D)** Maximum d-dimer levels measured in participants with LFT abnormalities (LFT#) in CHMI. D-dimer data are from 6 CHMI studies (n=81) with multiple samples measured during challenge infection. Error bars represent the median with inter-quartile range. ***p<0.001.

We hypothesized that inflammation, well known to be involved in malaria pathogenesis, might play a role in these abnormalities. In the imported uncomplicated malaria cases, C-reactive protein (CRP) was increased on the day of admission in patients with LFT abnormalities (p<0.001) (**figure 2C**). A statistically significant difference was found in maximum d-dimer levels in CHMI participants with or without LFT abnormalities (p<0.001) (**figure 2D**).

A more extended pro-inflammatory cytokine panel was measured in one CHMI study (CHMI-trans1, n=16) one day after treatment, showing a positive correlation between IFNγ, IL-6, and IL-8, and peak of the LFT abnormalities, p=0.039, p=0.049, and p=0.015, respectively (**S3 table**). A strong positive association was also found between the

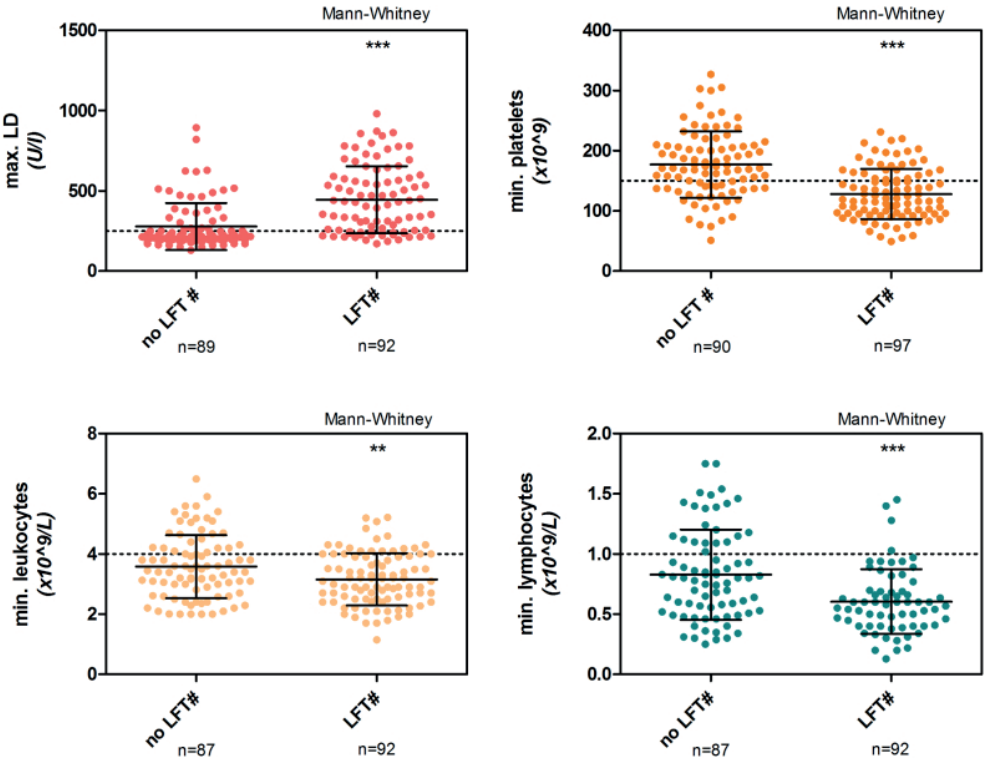

Figure 3 – Clinical laboratory parameters and liver function test (LFT) abnormalities in CHMI
Maximum lactate dehydrogenase (LD), and minimum platelets, leukocytes, and lymphocytes measured in CHMI participants without-, and with LFT abnormalities (LFT#). Error bars represent the mean with SD. **P=0.004; ***p<0.001.

cumulative inflammatory responses (IFNγ, MCP-1, IL-6, IL-8, IL-10, IL12p70, IL-17a, IL-18) and LFT abnormalities (r=0.65, *p*=0.008) (**figure 4**). In a second study (EHMI-8B study, n=7), a similar positive trend was found for d-dimer, CRP, IFNγ, and IL-6 values (**S3 figure**).

Finally, a number of anti-oxidant markers were measured to further define the nature of the induced inflammatory response in relation to LFT abnormalities. A significant reduction in the average of anti-oxidant gene expression SOD1 (*p*=0.03), SOD2 (*p*=0.001), NOS3 (*p*<0.0001), CAT (*p*<0.0001), and GTSK1 (*p*<0.0001) and an increase in expression of PRDX1 (*p*=0.007) was observed during the malaria infection (one day after treatment) (**S4 figure)**. The cumulative anti-oxidant response of these markers through transformation into a Z-score, showed a negative association between the anti-oxidant response and the LFT abnormalities (r=-0.63, *p*=0.001) (**figure 4**). All markers normalized by the end of the study.

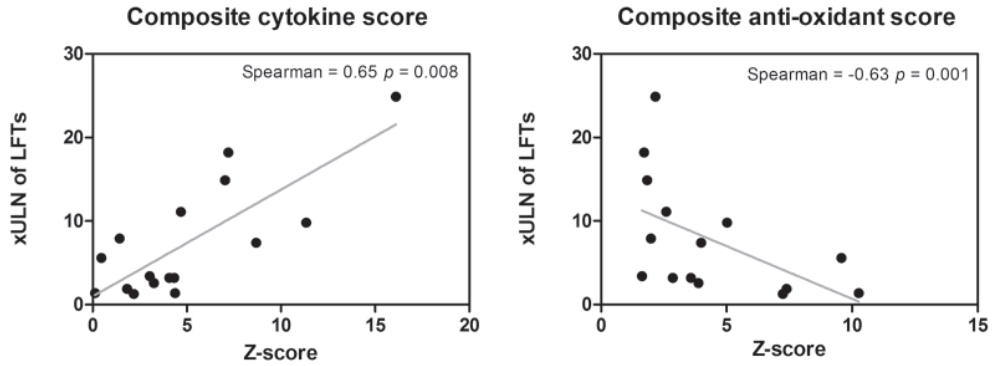

Figure 4 – Association between liver function test abnormalities, and cumulative cytokine levels and cumulative anti-oxidant gene expression
The cumulative cytokine score comprises IFNγ, MCP-1, IL-6, IL-8, IL-10, IL12p70, IL-17a, IL-18 and the cumulative anti-oxidant gene expression comprises SOD1, SOD2, NOS3, CAT, GTSK1, and PRDX1measured on 1 day after treatment (DT+1) in CHMI-trans1. Values are standardized as a Z-score. xULN of LFTs = x upper limit of normal range of liver function tests (LFTs). The xULN of LFTs values are the peak LFT abnormalities measured from day 2 after treatment and the samples available onwards.

Discussion

This study shows that abnormalities in liver function test (LFT) are a relatively frequent finding in uncomplicated falciparum malaria, and occur regardless of the choice of drug regimen. These abnormalities are transient in nature, resolving within 3-6 weeks. The typical pattern of LFT elevations is primarily limited to ALT/AST elevations, and to a lesser extent, to γGT and ALP, and peaks in particular two to six days after initiation of treatment. Furthermore, the study shows a significant association between LFT elevations and parasite load, inflammatory markers and reduced expression of oxidative stress markers. Early treatment based on qPCR thresholds can decrease the occurrence and severity of LFT abnormalities in CHMI.

Hepatic dysfunction is a well-known feature of severe malaria, contributing to clinically significant complications such as hypoglycaemia, metabolic acidosis, impaired drug metabolism, and finally organ failure (8, 12). Malaria-associated liver damage in uncomplicated malaria, however, has rarely been investigated so far (25). Observational studies are often restricted in number and limited to admission samples only(15), therefore, underestimating incidence and severity. Other studies do not differentiate non-severe from severe disease in their analysis (9, 14, 26). This study specifically advocates the relevance in relation to potential clinical implications in uncomplicated disease, which

constitutes the vast majority of clinical malaria cases. This study highlights that LFT abnormalities can be severe (up to > 20 xULN), but are apparently fully reversible upon parasitological cure, corroborating previous reports (25-27).

The observed liver injury seems to have a hepatocellular basis, as indicated by the disproportionate elevation in serum transaminases relative to alkaline phosphatase. Peak bilirubin levels precede antimalarial treatment in imported malaria cases but not CHMI. This suggests that these elevations are likely due to haemolysis leading from higher parasitaemia in these patients compared to CHMI volunteers. Similarly, Woodford *et al.* show that bilirubin elevations in clinical cases peak on admission day, resolving early after treatment in contrast to the later occurring peak of transaminases (14). The absence of clearly elevated bilirubin levels in CHMI adduces that these events may not result in a functional liver impairment, since conjugation of bilirubin occurred normally. Serum albumin or clotting factors were not tested in the current study, and therefore, it cannot be formally excluded that biosynthetic capacity of the liver may be compromised. The absolute increase in serum transaminases is of little prognostic value since the liver can recover from most forms of acute injury, due to its large regenerative capacity. In absence of symptoms such as haemorrhage or altered consciousness, significant hepatic dysfunction seems highly unlikely in patients without history of liver disease.

The potential contribution of infection transformation, from the liver stage to blood stage infection, to LFT abnormalities remains uncertain. However, similar LFT abnormalities are found CHMI studies with a blood stage challenge, where the liver stage of the parasite is circumvented (14). This, together with the observation that no substantial LFT abnormalities are found on day 6-7 (day of liver schizont rupture), makes it less likely that the liver stage can cause significant LFT abnormalities on its own. It is unknown if the liver stage could influence the severity of the observed abnormalities.

This study provides evidence that the underlying pathogenesis of liver injury in uncomplicated malaria conceivably differs from severe malaria. In severe falciparum malaria, tissue damage is thought to be at least partly related to decreased oxygen supply and disturbed metabolisms due to parasite sequestration in the microvascular capillary system (7, 17). However, in CHMI only very low parasite densities are present and sequestration likely limited. Systemic inflammation with leukocytopaenia, thrombocytopaenia, increased d-dimer, CRP, and IFN-γ concentrations is a typical hallmark of malaria even at the very low density of parasitaemias as observed after CHMI (28, 29). This study shows a clear relationship between these parameters and LFT abnormalities in falciparum malaria. Together with the timing of LFT abnormalities post treatment, and the presence of low-density parasitaemia, this suggests an inflammation-based mechanism of liver injury.

Similarly, lung injury with impaired alveolar-capillary function is described to deteriorate after antimalarial treatment, reflecting a prolonged inflammatory response to parasite killing (30). Histopathology of lung tissue in vivax malaria supports this hypothesis with an increase in alveolar-capillary monocytes and pulmonary phagocytic cell activity after initiation of treatment (31). An adjacent hypothesis describes platelet sequestration as a downstream promoter of liver injury by exacerbating local inflammation and leukocyte accumulation (32), which correlates with the observed thrombocytopaenia and leukocytopaenia. Interestingly, although inflammation-induced liver injury is common in other infectious diseases (e.g. extrahepatic bacterial infection and sepsis), it seems to predominantly have a cholestatic nature in contrast to uncomplicated malaria (33).

Systemic inflammation is the net result of an interplay between pro- and anti-inflammatory responses, and a shift towards a pro-inflammatory status can cause malfunctioning of the host's mitochondria in malaria, leading to organ damage (34). The resulting oxidative stress as observed in uncomplicated malaria is known to play a role in pathogenesis (35). For instance, lipid peroxidation and antioxidant enzyme activity associate with cholestatic jaundice in *P. vivax* patients (36), and oxidative stress causes hepatocyte apoptosis in murine malaria models (37, 38). In this study, we show that anti-oxidant gene expression is down-regulated for SOD1, SOD2, NOS3, CAT, and GSTK1 during infection, and up-regulated for PRDX1. This corroborates with data from rodent studies(39, 40) and a non-human primate study with *P. knowlesi* (41), where hepatic superoxide scavenging systems, glutathione S-transferase, superoxide dismutase and catalase, decrease during the course of a malaria infection. This net result may be reduced clearance of radicals and subsequent failure to protect cellular constituents from oxidative damage.

Our findings reflect a need to understand and reassess LFT abnormalities in malaria. This will have significant impact on clinical decision-making and in drug development, where they are often interpreted as drug-induced liver injury, particularly in malaria drug studies(27, 42). For example, drug-related serious adverse events in a recent multicentre trial comparing pyronaridine–artesunate or dihydroartemisinin–piperaquine versus current first-line therapies for uncomplicated malaria were associated mainly with increased liver enzymes (43). Incorrect attribution of liver injury to an antimalarial drug can lead to unnecessary discontinuation or shifts in drug regimens, or impede drug development. Given the diversity of drug regimens used, it is unlikely that the observed LFT abnormalities in this study are caused by antimalarials. Moreover, no marked deterioration of LFTs was observed after a second, higher drug dose (16). Our data does not support distinct differences between drugs and severity or occurrence of LFT abnormalities. Differences In line with findings from Woodford *et al.*, our data does not show a clear relation between the severity of abnormalities found, the pattern of

early- or delayed transaminase elevations, and the different drug regimens used in CHMI. Furthermore, the timing, varied and often limited use of supportive medication (such as paracetamol, known for dose-dependent hepatotoxicity) in CHMI does not support a clear relationship with LFT abnormalities. It seems similarly improbable that the observed injury is parasite strain-specific. Geographically distinct *P. falciparum* strains were used in CHMI, and the imported falciparum malaria cases were presumably caused by a wide diversity of strains. In addition, non-falciparum malaria (e.g. *Plasmodium vivax*) was also shown to increase LFT abnormalities.

The limitations of this study are related to the retrospective observational nature of some of the data. However, the longitudinal CHMI data from healthy participants excludes the majority of confounding factors, and provides a much more thorough description of LFT dynamics. Although, we observed differences between CHMI and naturally-acquired malaria cases, patients with sequential sampling data from the latter seem to have similar LFT dynamics, and affirm previous observations (14).
This study shows that liver function test abnormalities are an under-recognized but common feature of uncomplicated falciparum malaria. The pathophysiological basis is most likely a pro-inflammatory response associated with oxidative stress resulting in transient liver tissue injury. Given the rapid and spontaneous resolution, and the absence of clear functional liver impairment in uncomplicated malaria, these events are unlikely to result in permanent subclinical liver damage. Treatment should remain focused on fast parasite clearance, and a more expectative approach might be warranted to avert unnecessary discontinuation or shifts in antimalarials due to supposed drug-induced liver injury. Nevertheless, supportive drugs with hepatotoxic potential should be limited or given only with appropriate monitoring, and adjunctive therapy that mediates systemic inflammation and oxidative stress may have a potential role in helping to constrain malaria progress into severe disease. Clinical trials testing the safety and efficacy of novel antimalarials should consider the temporal relationship between LFT abnormalities and infection. Importantly, unnecessary early abrogation of further clinical development of promising drugs related to potential or supposed hepatotoxic effects should be carefully (re)considered.

Contributors
IJR, PJJG, and RWS designed the study, which was performed by IJR, LAS, QM, AJV, TB, RWS. IJR, GMJ, JW, LAS, and RK collected the data, and IJR, GMJ, JJH, PJJG, and RWS analysed and interpreted the clinical data. IJR, XZY, and MA performed, analysed, and interpreted the cytokine and oxidative stress markers assays, and supervised by AF and RWS. IJR wrote the first manuscript, which was critically reviewed and approved by all authors.

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Declaration of interests
All authors declare no competing interests.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Evidence before this study

We conducted an online literature search on liver function test (LFT) abnormalities in uncomplicated malaria using PubMed for articles published up to May 1st, 2018. Articles were searched by title and abstract using the terms “hepatic dysfunction” or “liver injury” or “liver function test” or “liver enzymes” or “transaminases”, with either “malaria” or “uncomplicated malaria” or “non-severe malaria” without language restrictions. Some articles cited in the screened publications were also included. We screened 58 articles, 26 of which described liver injury in malaria focusing on severe disease. Only four contained information on LFT abnormalities in uncomplicated disease specifically. Most other studies described hepatic adverse reactions in the context of antimalarial drugs. The most descriptive study was a recent retrospective study by Woodford *et al* that provides data on LFT dynamics. Ramirez *et al* described the proportion of LFT abnormalities on admission day in a case control study. In these studies, no clear associations were found between clinical and demographic factors, and LFT abnormalities in uncomplicated malaria. The pathophysiological basis of liver injury has only been described in animals to date.

Added value of this study

To our knowledge, this is the first study using longitudinal and prospective data from CHMI studies to characterize liver injury specifically in uncomplicated falciparum malaria. This study adds novel insights into clinical relevance, dynamics and pathophysiological basis of liver injury. We provide evidence that the underlying pathogenesis of liver injury in uncomplicated malaria is unlikely to be related to hepatotoxicity of antimalarials, and conceivably differs from that during severe malaria. Induced inflammation *per se* may be a major driver of liver injury since parasite load in CHMI is extremely low but sufficient to effect systemic inflammation and oxidative stress.

Implications of all the available evidence

Recognizing and understanding this relatively common phenomenon is of significant importance for both drug-related clinical decision making and development of antimalarial drugs. Incorrect attribution of liver injury to an antimalarial drug can lead to unnecessary discontinuation or shifts in drug regimens; instead, a more expectative approach might be warranted. Clinical trials testing the safety and efficacy of novel antimalarials should carefully (re)consider unnecessary early abrogation of promising drugs with supposed hepatotoxic effects. Supportive therapeutics with hepatotoxic potential during a malaria infection should, however, be limited or given with appropriate monitoring.

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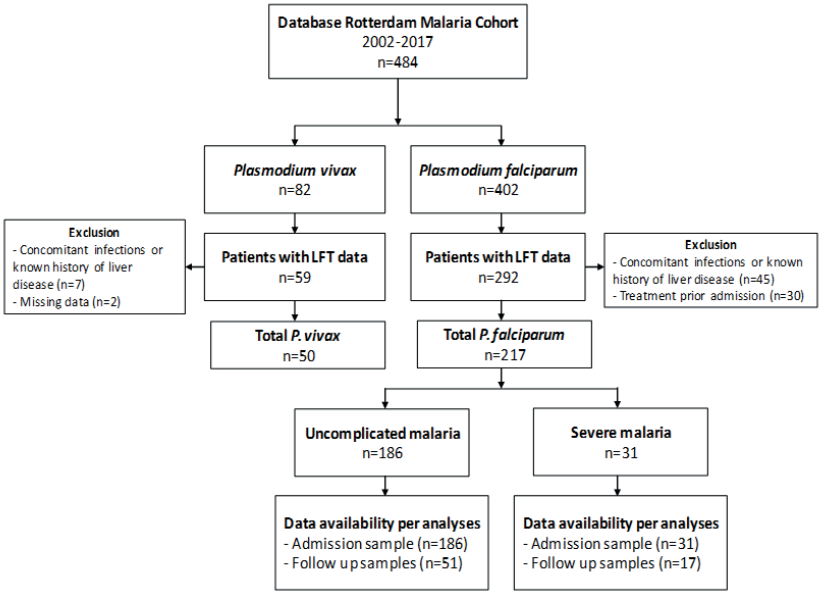
Supplementary data

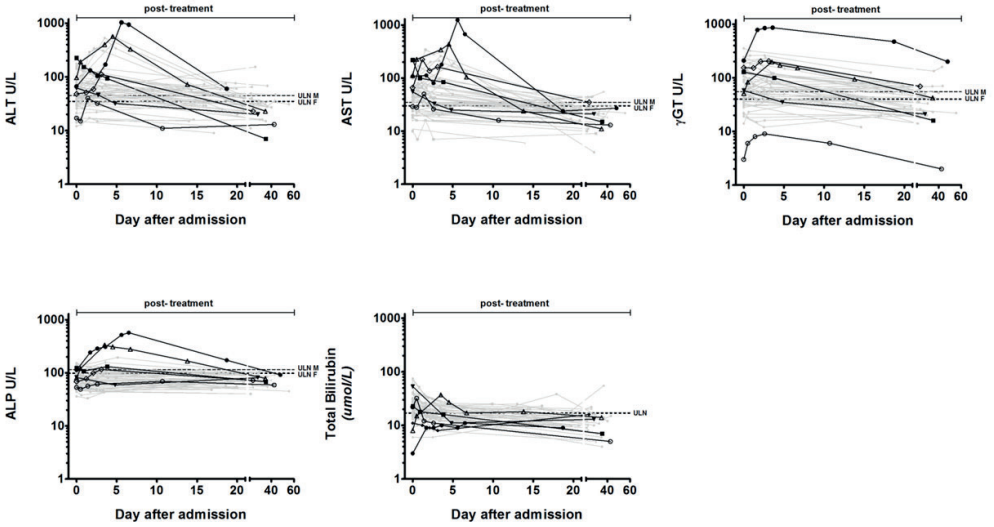
S1 Material. Oxidative stress analysis

Total nucleic acid was extracted from whole blood by using Magna Pure LC – High Performance kit. After RNA evaluation, cDNA was synthesized using the SuperScript® VILO™ cDNA Synthesis Kit (CAT # 11754250, Invitrogen, Thermofisher Scientific) according to the manufacturer´s instructions.

TaqMan gene expression Essays (Applied Biosystem) were used to measure antioxidant gene expression levels for Superoxide dismutases (SOD1, # Hs00533490_m1 and SOD2, # Hs00167309_m1), Nitric oxide synthase (NOS3, # Hs01574665_m1), Catalase (CAT, # Hs00156308_m1), Peroxiredoxin (PRDX1, # Hs00602020_mH) and Glutathione S-transferase Kappa (GSTK1, # Hs01114170_m1) on a QuantStudio5 qPCR instrument. The TaqMan® GAPDH Assay (CAT # 4485712, Applied Biosystem) was used as endogenous control and run in the same reaction to get more robust quantification. Each 20 µl reaction contained 5 µl cDNA, 10 µl TaqMan® Mutiplex Master Mix (CAT # 4461882, Applied Biosystem), 1 µl GAPDH Assay, 1 µl target gene Essay and ddH_2O. Thermal cycling condition was 95 °C for 20 s before running 45 thermal cycles (95 °C for 1 s and 60 °C for 20 s). ThermoFisher Clouds Software was used to obtain the C_T values. Gene expression was calculated as $2^{-\Delta CT}$, where ΔC_T = C_T of target gene – C_T of control gene (GAPDH).

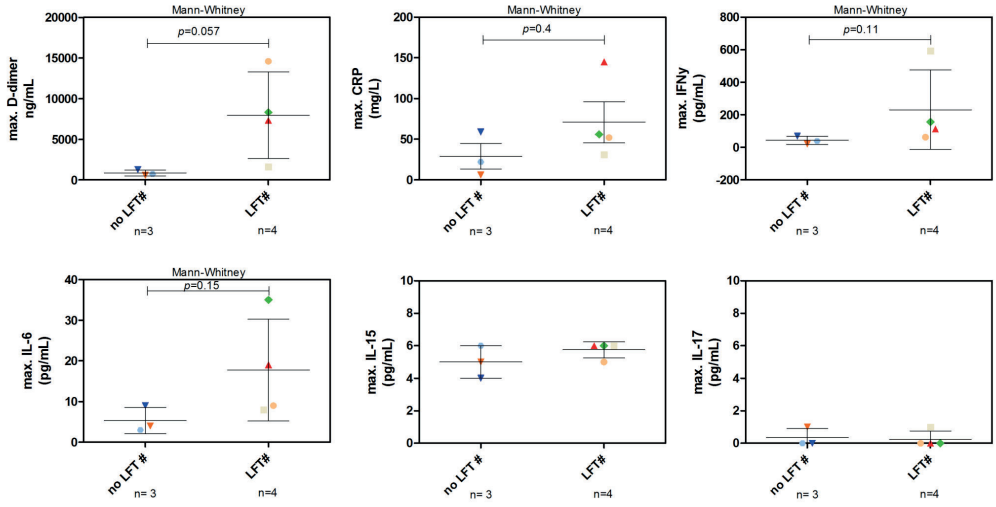


S1 Figure – Flowchart study profile imported malaria cases



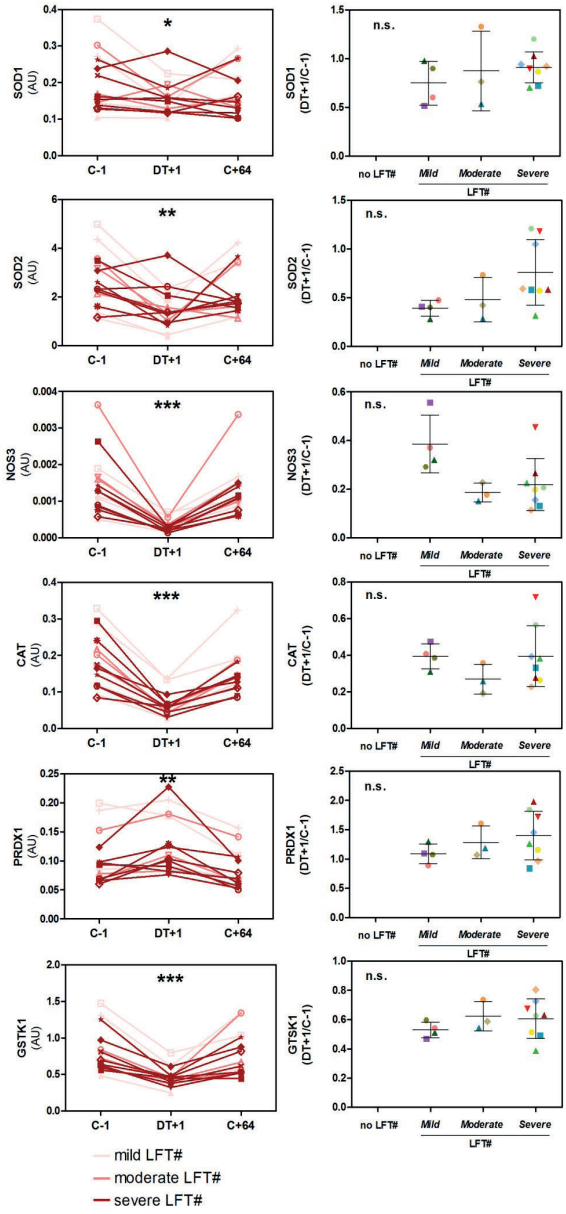
S2 Figure – Course of liver function test (LFT) levels of patients with imported falciparum malaria

Multiple LFT measurements of patients with imported uncomplicated falciparum malaria (n=51). ALT= alanine aminotransferase, AST= aspartate aminotransferase, γGT= gamma glutamyl transferase, ALP= alkaline phosphatase. Light grey lines are all individual patients used in the analysis. Black lines are six representative patients of all patients to ease interpretation.



S3 Figure – Association between cytokines and liver function test abnormalities

Maximum cytokine levels from EHMI-8b as measured during challenge infection. Coloured data points represent an individual participant. Error bars represent the mean with SD.



S4 Figure – Anti-oxidant gene expression during CHMI and liver function test (LFT) abnormalities in CHMI-trans1

C-1= day before challenge, DT+1= one day after treatment, C+64= 64 days after challenge. Significant differences between C-1 and DT+1 are determined by Wilcoxon matched-pairs signed rank test. *p<0.05; **P<0.01; ***p<0.0001. AU = Arbitrary Units, gene expression was calculated as $2^{-\Delta CT}$, where ΔC_T = C_T of target gene – C_T of control gene (GAPDH). Each individual color data-point corresponds to a participant. LFT#= liver function test abnormality; n.s.= not significant (tested by 1way ANOVA).

	No LFT#	LFT#	No LFT#	LFT#	p-value no LFT# v.s. LFT#	
	Severe P. falciparum		*P. vivax*			
	(n=4)	(n=27)	(n=27)	(n=23)	*p. falciparum*	*p. vivax*
Age	40 (32-44)	50 (40-56)	32 (25-43)	36 (29-49)	0·094	0·957
Gender			6 (22%)	7 (30%)	0·170 [a]	0·50 [a]
female	-	9 (33%)				
male	4 (100%)	18 (67%)				
Immunity					0·077 [a]	0·906 [a]
Unknown	-		8 (30%)	9 (39%)		
Non-immune	1 (25%)		15 (56%)	11 (48%)		
Partly	3 (75%)		3 (11%)	2 (9%)		
Semi	-		1 (5%)	1 (4%)		
Parasitaemia/µL	275·000 (108·325-465·000)	195·000 (108·100-396·000)	Not determined	Not determined	0·977	n/a
AST (U/L)	29 (25-34)	76 (50-111)	19 (14-25)	41 (31-54)	**0·008**	0·640
ALT (U/L)	28 (18-34)	52 (35-104)	21 (17-29)	50 (33-67)	**0·027**	**0·047**
γGT (U/L)	44 (26-49)	85 (52-154)	25 (15-30)	60 (34-86)	**0·033**	**0·047**
Alkaline phosphatase (U/L)	57 (51-65)	78 (68-98)	69 (64-79)	80 (68-101)	**0·023**	0·095
Bilirubin (total) (µmol/L)	55 (40-59)	58 (44-68)	20 (16-31)	24 (18-36)	0·498	0·198
LD (U/L)	-	418 (349-503)	--	-	-	-
Haemoglobin (mmol/L)	8·6 (7·8-9·9)	8·3 (5·2-9·2)	8·5 (7·4-8·9)	8·2 (7·8-8·9)	0·629	0·711
Leukocytes ($x10^9$/L)	6·2 (3·5-8·9)	6·9 (4·8-9·6)	5·3 (4·7-7·1)	4·9 (4·7-5·5)	0·476	0·870
Lymphocytes ($x10^9$/L)	1·0 (0·7-1·5)	1·1 (0·7-1·5)	1·0 (0·8-1·8)	0·9 (0·5-1·7)	0·971	0·667
Thrombocytes ($x10^9$/L)	97 (44-133)	37 (25-70)	95 (81-136)	89 (60-102)	0·137	0·701
CRP (mg/L)	178 (96-313)	165 (108-211)	69 (44-87)	79 (50-117)	0·883	0·779
Creatinine (µmol/L)	103 (97-114)	98 (89-124)	88 (71-98)	82 (70-91)	0·755	0·569
Urea (mmol/L)	5.3 (4·2-5·6)	7·2 (4·9-11·1)	4·7 (4·0-5·9)	4·9 (4·3-6·4)	0·082	0·078
Sodium (mmol/L)	132 (128-137)	132 (130-134)	136 (134-138)	136 (133-138)	0·977	0·424
Potassium (mmol/L)	3·6 (3·3-4·5)	3·8 (3·4-4·0)	3·9 (3·6-4·1)	3·8 (3·5-4·1)	0·932	0·285
Lactate (mmol/L)	-	2·1 (1·8-2·9)	1·3 (1·1-2·0)	1·3 (1·1-1·7)	-	0·923
Temperature (°C)	38·2 (37·1-38·8)	38·2 (37·6-39·8)	38·2 (37·3-39·2)	39·5 (38·1-40·8)	0·536	0·721

Treatment					0·53 [a]	0·565 [a]
atovaquone/proguanil	1 (25%)	2 (7·4%)	1 (4%)	-		
quinine	1 (25%)	8 (30%)	-	-		
artemether/lumefantrine	-	-	-	-		
artesunate	2 (50%)	17 (63%)	-	-		
chloroquine	-	-	21 (78%)	18 (78%)		
mefloquine	-	-	5 (19%)	4 (17%)		
unknown	-	-		1 (1 (4%)		
Severe *P. falciparum and P. Vivax (all)*	4 (12%)	27 (87%)	27 (54%)	23 (46%)	n/a	n/a
Mild LFT#	n/a	12 (44%)	n/a	21 (91%)		
Moderate LFT#	n/a	12 (44%)	n/a	2 (9%)		
Severe LFT#	n/a	3 (11%)	n/a	-		

All data are presented as median (IQR) or N (%). LFT#= liver function test abnormalities. Statistical differences are tested using Mann-Whitney or [a]Chi-square. *Clinical laboratory reference ranges can be found in S2 table.

S1 Table – *Plasmodium vivax* malaria, and severe falciparum malaria, and liver function test abnormalities on admission day of clinical cases with imported malaria

	Normal range male	Normal range female
AST (U/L)	<35	<30
ALT (U/L)	<45	<35
γGT (U/L)	<55	<40
Alkaline phosphatase (U/L)	<115	<100
Bilirubin (total) (µmol/L)	<17	
LD (U/L)	<250	
Haemoglobin (mmol/L)	8.6-10.5	7.5-9.5
Leukocytes (x10^9/L)	3.5-10.0	
Lymphocytes (x10^9/L)	0.5-5.0	
Thrombocytes (x10^9/L)	150-370	
CRP (mg/L)	<10	
Creatinine (µmol/L)	65-115	55-90
Urea (mmol/L)	2.5-7.5	
Sodium (mmol/L)	136-145	
Potassium (mmol/L)	3.5-5.1	
Lactate (mmol/L) (plasma)	0.5-2.2	

S2 Table – Clinical laboratory reference ranges

	IFNy		IL6		IL8		IL10		MCP1		IL12p70		IL17a		IL18	
	r	P	r	P	r	P	r	P	r	P	r	P	r	P	r	P
xULN of LFT#[a]	0·49	0·06	0·46	0·07	**0·65**	**0·007**	0·37	0·16	0·25	0·34	0·12	0·65	0·19	0·48	0·02	0·95
peak ALT	**0·52**	**0·039**	**0·50**	**0·049**	**0·60**	**0·015**	0·42	0·11	0·27	0·31	0·16	0·56	0·15	0·57	0·03	0·91
peak AST	0·34	0·20	0·46	0·07	**0·78**	**<0·001**	0·25	0·36	0·30	0·25	0·08	0·76	0·18	0·51	0·05	0·87
peak ALP	**0·56**	**0·023**	0·13	0·64	**-0·06**	0·82	0·29	0·28	0·07	0·80	0·10	0·72	-0·24	0·37	-0·25	0·34
peak yGT	**0·55**	**0·028**	0·25	0·34	0·34	0·20	0·38	0·15	0·44	0·08	0·03	0·91	-0·31	0·24	-0·14	0·61
peak Bilirubin	-0·15	0·57	-0·12	0·66	-0·34	0·20	-0·40	0·12	-0·12	0·67	-0·08	0·78	-0·29	0·27	0·03	0·91
peak. LD	0·25	0·36	0·46	0·08	0·44	0·09	0·34	0·20	0·18	0·51	0·50	0·05	0·07	0·80	0·11	0·70

[a]xULN of LFTs = x the upper limit of normal of liver function tests. **Bold/underlined** numbers are significant. r=Spearman rho.

S3 Table – Association between cytokines and liver function test abnormalities

Cytokine samples from CHMI-trans1 (n=16) measured on day one after treatment. Peak of ALT= alanine aminotransferase, AST= aspartate aminotransferase, γGT= gamma glutamyl transferase, ALP= alkaline phosphatase, LD= lactate dehydrogenase during infection. The LFTs from this study were measured from day 2 after treatment and the samples available onwards.

Chapter 6

Transforming Growth Factor-beta profiles correlate with clinical symptoms and parameters of haemostasis and inflammation in a controlled human malaria infection

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Abstract

Background

After a controlled human malaria infection (CHMI), presentation of clinical signs and symptoms and host responses is heterogeneous. Transforming growth factor-beta (TGF-β) is the first serum cytokine that changes in malaria-naïve volunteers after CHMI. We studied a possible relation between TGF-β changes, pro-inflammatory cytokines, activation of haemostasis and endothelial cells and clinical symptoms.

Methods

A panel of cytokines including TGF-β, and markers of activation of haemostasis and endothelial cells were measured in blood samples of 15 volunteers at baseline before CHMI and during CHMI at day of treatment. The fold change of the parameters on the day of treatment was calculated and examined for a significant alteration during infection.

Results

Nine of 15 volunteers showed a significant decrease in TGF-β compared to baseline, with concomitant increased concentrations of D-dimer ($p = 0.012$), Von Willebrand factor ($p = 0.017$), IL-6 ($p = 0.012$) and IFN-γ (0.028) and a significantly decreased platelet count ($p = 0.011$). In contrast, 6 of 15 volunteers showed sustained or increased TGF-β concentrations without change in the aforementioned parameters. The sustained responders presented with less moderate and severe clinical symptoms than the negative responders ($p = 0.036$) and had a higher baseline lymphocyte count ($p = 0.026$). TGF-β concentrations did not correlate with the parasitaemia on day of treatment.

Conclusion

Early decreases of serum TGF-β might function a marker for a pro-inflammatory host response and downstream clinical symptoms and pathology during CHMI.

Introduction

Malaria is still a major health concern with 438,000 deaths worldwide in 2015 (1). The parasitic disease is caused by *Plasmodium* spp. of which *P. falciparum* is the most pathogenic in humans. Contributing to its pathogenicity is adherence and sequestration of the infected erythrocytes to the vascular endothelium which can lead to obstruction of blood flow and a decline in oxygen delivery to the vital organs. Together with endothelial cell activation and inflammation this may eventually lead to multi-organ failure and death. However, the severity in clinical presentation in endemic areas differs substantially between individuals and is partly related to differences in their pre-existing immunity (2).

The host response to *P. falciparum* in malaria naïve individuals in controlled human malaria infection (CHMI) studies shows a heterogeneous pattern with differences in the number and severity of clinical symptoms (3). Also, the levels of coagulation- and endothelial cell activation and the magnitude and composition of the cytokine response (3, 4) differ between individuals infected with falciparum malaria during CHMI. Pro-inflammatory cytokines increase in a subset of volunteers (3), whereas in others an increase of the immune-regulatory TGF-β is observed. The increase in TGF-β can be as early as the end of the hepatic infection phase, or later, once parasites are detected in the blood stream (5). The aetiology of the heterogeneous immune response is unknown. Improved understanding of the heterogeneity is of interest as this initial immune response is responsible for the early control of blood-stage malaria (4, 6). A pro-inflammatory immune response, in which interferon gamma (IFN-γ) plays a major role, inhibits parasite replication during blood phase, whereas an initial increase of the immune-modulatory cytokine TGF-β correlates with a more rapid increase in number of infected erythrocytes and subsequently shorter pre-patent period (3, 5). This effect of pro- and immune-modulatory cytokines on parasitaemia is not limited to malaria, but also observed during other parasitic diseases such as leishmaniasis (7), infection with *Toxoplasma gondii* (8) and *Trypanosoma cruzi* (9). The role of pro- and immune-modulatory cytokines during more advanced malaria disease in clinical patients (10), is different from their function during early blood phase. Overproduction of pro-inflammatory cytokines during advanced clinical malaria can cause increased pathology leading to multi-organ dysfunction. In this situation immune-modulatory cytokines are protective and associated with increased survival (11, 12).

Although several studies demonstrated heterogeneity in distinct host responses in a CHMI (e.g. cytokines response, coagulation, endothelial activation) (3, 4, 13, 14), possible relationships between these distinct host responses remain elusive. Therefore, we investigated whether TGF-β profiles differ between naive volunteers and correlate with pro-inflammatory cytokines, parasite replication, clinical symptoms, endothelial cell activation and coagulation during CHMI.

Design and methods

Study populations

Details on the underlying CHMI study aim and design, characteristics of volunteers, as well as clinical and parasitological course have been described previously (15). Briefly, this CHMI study investigated the clinical and parasitological course of malaria after infection of healthy, malaria-naïve volunteers with the well characterized *P. falciparum* NF54 strain in comparison to two other strains (NF135.C10 and NF166.C8). The 15 volunteers that participated in that CHMI study were divided in 3 groups of 5 volunteers. Each group of CHMI volunteers was infected with 1 of the 3 *P. falciparum* strains by the bites of 5 infected mosquitos. Follow-up of CHMI volunteers started 5 days after infection by twice daily thick blood-smear analyses and assessment of clinical symptoms, in the morning and evening. Anti-malaria treatment with a 3-day curative regimen of Atovaquon/Proguanil 1000/400 mg (Malarone ®, GlaxoSmithKline), was started as soon as the thick blood-smear became positive. To study submicroscopic parasitemia, the presence of *P. falciparum* in peripheral blood was also analysed by quantitative polymerase chain reaction (qPCR) in stored blood samples, retrospectively. The Institutional Review Board of the Harbour Hospital and the Dutch Central Committee on Research involving Human Subjects approved the study protocol, including add-on studies. All CHMI volunteers gave written informed consent (NL41004.078.12).

Sample collection

Venous blood samples (EDTA-, serum- and trisodium citrate tubes) were collected and subsequently processed using standard laboratory procedures. Thick blood-smears were immediately prepared from EDTA-blood and subsequently examined. A positive thick blood-smear result was defined as ≥2 parasites per 225 high-powered microscopic fields.

Laboratory investigations

From all 15 CHMI volunteers, serum and citrate-plasma samples taken on the day before challenge (C-1) and the day of treatment (DT, i.e. the day of first thick blood-smear positivity), were examined for immunological, haemostasis and endothelial cell activation parameters. If a volunteer had a positive thick smear and was treated in the evening (DT), no serum and citrate-plasma samples were available as general laboratory investigations were performed only once a day. In these cases, the serum and citrate-plasma samples collected the next morning were used for analysis. Samples of DT (n=7) and samples of the next day (n=8) were combined for analysis. D-dimer was determined in trisodium

citrate plasma and the platelet count in EDTA-blood. Von Willebrand Factor (VWF) was measured with an in-house ELISA in citrate plasma as described previously (16).

IL-6, IL-12p70, TNF-α and IFN-γ concentrations were determined using high sensitive multiplex luminescent magnetic beads (R&D Systems, Inc.; Abingdon, United Kingdom) according to the manufacturer's instructions and analysed with a Luminex XMAP Multiplex reader. Total TGF-β concentrations were determined with ELISA (R&D Systems) according to the manufacturer's instructions.

Statistical analysis

Concentrations of the distinct parameters at C-1 and DT were compared with the Wilcoxon-Signed Rank test. For correlations, the Spearman's rho was used. As none of the parameters was normally distributed, data were presented as median with interquartile range unless stated otherwise. For comparison of concentrations of parameters between the groups of volunteers infected with the 3 different *P. falciparum* strains the Kruskal-Wallis test was used. All analyses were performed with SPSS version 24.0 (SPSS Inc, Chicago, IL, USA).

Results

TGF-β profile and characteristics of CHMI volunteers

The median age of the 15 CHMI volunteers was 22 years (range 18-36), 6 of them were female. One day before challenge (C-1) the full blood cell count, glucose concentration and renal- and liver function were all in the normal range. All volunteers developed parasitaemia after CHMI and received antimalarial treatment upon microscopic detection of *P. falciparum* parasites in a thick blood smear. Retrospective analysis with qPCR showed a median parasitaemia of 33 parasites/mL (IQR: 10-62) on DT.

Volunteers showed a heterogeneous response in serum TGF-β concentrations and could be divided in two groups; *i)* CHMI volunteers with a decrease in TGF-β concentration between C-1 and DT (9/15, median 1.56-fold reduction (range 1.10-2.25) were defined as TGF-β negative responders) and *ii)* CHMI volunteers with a stable or increased TGF-β concentration (6/15, median 1.29-fold increase (range 1.02-4.39) were defined as TGF-β sustained and/or positive responders (**Figure 1, Table 1**)). The TGF-β concentration was lacking for one of the volunteers on DT, but the volunteer was regarded as a TGF-β negative responder, based on measurement 2 days after treatment. None of the parameters

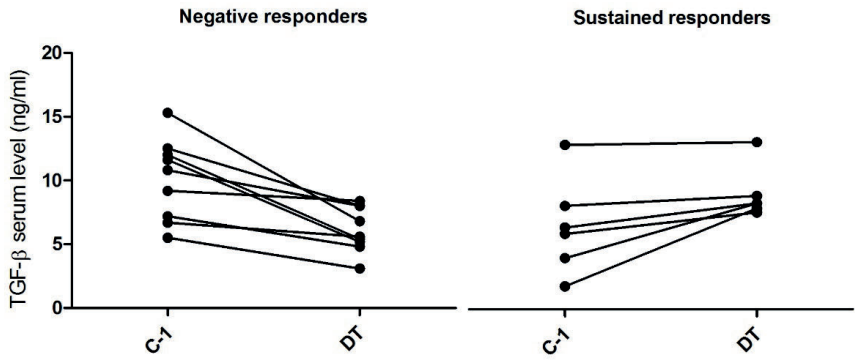

Figure 1 – Serum TGF-β concentrations before challenge and on day of treatment
C-1, day before challenge; DT, day of treatment. TGF-β, transforming growth factor beta.

	TGF-β negative responders	TGF-β sustained responders	p-value
Number of volunteers	9	6	NA
TGF-β (ng/mL) C-1	10.8 (6.9-12.3)	6.1 (3.4-9.3)	0.113
TGF-β (ng/mL) DT	6.2 (5.3-8.0)	8.3 (7.8-10.0)	0.043
Age (years)	22 (19-23.5)	20.5 (18.8-24.8)	0.864
Male/female (%)	66/33	50/50	0.622[a]
Time towards positive qPCR (days)	6.5 (6.5-7.0)	6.5 (6.5-7.0)	0.689
Time towards positive thick smear, DT (days)	8.5 (7.0-10.5)	7.0 (6.9-9.0)	0.328
Parasite count at first positive qPCR (parasite/mL, qPCR)	1.1 (0.1-14.5)	3.0 (0.5-25.1)	0.456
Parasite count on DT (parasite/mL, qPCR)	18.2 (7.9-57.7)	33.5 (13.2-85.0)	0.689

Table 1 – Characteristics of CHMI volunteers in TGF-β negative and sustained responders
Clinical and parasitological characteristics for TGF-β negative responders and TGF-β sustained responders at baseline (C-1). Data shown as median with interquartile range (IQR), Mann-Whitney test, unless otherwise stated. *C-1; 1 day before challenge, DT; day of treatment, TGF-β, transforming growth factor beta. qPCR; quantitative polymerase chain reaction.* [a]Fisher's exact test

(**Table 2**) showed any significant difference in absolute values between TGF-β negative and sustained responders at baseline (C-1) (not shown), except the absolute lymphocyte count, which was significantly lower ($1.9x10^9$/L; IQR 1.7-2.1) in the negative responders than in the sustained responders ($2.2x10^9$/L; IQR 2.0-2.5) (p=0.026).

Pro-inflammatory cytokine concentrations of CHMI volunteers

The profiles of circulating pro-inflammatory cytokines differed between the volunteers and correlated with the TGF-β profiles. For the TGF-β negative responders the IL-6 and IFN-γ concentrations were significantly increased on DT compared to C-1, whereas none of these parameters changed in the TGF-β sustained responders (**Table 2**). In both the TGF-β negative and sustained responders the lymphocyte count was decreased, the IL-12p70 concentrations were constant and TNF-α concentrations were significantly increased on DT compared to C-1. The levels of neither IFN-γ nor IL-6 on DT correlated with parasitaemia. However, a negative correlation was observed between parasitaemia upon first detection by PCR and the IL-6 peak (as measured upon first detection by PCR or on DT) (-0.642; p=0.013).

	TGF-β negative responders			TGF-β sustained responders		
	C-1	DT	P-value	C-1	DT	P-value
IL-6 (pg/mL)	Below detection limit	2.3 (1.4-8.1)	0.012	Below detection limit	1.22 (0.9-2.2)	0.109
IL-12p70 (pg/mL)	Below detection limit	6.9 (5.8-14.2)	0.063	Below detection limit	5.8 (5.8-6.7)	0.317
IFN-γ (pg/mL)	Below detection limit	13.5 (1.9-25.4)	0.028	Below detection limit	2.2 (0.3-6.2)	0.109
TNF-α (pg/ml)	4.3 (3.9-5.1)	7.9 (5.8-14.0)	0.008	3.7 (2.4-6.4)	4.7 (2.8-15.7)	0.027
Lymphocytes (*10^9/L)	1.9 (1.7-2.1)	0.8 (0.6-1.2)	0.011	2.2 (2.0-2.5)	1.8 (0.9-1.9)	0.028
Platelets (*10^9/L)	248 (194-263)	165 (141-244)	0.011	196 (170-247)	183 (168-248)	0.917
D-dimer (mg/L)	0.23 (0.19-0.35)	1.57 (0.45-2.04)	0.012	0.22 (0.19-0.26)	0.27 (0.24-0.31)	0.092
VWF (μg/mL)	12.2 (9.3-13.4)	19.5 (13.1-24.8)	0.017	9.4 (7.7-11.0)	11.1 (6.5-14.1)	0.753
Temperature (^oC)	36.1 (35.7-36.4)	37.3 (36.9-38.3)	0.028	35.9 (35.6-36.3)	36.5 (36.1-36.7)	0.173

Table 2 – Immunological and hematological parameters in TGF-β negative and sustained responders

The Wilcoxon signed rank test was used for comparison between C-1 and DT. *C-1; 1 day before challenge, DT; day of treatment, VWF, Von Willebrand factor.* Data shown as median with interquartile range (IQR).

Haemostasis and endothelial cell activation of CHMI volunteers

Endothelial cell activation and coagulation were activated in a subset of volunteers: a 50% increase on DT compared to C-1 concentrations was observed in 4 of 15 volunteers for VWF (3 of 4 were TGF-β negative responders) and for 6 of 15 volunteers for D-dimer (all of them were TGF-β negative responders). Both parameters strongly correlated with each other (Spearman's rho: 0.753; p-value:0.002). The pro-inflammatory cytokine TNF-α also correlated positively with both VWF (0.538; 0.047) and D-dimer (0.520; 0.047). TGF-β negative responders showed increased concentrations of D-dimer and VWF, as well a significantly decreased platelet count on DT compared to C-1. In contrast none of these parameters changed significantly in the sustained TGF-β responders.

Clinical and parasitological parameters correlate with TGF- β profiles

Finally, we investigated whether the pro-inflammatory profile in the TGF-β negative responders was associated with clinical symptoms. We found a higher body temperature and more episodes of fever (p=0.036) (**Table 3)** in the TGF- β negative responders compared to the sustained responders; none of the other clinical symptoms differed between the groups. The number of fever episodes correlated strongly with the IFN-γ concentration on DT (Spearman's rho 0.757; p=0.002). The number of grade 2 and 3 clinical symptoms together (p = 0.036) was significantly higher in the TGF-β negative responders. However, the parasitaemia did not show a significant difference on the day of the first positive PCR and on DT (**Table 1**). Also, parasite growth (defined as: (parasitaemia DT – parasitaemia on first positive PCR)/days between DT and first positive PCR) did not differ between the groups (p=0.105). No differences in TGF-β dynamics between the volunteers infected with the distinct *P. falciparum* strains (NF54, NF135 and NF166, respectively) were observed (Fisher's exact test: p=1.000).

Discussion

Here we show that the TGF-β profiles are associated with heterogeneity in host responses observed in controlled human malaria infection study (CHMI). Previously it has been shown that TGF-β is the first cytokine that increases detectably after *P. falciparum* infection in a subset of volunteers in a CHMI (3, 5). We confirmed this TGF-β increase in a subset of volunteers after infection with *P. falciparum.* while we found a TGF-β decrease in others. The malaria naïve volunteers infected with *P. falciparum* with a decrease in TGF-β concentration showed *i*) a higher degree of endothelial cell activation. *ii*) a higher degree of coagulation activation. *iii*) a higher body temperature. *iv*) a higher

Adverse Events Average episode per volunteer	Sustained responders (n=6)				Negative responders (n=9)				
	Grd 1	Grd 2	Grd 3	Total	Grd 1	Grd 2	Grd 3	Total	p-value
Headache	0.7	0.8	-	1.5	0.8	0.8	-	1.6	NS
Malaise	0.5	0.3	-	0.8	0.2	0.2	0.3	0.8	NS
Fatigue	0.5	0.3	-	0.8	0.2	0.2	0.1	0.5	NS
Myalgia	0.7	0.2	-	0.9	0.7	0.2	-	0.9	NS
Chills	0.3	0.2	-	0.5	0.3	0.2	-	0.5	NS
Common cold	0.5	-	-	0.5	0.2	-	-	0.2	NS
Fever	0.3	-	-	0.3	0.1	0.9	0.2	1.2	0.036
Abdominal pain	0.2	-	-	0.2	0.2	0.2	-	0.4	NS
Nausea	-	0.2	-	0.2	0.6	0.3	-	0.9	NS
Diarrhoea	0.2	-	-	0.2	0.3	-	-	0.3	NS
Decreased appetite	0.2	-	-	0.2	0.1	-	-	0.1	NS
Dyspnoea	0.2	-	-	0.2	-	-	-	-	-
Swelling jaw	-	-	-	-	0.2	-	-	0.2	-
Blurry vision	-	-	-	-	0.1	-	-	0.1	-
Orbital oedema	-	-	-	-	0.1	-	-	0.1	-
Lower back pain	-	-	-	-	-	0.1	-	0.1	-
Total number of episodes per volunteer (median, IQR)	4.0 (1.8-6.5)	1.0 (1.0-3.5)	-	6.0 (6.8-9.0)	4.0 (4.0-5.0)	3.0 (2.0-4.0)	0.0 (0.0-1.0)	8.0 (6.5-10.0)	NS
Duration op episodes (days) (median, IQR)	10.5 (2.1-19.3)	4.6 (1.4-8.1)	-	13.7 (7.2-24.6)	4.4 (3.0-5.7)	6.6 (3.5-10.7)	0.0 (0.0-5.0)	12.5 (8.6-19.8)	NS

Table 3 – Clinical symptoms in TGF-β negative and sustained responders
Grade 1. Mild; no interference with daily life; Grade 2. moderate: some interference with daily life; Grade 3. severe: requiring bed rest. IQR; interquartile range. NS; not significant.

number of clinical symptoms compared to the volunteers with a sustained or increased TGF-β concentrations. These results suggest that the TGF-β profile is able to define two distinct host-response types with up regulated pro-inflammatory cytokines. activation of endothelial cells and coagulation with relatively more clinical symptoms. The second profile is characterized by stable or early increased TGF-β concentrations with a modest increase of pro-inflammatory cytokine concentrations. a lower degree of endothelial cell activation and coagulation and less clinical symptoms. As the CHMI volunteers had to be treated relatively early after infection compared to malaria patients in endemic areas. the observed differences in host response could not be followed until advanced or even severe malaria would occur.

Both activation of endothelial cells and formation of coagulation products have impact on malaria pathogenesis (17, 18). They are upregulated in response to pro-inflammatory responses (17, 18) as illustrated by the positive correlation of TNF-α with VWF and D-dimer. Pro-inflammatory cytokine production is downregulated by TGF-β. by inhibiting type-1 helper T-cell activation (19). TGF-β negative responders have a higher degree of endothelial cell activation and coagulation than sustained responders. This shows that the early immune-modulatory cytokine response is associated with upstream clinical pathology during malaria. Higher serum TGF-β concentrations are protective during clinical malaria being associated with a lower number of clinical symptoms (12). However. higher serum TGF-β concentrations are only beneficial during clinical malaria and the timing of release seems essential for the subsequent downstream effects. Whereas high serum TGF-β prevent over activation of the pro-inflammatory response during clinical disease in malaria endemic areas. an anti-inflammatory environment during early disease might result in impaired ability to control parasite replication during the blood phase. as shown in a mouse model (20). Mice infected with a lethal *P. yoelii* strain had an increase in TGF-β concentrations and were less able to kill parasites in the days after infection. which resulted in a major rise in parasite number and death (20). TGF-β did not increase in mice infected with a nonlethal *P. yoelii* strain early during the infection (20). We did not find a correlation between parasite replication and TGF-β concentrations. The time period of parasitaemia in this CHMI might be too short to measure differences in parasite replication as one cycle takes 44 hours (21). CHMI studies with a longer time to treatment can be used for evaluation of cytokine responses on the parasite growth. Interestingly, a negative correlation was observed between the IL-6 peak concentration and the first wave of parasitaemia post-infection, which may suggest a direct effect of IL6 on parasite release from the liver. Indeed, IL6 has been shown to block parasite liver-stage development in murine malaria models (22).

The aetiology of the observed differences in initial responses upon a *P. falciparum* infection is unknown. However, difference in lymphocyte count at baseline between TGF-β negative and sustained responders are suggestive for pre-infection differences, which have been shown to be present in healthy individuals (23). Different gene expression patterns in a broad diversity of populations worldwide were found to be predictive for immune responsiveness (24) and the lymphocyte/neutrophil ratio predicts cytokine receptor activity (24). Genetics, age, previous infections and seasonal changes do affect this immune set-point (23, 24). CHMIs appear to be a valuable tool to elucidate the relation between the baseline immune responsiveness and initial host response after infection and to evaluate the implication for immunization strategies.

Platelet count decrease was previously suggested to be due to the differences in TGF-β concentrations during clinical disease in children in a malaria endemic area (12) as TGF-β is stored in platelets and released upon their activation (25). In our study. we cannot confirm this correlation or find an increase is platelet activation markers (platelet factor 4 in EDTA-blood or hydroxy thromboxane b2 in urine) (data not shown). In addition. the TGF-β changes in our study are observed before treatment whereas the platelet count starts to decrease shortly afterwards. as shown before (13). The combined data strongly suggest that the TGF-β changes in our study are not caused by platelet activation. Also. the number of parasites emerging from the liver are not likely to result in the different profiles as they did not correlate with TGF-β levels.

Although our data generally corroborate those from previous studies. conclusions on the effect of TGF-β concentrations on pathology and clinical symptoms should be drawn with caution due to the small sample size. In this study 3 groups of volunteers were infected with 3 distinct *P. falciparum* strains. Although the prepatent period was longer and the first parasitaemia lower after infection with NF54 compared to NF135.C10 and NF166. C8 (15), no correlation between prepatent period, parasitaemia and cytokine levels was observed. Also, the overall number of adverse events and clinical symptoms did not differ between the groups. As cytokine concentrations are known to correlate with clinical symptoms, this is in line with our finding of an equal distribution of pro-inflammatory and immunomodulatory volunteers over the 3 distinct strains. In addition, previous research shows different TGF-β dynamics in a group of CHMI volunteers infected with a single strain (NF54). So, there is no indication of a confounding relationship between Plasmodium falciparum strain and a pro-inflammatory and immuno-modulatory host response.

In conclusion, the host response in malaria naïve volunteers upon infection with *P. falciparum* can be divided in 2 groups: those with a TGF-β decrease together with an up regulation of pro-inflammatory processes resulting in symptomatic disease and those with a sustained or increased TGF-β concentrations without a significant up-regulation of pro-inflammatory processes and only mild malaria symptoms. This early stage immune response correlates with the extent of endothelial cell activation and coagulation and might originate from the immune responsiveness at baseline. CHMIs are highly valuable tools to further evaluate the effect of pre-infection immune responsiveness on the host response after infection and early TGF-β serum concentrations might function as a marker for downstream clinical symptoms and pathology.

Authors' contributions

GMDJ. JJVH. PJGV and AV conceived of the study. GMDJ performed the laboratory experiments together with RK. Haematological experiments were supervised by RU and cytokine concentrations were determined in the laboratory of WD. GMDJ wrote the manuscript which was reviewed by RWS. JVH. AV. PJVG. RK. MMC. RU. LW. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

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Chapter 7

Baseline immune activation status is associated with differential activation patterns and induction of protective immunity in the Controlled Human Malaria Infection model

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Abstract

Background

Immune activation status is heterogeneous in the steady-state amongst healthy individuals and co-determines host responses to infections. This study investigated the relationship between a number of pro- and anti-inflammatory cellular immune markers and plasma TGF-β concentrations in malaria naïve Dutch volunteers at baseline and their dynamics during Controlled Human Malaria Infection (CHMI).

Methods

Single CHMIs; circulating TGF-β concentrations and the percentage of *in vitro* IFN-γ producing lymphocytes and regulatory T-cells were determined in blood samples before and after infection (n=35), in relation to parasitaemia. *Sporozoite immunization studies;* In 30 participants, TGF-β concentrations were measured at baseline and after the first immunization, in relation to parasite dynamics.

Results

After a single infection, volunteers with high TGF-β concentrations at baseline tended to show a decline after infection (negative responders) (n=24); in contrast, lower concentrations at baseline were associated with increased/sustained TGF-β concentrations (n=11) (p=0.001) (sustained responders). The percentage of regulatory T-cells (Treg) correlated negatively with TGF-β concentrations at baseline (r = -0.335; p=0.049) and induction of Treg correlated positively with the rate of parasite growth (r=0.549; p=0.002). The negative TGF-β responders had higher *in vitro* IFN-γ production by lymphocytes after treatment than the TGF-β sustained responders (p=0.033). Rapid acquisition of sterile immunity after sporozoite immunization correlated with higher baseline concentrations of TGF-β (p=0.028) and with a TGF-β decrease during the first immunization (p=0.032).

Conclusion

Low TGF-β concentrations at baseline predict an increase in anti-inflammatory immune responses after a single infection and slower induction of protective immunity after sporozoite immunization in the CHMI model.

Introduction

Healthy malaria naïve participants of controlled human malaria infection (CHMI) studies show varied severity of clinical symptoms and cytokine dynamics following infection (1). A subset of CHMI participants shows an early increase of pro-inflammatory cytokines (2) especially IFN-γ (3), which was associated with more severe clinical pathology (e.g. fever and coagulation activation) and inhibition in the rate of parasite growth (4). NK cells and T-cells, especially the Th1 subset, are the main producers of IFN-γ during early malaria infection (2, 5). A second subpopulation of CHMI participants typically displays an increase in TGF-β (1). In contrast to IFN-γ, TGF-β exerts multiple immune-modulatory effects including induction of regulatory T-cells (Treg) and inhibitions of Th1 differentiation, resulting in reduction of pathology e.g. by reducing fever (6) and by hampering the activation of coagulation and endothelial cells (1, 7-9). A likely source of TGF-β within the peripheral blood during malaria infection are CD14+ monocytes (4). However, other cells, including hepatocytes and tissue macrophages might also contribute to TGF-β production (10). The reasons for the variation of TGF-β kinetics during CHMI are not understood. While genetic differences may play a part, baseline immune activation status (the threshold for activation of immune cells) in the steady-state may also affect immune outcomes upon infection or immunization. In addition, previous exposure to particular immunogens may result in increased non-specific innate immune responses upon encountering a heterologous pathogen (11-13). This was shown when malaria naïve healthy participants received BCG-vaccination prior to P. falciparum experimental infection, which resulted in an increase in clinical symptoms (e.g. fever) and rapid monocyte activation compared to non-vaccinated participants. In addition, in a previous study we demonstrated that participants with an immune modulatory skewed cytokine profile (high TGF-β during infection) present with higher baseline lymphocyte counts (1). This may suggest that heterogeneous immunity and pathology in CHMI may be co-determined by immune status prior to infection. Here we studied the possible relationship between circulating TGF-β concentrations, Treg percentages, and the capacity of lymphocytes to produce IFN-γ upon in vitro stimulation with parasitological and clinical responses in the CHMI model.

Methods

Study design and approval

Samples were collected from four (A-D) CHMI studies (Study A, NL41004.078.12; Study B NL48704.000.14; Study C NL48301.091.14, Study D, 48732.091.14). Participant characteristics, clinical, and parasitological course have been described previously (14-16) (NCT02080026). Briefly, in study A (15), 15 study participants were infected with *P. falciparum* parasites by 5 infected mosquito bites. In study B (14, 15), 24 participants were allocated over 6 groups and exposed to 1, 2 or 5 bites of *P. falciparum* infected mosquitoes. Only the 20 participants who developed parasitaemia in study B were included in our current study. Twice-daily parasitaemia monitoring with thick blood-smear analyses (study A) or quantitative polymerase chain reactions (qPCR) (study B) started 5 days after infection. Study participants received anti-malaria treatment with a 3-day curative regimen of Atovaquon/Proguanil 1000/400 mg (Malarone ®, GlaxoSmithKline) upon a positive thick smear (study A) or on 2 consecutive qPCR positive whole blood samples with >500 parasites/mL (study B).

Studies C (n=8) (NCT02080026) and D (n=24) (16) followed the 'Chemoprophylaxis with Sporozoites (CPS)' immunization protocol. Study participants were immunized three or four times (time-intervals between each immunization is 4 weeks) by bites of 15 *P. falciparum* infected mosquitoes while receiving a prophylactic dose of chloroquine (300 mg once a week). Ten weeks (study C) or fourteen weeks (study D) after discontinuation of chloroquine prophylaxis, the subjects were challenged with bites of 5 *P. falciparum* infected mosquitoes. Subjects were checked twice daily for blood parasitaemia as measured by qPCR from days 5 to 21 after challenge and treated with Atovaquon/Proguanil at the end of study (day 28) or earlier if parasitaemia rose above 100 *Pf*/ml (17).

In all four studies, symptoms were monitored and clinical laboratory parameters were determined during all control visits. Parasitaemia was measured by qPCR as described previously (17). The Institutional Review Board of the Harbour Hospital (Study A and B) and Radboudumc (C and D) and the Dutch Central Committee on Research involving Human Subjects approved the study protocols and add-on studies. All CHMI participants provided written informed consent.

Sample collection

From studies A and B, serum samples were collected and stored at -80°C and venous whole blood samples were collected in Vacutainer Cell-Preparation Tubes (CPT, Becton Dickinson)

on baseline and 2 (Study A) or 3 days (Study B) after start of treatment. Peripheral blood mononuclear cells (PBMCs) were isolated and frozen in dimethyl sulfoxide supplemented with 10% foetal calf serum and stored in liquid nitrogen. From studies C and D, citrate plasma samples were collected in a similar manner one day before immunization 1 (I1-1) and one day before the second immunization (I2-1) and stored at -80°C.

Cytokine measurements (all studies)

TGF-β concentrations were determined by ELISA (R&D Systems) according to the manufacturer's instructions in baseline samples from all 67 participants (study A-D). In addition, TGF-β was measured in studies A and B (infection studies) on the day of treatment decision (DT), and in studies C and D (CPS immunization) one day before the second immunization (I2-1), which was 28 days after the first immunization.

Measuring IFN-γ production in PBMCs of CHMI volunteers

For study B, cryopreserved PBMCs from baseline and three days after treatment were thawed and resuspended in RPMI 1640 culture medium (Gibco) supplemented with 2 mM glutamine, 1mM pyruvate, 50 µg/ML gentamycine and 10% pooled A+ human serum (Sanquin, Nijmegen, the Netherlands) and transferred into 96-well round-bottom plates (Corning) at a concentration of $5x10^5$ cells in 100 µL/well for stimulation. Forty µg/mL brefeldin A (Sigma) and 5.3 µg/mL monensin diluted in 50 µL were added to each well (resulting in a final concentration of 10 µg/mL and 2µM, respectively) 3-4 hours prior to staining. Stimulations were carried out with 1 ng/mL PMA and 1µg/mL iomomycin for 4 hours at 37°C and 5% CO_2. For the stimulation assays, PBMC samples collected at the same CHMI time point were examined simultaneously. After stimulation the PBMCs were stained for the extracellular markers CD3–APC-eFluor780, CD4–AF647 and CD56-PerCP-Cy5.5. PBMCs were then permeabilised with the FoxP3 Fix/Perm Kit (eBioscience) and stained for intracellular IFN-γ (IFNγ-PeCY7; eBioscience). All flow cytometry was performed on a 10-colour Beckman Coulter Gallios and analysed using FlowJo software (version 10.0.8, Tree Star). Staining panels and gating strategy are shown in Supplementary data 1 and 2.

Phenotyping of monocyte and Treg populations in CHMI participants

For each participant in studies A and B a sample from baseline and three days after treatment was thawed and cells were immediately stained for phenotyping. Samples of the same participants of the two time points were tested simultaneously (for colour panels and gating strategy see Supplementary data 1 and 2). Staining panels and gating strategy are shown in Supplementary data 1 and 2.

Statistical analysis

The Wilcoxon-Signed Rank test was used for comparison of concentrations of the same parameter in one study participant over time. For independent comparison the Mann-Whitney test was used. For correlations, Spearman's rho was used. As none of the parameters were normally distributed, data were presented as median with interquartile range unless stated otherwise. For comparing distributions of parameters between the groups of CHMI participants infected with the three different *P. falciparum* strains the Kruskal-Wallis test was used. The Fisher's exact test was used for categorical data. All analyses were performed with SPSS version 24.0 (SPSS Inc, Chicago, IL, USA).

Results

Volunteer characteristics

A total of 35/39 CHMI participants across two separate CHMIs (Study A and B) developed parasitaemia and were included in this analysis (Table 1). In study C and D (CPS immunization studies) 32 participants successfully completed three rounds of immunisation with 15 *P. falciparum* infected mosquitoes under chloroquine prophylaxis. Two volunteers from study D were not included in the analysis due to absence of samples. During the first CPS immunization, all 30 included participants developed transient parasitaemia under chloroquine prophylaxis. However, after the second immunization only 16/30 included participants developed parasitaemia (slow responders) and 14/30 remained aparasitaemic (fast responders). An overview of the studies and data on parasitaemia is listed in **Table 1**.

Kinetics of circulating TGF-β and monocyte function during CHMI

TGF-β blood concentrations decreased in 24/35 participants after P. falciparum infection compared to baseline (median 0.6-fold; IQR 0.4-0.8; 'negative responders') and remained constant or increased in 11 participants (1.6-fold increase; 1.3-2.1; 'sustained responders') (**Figure 1A**). The circulating TGF-β concentration at baseline was significantly higher in the TGF-β negative responders (11.2 ng/mL; 7.1-14.4) compared to the TGF-β sustained responders (5.1 ng/mL; 3.6-8.1) (p=0.0010) (**Figure 1A**). As TGF-β inhibits inflammatory immune responses, we investigated whether the ability of lymphocytes to produce IFN-γ, which is the most important pro-inflammatory cytokine during malaria, differed between TGF-β negative and sustained responders. Indeed, negative responders had a higher percentage of IFN-γ producing lymphocytes than the sustained responders after treatment (p=0.033) (**Figure 1B**).

Study	A TIP3	B BMGF2a	C BMGF1	D BMGF2b
Number of participants	15	20/24	8	22/24
Study type	CHMI	CHMI	Immunization (3x), subsequent controlled homologous infection	Immunization (3x), subsequent controlled heterologous infection
Study design	3 groups of 5 participants. Each group was infected with bites of 5 mosquitoes (NF54, NF135.C10 or NF166.C8)	6 groups of 4 participants. Each group was infected with bites of 1,2 or 5 mosquitoes (n=12 NF135.C10 or n=12 NF166.C8	Immunization: bites of 15 infected mosquitoes (NF54) during chloroquine prophylaxis.	Immunization: bites of 15 infected mosquitoes (NF54) during chloroquine prophylaxis.
Treatment criteria	Positive thick smear	2 consecutive qPCRs >0.5 parasites/µL	NA	NA
Parasitaemia on day of treatment (parasites/µL) (median, range)	33 (0.68-155)	7 (0.91-39)	NA	NA
Time to day of treatment (median, range)	8.0 (6.5-10.5)	7.3 (7.0-11.0)	NA	NA
DT TGF-β concentration (ng/mL)	7.9 (5.6-8.4)	6.6 (5.8-7.9)	[1]0.4 (0.4-0.6)	[1]0.4 (0.4-0.5)

[1]One day before immunization 2 instead of day of treatment (DT).

Table 1 – volunteer and study characteristics

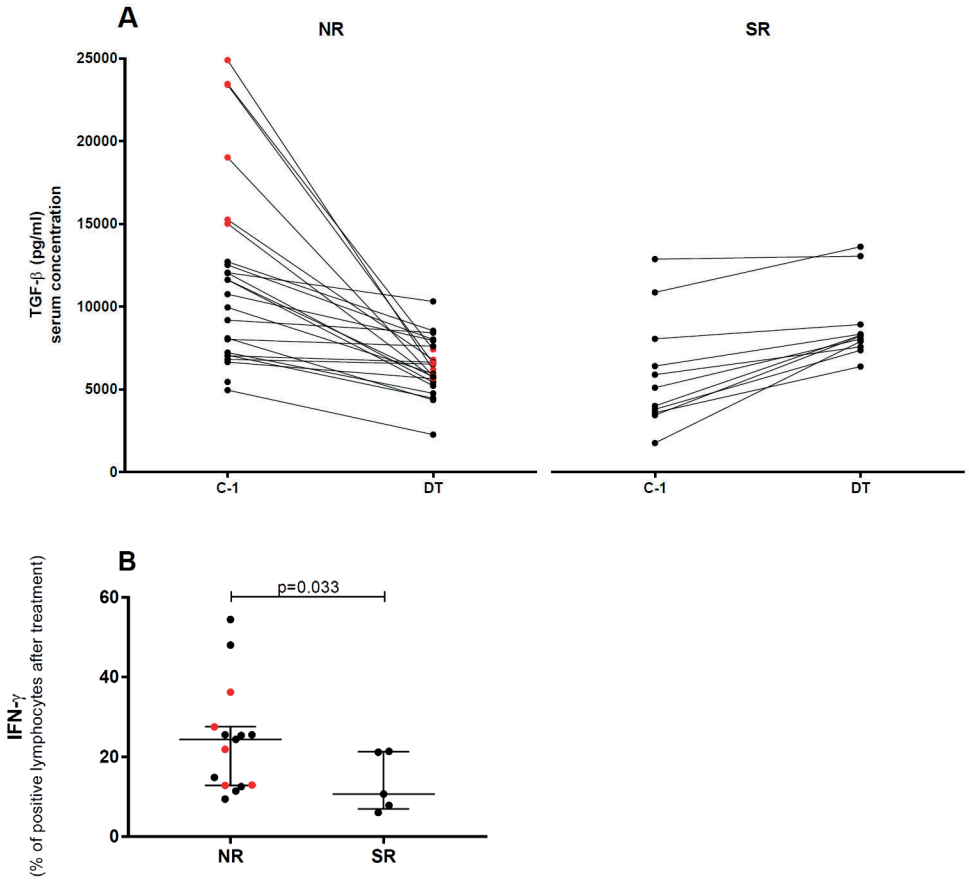

Figure 1 – Cytokine profiles in a CHMI
(A) TGF-β blood concentrations in negative responders and sustained responders of CHMI study A and B**.** The 6 participants with highest TGF-β blood concentration at baseline are shown in red**.** **(B)** IFN-γ production by PMBC of sustained and negative responders of study B collected at DT after *in vitro* stimulation by PMA/ionomycin. *C-1; 1 day before challenge; DT, Day of treatment***.** *NR, negative responders; SR, sustained responders; TGF-β, transforming growth factor-beta*

The six participants with the highest TGF-β blood concentrations at baseline (**Figure 1, red dots**) showed the largest decrease in TGF-β after CHMI, with a TGF-β ratio of DT/baseline of 0.31 (IQR: 0.27-0.40) compared to the other 18 negative TGF-β responders (0.77 (0.57-0.95) (p<0.001)). Monocytes are a major source of TGF-β and the six participants with the highest TGF-β concentration at baseline showed significantly higher peripheral blood monocytes numbers than TGF-β negative responders (p=0.008) (**Figure 2A, red dots**). While as a whole the total group of negative responders displayed equal monocyte numbers to the sustained responders (**Figure 2A**), the TGF-β/monocyte ratio was clearly different

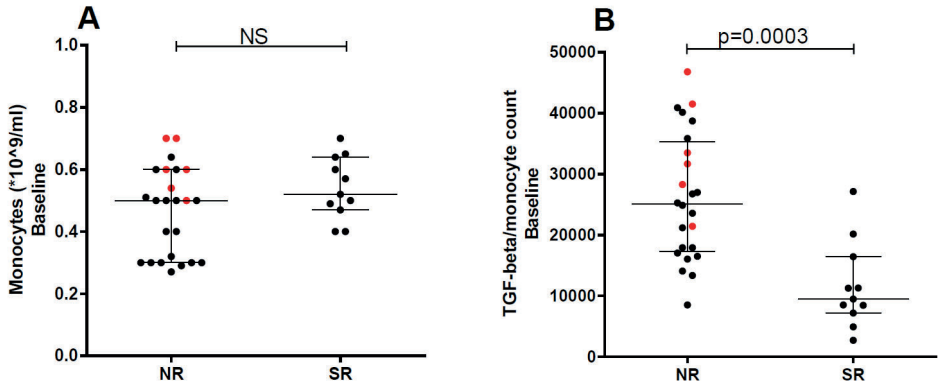

Figure 2 – Monocyte counts in TGF-β negative and sustained responders
(A) Monocyte count in TGF-β negative and sustained responders. **(B)** The TGF-β/monocyte ratio in TGF-β negative and sustained responders. The 6 participants with highest TGF-β blood concentration at baseline are shown in red**.** *NR, negative responders; SR, sustained responders; TGF-β, transforming growth factor-beta*

(p=0.0003) (**Figure 2B**). This suggests that there is a subset of monocytes producing high levels of TGF-β. concentration.

Treg induction is associated with attenuation of IFN-γ mediated antimalarial immunity during CHMI

TGF-β is known to induce Treg, but a negative correlation was found between TGF-β plasma concentrations and the percentage of Treg at baseline (Spearman's rho: -0.335, p=0.049) (**Fig 3A**). However, an increase in Treg percentage was strongly associated with both an increase in parasitaemia over time (parasitaemia on day of treatment compared to parasitaemia on first positive qPCR) (Spearman's rho: 0.549, p=0.002) (**Fig.3B**) as well as the parasitaemia on the day of treatment (Spearman's rho: 0.524, p=0.002) (**Fig.3C**). As IFN-γ producing lymphocytes are inhibited by Treg, we looked for possible correlations between IFN-γ production of in vitro stimulated lymphocytes and induction of Treg. An increase in the percentage of Treg over time (2 or 3 days post treatment versus baseline) was clearly associated with a decrease in the percentage of IFN-γ producing lymphocytes over the same time period (Spearman's rho: -0.507, p=0.027) (**Fig. 3D**). The increase of Tregs was only observed in a proportion of CHMI participants (17/31); no correlation was observed between TGF-β and Treg kinetics (Fisher's exact test p≥0.05). Altogether, these data suggest a complex interplay between immune cells and cytokines in which immuno-modulatory responses are associated with inhibition of pro-inflammatory cytokine responses and loss of ability to inhibit parasite growth.

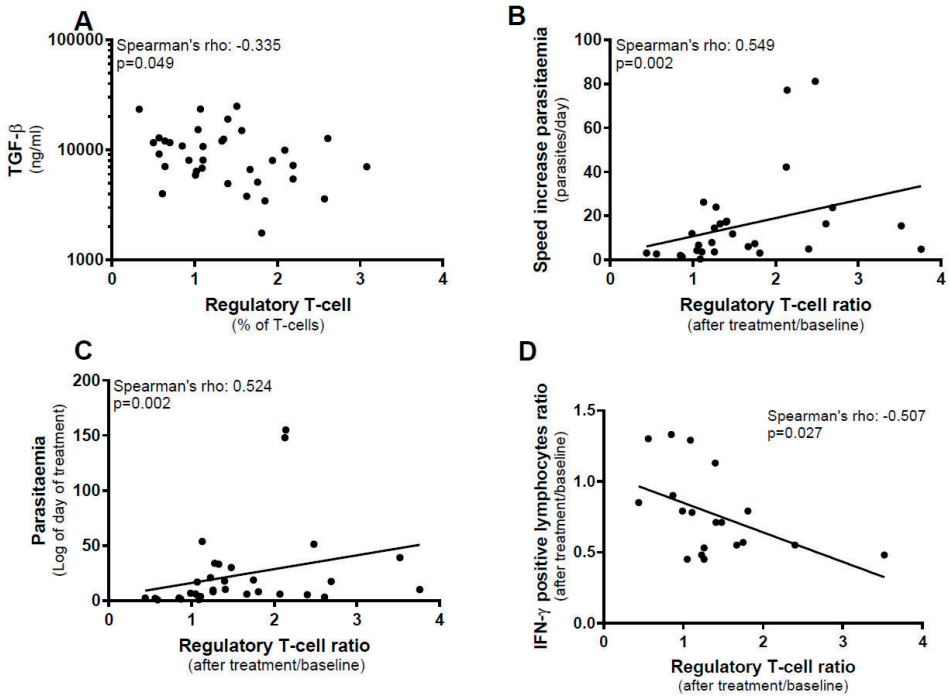

Figure 3 – Correlations of regulatory T-cells with parasitaemia
(A) Correlation between TGF-β concentration correlates and Treg (% of T-cells) on baseline (Study A and B) (p=0.049). **(B)** Correlation between Treg ratio (post-treatment/baseline) and speed increase of parasitaemia ((parasitaemia on day of treatment – parasitaemia at baseline)/time to DT)) (spearman's rho; p value; 0.549; 0.002). **(C)** Correlation between Treg ratio (post-treatment/baseline) and parasitaemia on day of treatment (Study A and B). **(D)** Correlation between Treg ratio and IFN-γ ratio. *Treg, regulatory T-cell.*

Baseline TGF-β and TGF-β dynamics correlate with induction of protection in CPS immunization

Since plasma TGF-β concentrations at baseline differentiated pro-inflammatory and immuno-modulatory profiles after CHMI, we also studied a possible association with acquisition of protective immunity after CPS immunization. Thirty-two CPS immunized volunteers were divided into fast responders and slow responders based on the number of immunizations required to become sterilely protected against a homologous challenge infection (14). TGF-β levels at baseline were significantly higher in fast responders (median; IQR) (448 pg/ml; 389-589 pg/ml) compared to slow responders (376 pg/ml; 332-426 pg/ml) (p=0.028) (**Fig. 4A**). Next, volunteers were divided into pro-inflammatory and immuno-modulatory groups as described previously (1). As in studies A and B, the

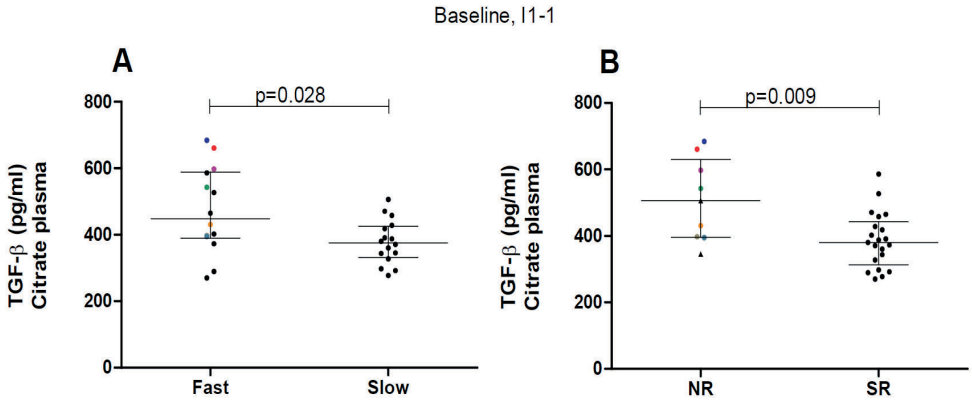

Figure 4 – Plasma TGF-β concentration in slow and fast responders during immunization
(A) TGF-β citrate plasma concentrations in slow and fast responders at baseline (p=0.028) **(B)** TGF-β citrate plasma concentrations in TGF-β negative and sustained responders. (Study C and D). *Dots with the same colour in A and B represent the same CHMI participant I1-1, 1 day before immunization 1. NR, negative responders; SR, sustained responders; TGF-β, transforming growth factor-beta*.

baseline TGF-β concentration was higher in negative responders (median, IQR) (507 pg/ml, 396-629 pg/ml) than in sustained responders (381 pg/ml, 313-443 pg/ml) (p=0.009) (**Fig. 4B**). Participants that showed a TGF-β decrease during immunization (negative responders) were significantly more likely to be fast responders (7/9; 78%) compared to participants with a TGF-β decrease during immunization (sustained responders) (7/21; 33%) (Fisher's exact test; p=0.032). The combined data suggest that the immune response during immunization correlates with patterns of immune function that are present prior to immunisation and affect acquisition of sterile immunity.

Discussion

In this study the pre-infection immune activation status of individuals was investigated by the measurement of: 1) TGF-β blood concentrations and 2) Treg percentage. These baseline markers differ significantly between CHMI participants with either a pro-inflammatory or an immune-modulatory cytokine response during infection in this study. Therefore, pre-infection immune activation status of an individual may co-determine the final immune response upon malaria infection in a non-antigen specific manner and may, therefore, partly explain heterogeneity. In addition, Treg induction was found to correlate negatively with IFN-γ production capacity during infection. As Treg are well recognized for their capacity to downregulate a pro-inflammatory Th1 response, this might explain

the positive correlation between Treg and parasitaemia. T-reg inhibition of Th1 response resulting in limited IFN-γ production might decrease parasite growth inhibition and result in a higher parasitaemia.

During CPS-immunization, low baseline TGF-β blood concentrations correlated with more rapid acquisition of immunity: study participants with sustained or increased TGF-β concentrations after immunization were more likely to be slow responders. Such correlations between pre-infection immune status and responses during infection may predict whether an individual will develop sterile immunity or the number of immunizations needed to reach sterile immunity.

The etiology of these differences in pre-infection TGF-β concentrations between the study participants is unknown. Multiple cell types produce TGF-β, with Treg and monocyte-derived macrophages as the primary producers. Previously, monocytes stimulated with *P. falciparum* infected erythrocytes were found to produce TGF-β (4). However, these in vitro data do not exclude other cellular source of TGF-β *in vivo*, as have been suggested (10). Although we could not measure intra-cellular TGF-β in monocytes of healthy volunteers after CHMI (data not shown) the TGF-β/monocyte count ratio at baseline could discriminate between pro-inflammatory and immuno-modulatory participants better than the TGF-β concentration in isolation, emphasizing the role of monocytes as TGF-β producers and their importance for control of immunity.

Tregs are also TGF-β producers and in a positive feedback loop, TGF-β is known to induce Treg differentiation (19). However, we observed an unexpected negative correlation between TGF-β and Treg ($CD25^+CD4^+FOXp3^+$ T-cells). This apparently contradictory negative correlation may relate to different Treg subsets as TGF-β is required for Treg generation in the thymus (tTreg) but not for Treg induction in the peripheral blood (pTreg) (20, 21). Second, low ubiquitination of FOXp3+ Treg is a major driving force for FOXp3+ stability, which is linked to suppressive potential of Treg (22) and might explain the negative correlation between Treg and TGF-β in our study. However, an increase in Treg proportions was not observed in all CHMI participants. Further evaluation of the different Treg subsets and their level of ubiquitination in the sustained and negative responders is of interest for increase understanding of the heterogeneity in host response.

In previous malaria studies, the presence of Treg with inhibitory properties was associated with lack of upregulation of the cytotoxic marker CTLA-4 (23). Others also found that low Treg numbers were associated with improved protection after immunization (24). This is in line with our observation that the percentage of induced T-reg strongly correlated to parasite growth rate, as was shown before (4). The cause of these differences in T-reg

induction between individuals is unclear so far. However, for T-reg a specific epigenetic signature seems necessary for long-term stable commitment (25). Epigenetic signatures (specific DNA methylation and histone modification) are known to be modulated by the number of pathogen encounters as well as the type of pathogen encountered (26). For example, helminth infections are known to skew the host response towards an immune-modulatory direction (27) resulting in a decreased pro-inflammatory immune response upon *Plasmodium* spp. infection, and to vaccinations or infections other than malaria (28-30). The relation between the baseline immune activation status, initial TGF-β response upon immunization, and immunization efficacy was shown in this study. A low TGF-β blood concentration and an immune-modulatory cytokine profile during immunization was associated with a high number of immunizations needed to become sterile protected. A pro-inflammatory response seems to be necessary for the induction of immune memory (31). Therefore, TGF-β blood level might represent a marker that predicts malaria immunization efficacy at an early stage.

Possible limitations of the study are the small sample size per study and differences in *Plasmodium falciparum* strain used in the distinct studies. Despite the differences in study design, namely different strains, numbers of mosquito bites, or timepoints of sample collection, all four studies clearly identified two type of responses related to TGF-β kinetics (negative and sustained responders), in a total of 67 individuals. This data further strengthens the existence of two different TGF-β response groups in case of malaria infection (1, 2). Although these data are in line with previous studies, conclusions should be drawn with caution as our study is of observational nature, thus showing correlations rather than direct causality. Lastly, the subgrouping of CHMI participants based on their TGF-β response is sufficient to categorize differences in host responses. However, even within these subgroups (negative and sustained TGF-β responders) the immune response may still be heterogeneous (1, 2). The pro-inflammatory or immune modulatory responses upon a malaria infection are therefore rather a scalable than a binary outcome. Further definition of the immune activation status by e.g. RNA-sequencing and epigenetic studies that compromise multiple pro-inflammatory and immune-modulatory pathways could add to the current knowledge. However, it is unlikely that a single baseline marker will predict the clinical disease course or immunization efficacy, and any functional read-out for immune activation status should be evaluated for its predictive value.

Taken together, these data showed that baseline differences in immune activation status correlate with host responses after P. falciparum infection and with immunization efficacy in CHMIs. The effects of pre-infection immune activation status on responses to a pathogen have been shown in rodents, where immune-modulatory compounds (e.g. FTY720, rosiglitazone or inhalation of nitric oxide) administered prior to malaria infection resulted

in reduced pathology (32). In humans it was also shown that heterologous modification of immune activation status affects the course of malaria disease, as a decrease of infection related mortality was observed after a BCG-vaccination (12, 13). Further analysis of how baseline immune activation status can predict clinical and vaccination outcomes could improve understanding and development of vaccination strategies, paving the way for nonspecific modification of immune activation status to increase vaccine efficacy.

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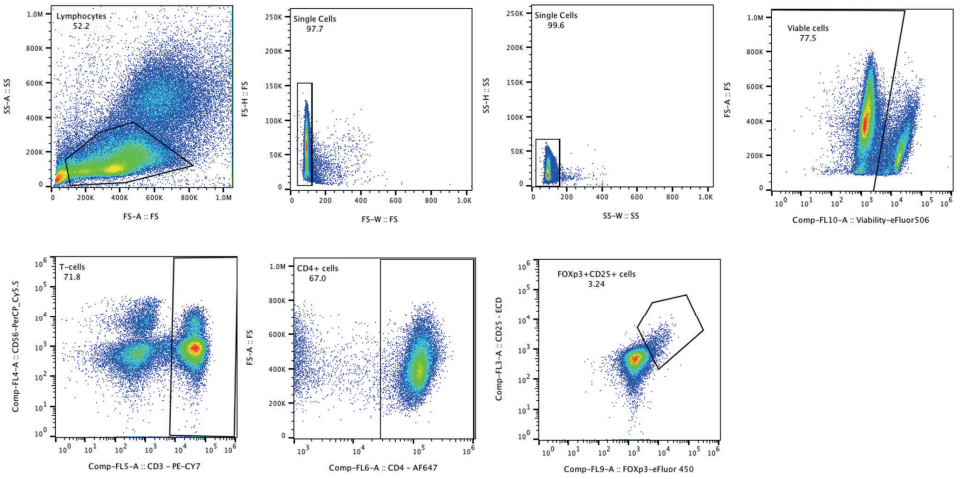
Supplementary data

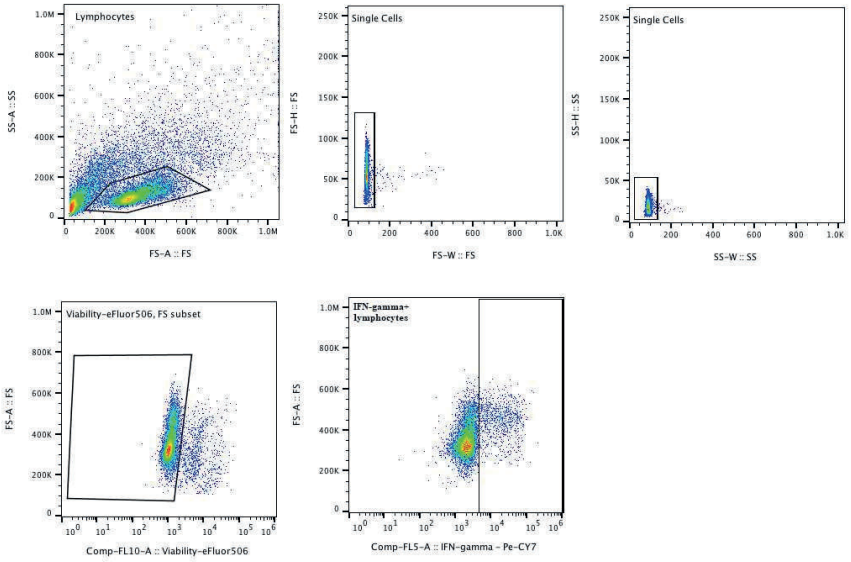
All panels	Marker/stimulus	Colour	Dilution	Clone	Firm
	Viability	eFlour506	500		Biolegend
Panel regulatory T-cells	**Marker/stimulus**	**Colour**	**Dilution**	**Clone**	**Firm**
	CD3	PE-CY7	640	OKT3	BioLegend
	CD4	AF647	640	OKT4	BioLegend
	CD8	AF700	2000	HIT8A	BioLegend
	CD56	PerCP-CY5.5	100	HCD56	BioLegend
	CD25	EDC	20	B1.49.9	Beckman Coulter
	FOXp3	eFlour450	80	PCH101	ebioscience
Panel after stimulation Intracellular IFN-γ	CD3	APC-eFluor780	640	SK7	ebiosciences
	CD4	AF647	640	OKT4	BioLegend
	CD8	AF700	2000	HIT8A	BioLegend
	CD56	PerCP-CY5.5	100	HCD56	Biolegend
	IFNγ	PE-CY7	100	4S.B3	Biolegend
Stimulation	Brefeldin A	na	10 µg/mL	na	Sigma
	Ionomycin	na	1 µg/mL	na	Sigma
	phorbol 12-myristate 13-acetate	na	1 µg/mL	na	Sigma-Aldrich

Supplementary data 1 – FACS panels

Regulatory T-cells



IFN-γ positive lymphocytes



Supplementary data 2 – Gating strategy

Chapter 8

Summarizing discussion and future perspectives

General discussion

Malaria vaccine development has been a highly important research topic for decades, as a successful vaccine may decrease the global disease burden. However, the currently designed candidate vaccines are lacking efficacy, partly explained by a substantial limitation in knowledge on what kind of immune response will result in sterile and durable protection after immunization. For malaria vaccine research, Controlled Human Malaria Infection (CHMI) studies are a valuable tool for both *i*) examination of efficacy of candidate malaria vaccines and for *ii)* investigating the host response after infection and immunization in a highly controlled setting (1).

Part I: Clinical malaria vaccine development

Establishment of sterile protection: pre-erythrocytic vaccines

The possibility to reach protection to *P. falciparum* was shown after immunization with the pre-erythrocytic *RTS,S* vaccine, which combines the highly conserved *P. falciparum* sporozoite surface protein, Circumsporozoite Protein (CSP), linked to hepatitis B virus surface antigen virus-like particles (2). This recombinant protein vaccine induces high antibody titers against CSP (3-5), thereby preventing sporozoites to infect hepatocytes. Although this vaccine strategy resulted in immunity in a subset of healthy malaria naïve volunteers shortly after they were immunized (3-5), this sterile protection was not long lasting. During a re-challenge after 5 or 6 months, most volunteers developed parasitaemia and had to receive anti-malarial treatment (6, 7). Also, phase III trials in malaria endemic areas showed a rapidly waning protective immunity within a year after vaccination (8-13). As the subunit vaccines did not result in long-lasting anti-malarial immunity, interest has risen for whole parasite vaccination, which exposes the immune system to a broader array of parasite antigens. Several approaches for attenuation of sporozoites have been investigated in order to immunize humans with whole parasites without resulting in malaria blood stage infection; e.g. attenuation by co-administration of anti-malaria drugs, radiation of sporozoites or genetic modification.

Chemoprophylaxis and sporozoites

Encouraging results were observed in healthy volunteers whom were 3 times immunized by the bites of 15 *P. falciparum* infected mosquitoes while receiving chloroquine prophylaxis (CSP immunization). Upon challenge to the same *P. falciparum* strain (homologous challenge) by 5 infective mosquito bites none of the volunteers developed blood parasitaemia (14). Also, the majority of volunteers was still protected against a re-challenge 28 months

after the initial immunization, showing that long-lasting sterile protection to *P. falciparum* is possible (15). It is thought that exposure to many antigens of a high number of viable *P. falciparum* sporozoites (16) combined with complete development of liver schizonts is required for induction of protective immunity (17-19). However, the CSP-immunization strategy is logistically challenging to deliver to a high number of individuals. In addition, a high compliance in taking antimalarial medication is essential to prevent breakthrough infections, this seems not feasible to execute on population-based level. For these reasons, attenuation of parasites without losing immunogenicity has been a major research topic during the last decade.

Radiation-attenuated parasites

Attenuation of parasites can be also established by radiation of live sporozoites, which causes random mutations in the DNA. These radiation-attenuated sporozoites (RAS) can invade hepatocytes but arrest early during liver stage, and therefore, do not result in blood stage malaria. Infection by intravenous administration of radiated sporozoites resulted in homologous (20-22) and partly in heterologous (23) protection against falciparum malaria in malaria naïve, healthy humans. However, field studies in malaria pre-exposed individuals, provided disappointing results as 66% of immunized volunteers was not protected against malaria (24). Although these studies have an excellent safety record, immunogenicity of RAS is suboptimal and high doses of cryopreserved sporozoites are needed to be administered intravenously (21). It has been shown that human individuals need to be exposed to more than 1,000 bites of *P. falciparum*-infected and irradiated mosquitoes (25) to reach a suboptimal level of protection against malaria. For effective immunization a product with a higher immunogenicity is necessary.

A novel pre-erythrocytic vaccine strategy: attenuation by genetically modification

New promising techniques have made gene modification in *Plasmodium* parasites possible and genes essential for liver development can be deleted. Genetically attenuated parasites (GAPs) have several advantages over RAS: *a)* the product has a higher level of homogeneity (26), *b)* GAPs expose the host to a broader antigen repertoire as they can replicate in the liver (19, 27), *c)* a lower number of sporozoites is necessary to induce protective immunity after replication in the liver (14, 27, 28), *d)* repeats of highly immunogenic genes can be inserted to induce a robust immune response. Candidate GAP-products are currently under development and tested for their infectivity and safety as after administration parasites should not emerge from the liver into the blood (26, 29). A GAP candidate in which p52 and p36 were lacking caused a protective immune response in mice in absence of blood parasitaemia (30), but resulted in a breakthrough infection in 1 of the 6 CHMI participants

(31). Taken together, despite these encouraging developments, generation of a safe product for immunization of humans remains challenging and the effect of administration of GAP in vulnerable populations is still a not known. Moreover, probably multiple genes need the be removed to establish sufficient safety of the immunizing agent. Removal of an additional gene ($p52^-$/$p36^-$/sap1-) could establish complete protective immunity in mice and was found to be safe in human as no breakthrough infection occurred (32).

A novel immunization GAP platform: Plasmodium berghei

Chapter 2 describes a novel approach in which *P. berghei* with an inserted *P. falciparum* CSP-gene repeat was used for immunization (*PbVac*). *PbVac* parasites can invade human hepatocytes, but they cannot replicate in human erythrocytes (33) making it a safe method of immunization with viable parasites (34). Although four immunizations with *PbVac* did not result in protection during a CHMI in healthy malaria naïve volunteers, a delay of 2.2 days in patency and a decrease of 95% in parasite liver burden was observed. Further analysis showed a dose-dependent cellular immune response and the production of *Pf*CSP-dependent antibodies that can block *P. falciparum* invasion in liver cells *in vitro*. The explanation for this clear functional biological effect of *PbVac* is not fully elucidated yet. In previous studies, the immune response in malaria naïve volunteers upon immunization with whole sporozoites varied tremendously with variation in the number of elicited antibodies and differences in contributing immunological mechanisms (35). A number of studies showed a major contribution of cellular responses (36) for establishment of protective immunity after GAP immunization (37), RAS immunization (38-40) and CSP immunization (41, 42), whereas in other studies CSP-antibodies were found to play a crucial role for establishment of anti-malarial immunity (5, 43-45). All these studies focused on cellular and humoral markers as measured in the peripheral blood. However, studies in mice showed that resident memory CD8+ T-cells in the liver that can recognize *Plasmodium spp*. (46-48), were responsible for the protective immunity after *P. berghei* immunization (49) and are the first line of defence after challenge (48, 50).
Remarkably, during the first *PbVac* immunization, a decrease in the number of monocytes was observed which was associated with Monocyte Chemoattractant Protein-1 (MCP1) concentrations. In addition, the IFN-γ and IL-6 concentration after vaccination were negatively associated with the prepatent period during challenge. These observations suggest a possible recruitment of immune cells to the liver, which may contribute to the development of protective tissue-resident immune responses. As liver immunity seems to be highly important for the development of protective immunity future studies should focus on the contribution of liver immune cells, although this is clearly a major challenge. Fine needle biopsy may be used for study purposes and has been found to be relatively safe, but given the 0.9% adverse event rate may be not safe enough to perform

in healthy volunteers (51). In addition, the number of cells harvested by this technique are too low and not representative for the immunological processes going on in the entire liver. Development of a proper model which represents the human liver is highly needed and currently under research (52, 53). Promising are mice models with humanized livers in which *P. falciparum* can replicate to test immune responses, antimalarial drugs and vaccinations.

Taken together, this novel vaccination strategy of *P. berghei* as an immunization platform is promising and should be explored further. Adding additional *P. falciparum* genes that are expressed during the pre-erythrocytic stages to the *P. berghei* backbone may elicit a broader protective immune response and warrant further investigation. As the level of elicited *Pf*CSP antibodies depend strongly on the number of effective mosquito bites, increasing the number of mosquito bites might be suitable in an experimental setting but this is not feasible for commercial production. Questions on upscaling of GAPs need to be answered and techniques to produce cryopreserved *Plasmodium* parasites need to be explored. Also, the route of administration of parasites is important to take into account as intradermal immunization, but not intravenous infection, has been shown to induce an immune suppressive response that hampers vaccine efficacy (54).

Transmission-blocking vaccines

In addition to vaccines that result in immunity against the malaria parasite in humans, vaccines that block transmission from humans to mosquitoes are gaining interest in the last decade (54). These transmission blocking vaccines elicit antibodies against proteins that are expressed by the sexual parasite stages in the midgut of the mosquito or in the human host. These antibodies are taken up by the mosquito when it takes a blood meal and subsequently prevent development and maturation of the gametocytes in the mosquito vector. Although benefit for the vaccinated individual is only indirect, this approach increases herd immunity as it affects the transmission reservoir by interrupting transmission of parasites of both symptomatic and asymptomatic human *Plasmodium* parasite carriers. To evaluate transmission blocking interventions the current CHMI model needs to be adjusted to test transmission of *Plasmodium* parasites from humans to mosquitoes. This model should result in a high number of viable fertile gametocytes without compromising the safety of CHMI volunteers. In addition, the transmission rate of mosquito's needs to be sufficient for testing transmission blocking interventions (TBIs) in a small number of healthy volunteers. For this purpose *Reuling et al* (55) used different antimalarial drug regimens after infection of healthy volunteers by mosquito bites to induce gametocyte carriage of which a subcurative dose with piperaquine resulted in high gametocyte counts. However, gametocytaemia was too low for effective transmission of *P. falciparum* from humans to mosquitoes and the volunteers already experienced severe

clinical symptoms (**Chapter 5**). The transmission model was adjusted by *Collins et al* (56): healthy volunteers were infected by intravenous inoculation of *Plasmodium* infected erythrocytes instead of by mosquito bites. This resulted in a high asexual parasitaemia, gametocytaemia and high transmission rates without severe clinical symptoms. In **Chapter 3** both methods of infection were compared in a single CHMI study. Additional subcurative treatment with piperaquine was given to increase the duration of asexual parasitaemia thereby also increasing the gametocytaemia. It was clearly shown that blood stage infection induced higher gametocytaemia with only a limited number of adverse events. An even higher mosquito infection rate can further optimize the model. As asexual blood stage parasitaemia and gametocytaemia correlate with each other, this can be established by increasing the asexual parasitaemia during CHMI. However, this will also compromise the safety of the healthy malaria naive individuals that participate in these trials. Individuals living in malaria endemic areas are known to have a relative mild clinical disease despite a high parasite burden (57). Establishing a transmission model in these populations is of interest, but, pre-existing immunity on gametocyte generation is not completely understood and an immune tolerant response might result in a low gametocyte concentration. A study with the current model in individuals from both a high and low endemic area may elucidate in what population the gametocyte concentration will result in a 100% mosquito infection rate allowing testing of transmission blocking interventions in a small sample size.

Route of administration of parasite effects the malaria infection

The higher number of adverse events after infection by mosquito bites compared to infection by an inoculum with infected red blood cells despite a similar level of parasitaemia, as described in **Chapter 3,** is striking. Volunteers experienced headache, fever and had an increase in inflammation parameters in the peripheral blood after infection by mosquito bites, whereas these alterations were very limited after blood stage infection. Several hypotheses may explain this difference. First, the initial low parasitaemia after blood stage infection might result in a different immune response than after infection by mosquito bites which result in a higher initial parasitaemia. The inoculum contained 2.800 sporozoites which is 0.5-0.6 parasites/ml after administration (in a human with 5L blood) whereas after infection by sporozoites thousands of merozoites are released into the bloodstream resulting in a much higher parasitaemia at once. This relative high parasitaemia results in a pro-inflammatory response with an IFN-γ increase (58). The initial response upon a relatively low parasitaemia might instead result in an induction of immune tolerant mechanisms such as regulatory T-cells (59) and loss of γδ T cells (60). Thereby it might imitate the host response in individuals that have experienced multiple malaria episodes and are known to have a regulatory immune response upon a malaria infection.

Another difference is the passing of the liver stage after infection by an inoculum with infected red blood cells in contrast to infection with mosquito bites. The immunological processes in the liver after a natural infection are still unclear, but seem to be important for the development of protective immunity (61). Further research on these differences in clinical symptoms and parasitological kinetics should give knowledge on the induction of immune tolerance and protective immunity.

Part II: Heterogeneity in host responses after *Plasmodium* spp. infection

Malaria patients in endemic areas: a heterogeneous group

Many previous studies have focused on finding a predictive marker for protection after immunization (35) or for prediction of disease severity during clinical malaria (62). Biomarkers predicting both disease outcome and immunization efficacy have been described (35, 62). However, studies conducted in different populations and different age groups described varying levels of sensitivity and specificity for predicting morbidity and mortality or protection after immunization. The different outcomes depend partly on differences in pre-existing immunity between populations correlating with *P. falciparum* transmission rates (63). This effect on pre-existing immunity was very clearly shown in a study comparing malaria patients from areas with high and low *P. falciparum* transmission. Whereas after stimulation with PMA PBMCs of people living in low endemic areas produced high levels of pro-inflammatory cytokines, PBMCs of people living in high endemic areas produced only very limited pro-inflammatory cytokines (63). In **Chapter 4** the problem of using a single marker for prediction of disease outcome or differentiation between diseases in different populations was shown for angiopoietin-1 and angiopoietin-2 as biomarkers. The sensitivity and specificity of these biomarkers showed variability between study populations in malaria endemic areas possible due to differences in malaria immunity. In addition, prediction of malaria disease outcome at hospital admission could have consequences for treatment strategies. Such treatment strategies are not available yet and multiple promising studies in mice have not resulted in a strategy with reduction in mortality or morbidity in humans (64). Most of the studies are targeting a single pro-inflammatory protein or aim to activate a single anti-inflammatory process (e.g. by an increase of nitric oxide or increase in angiopoietin-1). In my opinion, it is questionable whether the complexity of malaria disease is suitable for such a focused approach. Interfering with multiple processes that are involved in the pathophysiology of malaria (e.g. coagulation, endothelial cell activation, immune paralyses or over-activation) seems necessary.

CHMI participants: a homogenous group with a heterogeneous host response

Selecting and screening healthy volunteers for a CHMI study, aims to result in a highly comparable group. Because of the homogeneity of these groups and the highly controlled standardized study protocols, only a small number of volunteers needs to be included to demonstrate differences between distinct interventions. All included volunteers are healthy malaria naïve individuals, in contrast to individuals in malaria endemic areas with varying levels of immunity to *Plasmodium* spp. **Chapter 5, 6 and 7** describe the host response to the *P. falciparum* infection which is still heterogeneous despite the absence of pre-existing anti-malarial immunity. Taken the different chapters together, differences are observed in the number and severity of clinical symptoms (**Chapter 5 and 6**), cytokine responses (**Chapter 5, 6 and 7**), coagulation (**Chapter 6**), endothelial cell activation (**Chapter 6**), body temperature (**Chapter 6**), alterations platelet count and liver enzymes (**Chapter 5 and 6**), parasite growth (**Chapter 6**) and speed of acquisition of immunity (**Chapter 7**). In general, our CHMI participants can be divided in 2 groups: those with an initial pro-inflammatory and those with an anti-inflammatory response. The pro-inflammatory responders experience clinical symptoms and an increase in pro-inflammatory cytokines (e.g. IFN-γ), coagulation and endothelial cell activation and laboratory abnormalities such as liver function parameters. The anti-inflammatory responders experience only mild clinical symptoms and have an increase of anti-inflammatory cytokines (limited activation of coagulation and endothelial cells and limited increase in laboratory abnormalities, for instance, no abnormalities in liver function parameters). The pro-inflammatory phenotype is known to result in inhibition of parasite growth (65), but without a feedback mechanism, the inflammation can result in tissue and subsequent multi-organ damage. On the other hand, the anti-inflammatory responders experience limited clinical symptoms during early disease, but the absence of inflammation results in a lack of parasite growth inhibition (65). As CHMI participants are treated in an early phase of disease, no conclusions about causality can be drawn between the early host response in CHMIs and disease severity during advanced clinical disease as they cannot be compared. However, it can be speculated that the initial lack of parasite growth inhibition during malaria with an initial anti-inflammatory response will change in time into a strong pro-inflammatory response with clinical symptoms when a certain threshold of parasite density is reached.

The effect of the immune activation status on the host response after a P. falciparum infection

As the initial cytokine response correlates with early clinical pathology (58, 65) and increase in parasitaemia (65), the diversity in host response in a homogenous group of CHMI

participants is of great interest. Multiple studies that investigated malaria vaccine efficacy, found a difference in the initial immune response between individuals who developed protective immunity after immunization and those who did not (35). **Chapter 7** describes that this initial immune response upon infection was associated with the baseline immune activation status: a higher level of activation results in a pro-inflammatory response upon malaria infection whereas a more immune-modulatory immune response is associated with a lower baseline immune activation status. The baseline immune activations status was associated with the number of immunizations needed for acquisition of protective sterile immunity.

Pre-infection immunity is also known as innate immune memory or trained immunity (66). The host immune response is classically divided in an innate and adaptive response in which the innate response is typically fast and non-specific and the adaptive slow and specific. In addition, the adaptive immune response leads to immune memory and can react fast with high specificity upon a subsequent infection with the same pathogen. However, this paradigm is challenged by relative recent results showing adaptive characteristics of the innate immune response. After encountering pathogens or after vaccinations, the initial host response shows an increased responsiveness to a secondary stimulus, which is not directed to a specific pathogen (66). An example is the innate immune memory induced by BCG vaccination which results a long-lasting Th1 skewed immune response (67).

The literature on the effect of the innate immune memory on the immune response during a malaria infection is limited (68). One study showed a negative association between parasitaemia and an early NK-cell activation in a subset of CHMI participants that received a BCG-vaccine before challenge (68), but not in the individuals that did not receive the vaccination. In addition, in a malaria endemic area a lower number of malaria episodes in children correlated with the presence of the BCG specific scars, classically present on left upper arms (69). The BCG-vaccine induces immunological activation (trained immunity) results in a decrease in non-tuberculosis mortality in newborns (70). In adults pro-inflammatory cytokine production increases after stimulation of PBMCs with non-TB stimuli in recent BCG vaccinated adults (71). Remarkably, after CHMI following a BCG-vaccine, two groups were observed within the BCG-vaccinated individuals similar to the groups found in the CHMIs described in **Chapter 6 and 7**: those with and without an IFN-γ increase upon *P. falciparum* infection. These differences observed in a number of studies are highly interesting and it seems that multiple pro- and immune modulatory pathways together are balancing and result in a final immune response. BCG vaccination might force an individual in a more 'tolerant' or more 'memory' direction (72), thereby affecting the innate immune response during the subsequent CHMI.

Chapter 7 shows that the immune activation status before immunization is associated with to the initial cytokine response and to the number of immunizations needed for the acquisition of protective immunity. These aspects need to be taken into account in the development of vaccine regimens. Multiple malaria episodes result in a tolerant immune response against *P. falciparum* as shown by the lack of pro-inflammatory cytokines after infection. In addition, areas where helminth infections are prevalent are overlapping with countries in which malaria is endemic (73, 74). Consequently, co-infections with both parasites occur often and interact. As helminth infections up regulate an Th2-type immune response, the pro-inflammatory Th1 response is suppressed (75-77). However, studies are not conclusive on whether the helminth infections have a protective or harmful effect on clinical malaria disease (74). Comparison of these studies is not possible due to difference in measurement of parasite stage within in the host, age, nutrition state and previous helminth infections. The decreased pro-inflammatory immune response is not specific for malaria vaccines, also diphtheria antitoxin (78) and tetanus antitoxins (79, 80) production upon vaccination is decreased during a helminth infection. This underlines the importance of further analysis of the relation between helminth infections, immune activation status, pre-existing immunity and cytokine response. Especially as limited progress in clear understanding of the effect of helminth infections on the host response upon malaria is made, while it might have major implications. Determination of the immune activation status before immunization might lead to a prediction of the host immune response and thereby of vaccine efficacy if the baseline immune activation status can be altered. This might be done by treatment of helminth infections (81), but also malaria vaccination in the first 3 months after BCG vaccination might lead to a stronger pro-inflammatory immune response and higher vaccination efficacy. In addition, this might lead to an alteration of vaccination schedules and programs in which a part of the populations might be selected to receive additional immunizations.

Determination of the immune activation status

Questions on how immune activation status can be measured are remaining. In **Chapter 7**, the baseline TGF-β concentration was different between pro- and anti-inflammatory responders after infection and between fast and slow responders after immunization. Participants with high TGF-β concentrations before infection showed a decrease in TGF-β concentrations during infection combined with a pro-inflammatory immune response. These individuals have a high level of immune activation status (the immune system is easily triggered to react with a pro-inflammatory response) and high levels of anti-inflammatory cytokines might be needed to keep the immune systems in a non-active steady state. A functional read-out in which baseline immune cells are stimulated and cytokine production is measured, might have more potential as it demonstrates the final

effect of the different pathways involved. Such a test-type is likely to show the level of immune activation before vaccination in malaria endemic areas or before challenge in a CHMI. From these results priming into a direction of higher immune activation or selecting individuals that need an adjusted vaccination schedule or adjuvant can be considered.

Final conclusions and future perspectives

The work in this thesis contributes to an improved understanding of the causes and effects of the heterogeneity in the host immune response after a *P. falciparum* infection on the disease severity and acquisition of immunity after vaccination. This thesis showed that an initial pro-inflammatory immune response after infection is associated with a higher baseline immune activation status and with more clinical symptoms during CHMI and a quicker acquisition of immunity after immunization than an initial immune-modulatory response with a TGB-β increase. The effect of the immune activation status on vaccine efficacy is not malaria specific and testing whether the baseline immune activity affects the vaccine efficacy as measured by activation markers of functional assays can also be tested after administration of e.g. hepatitis B vaccine. In addition, administration of a malaria vaccination in the first 3 months after BCG-administration in children might be interesting to evaluate as the active immune system after the BCG-vaccination might increase malaria vaccine effectivity. Understanding the immune response after immunization might result in 'personalized vaccination' in which an adjusted vaccination schedule can be given according to the baseline immune activation status. A second application can be priming the immune system before immunization to increase vaccine efficacy.
Additionally, the research described in this thesis showed that the use of *P. berghei* GAPs has high potential as vaccination strategy and should be evaluated for further improvement e.g. by inserting more repeats or preserved *P. falciparum* genes.

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Samenvatting

Malaria is een infectieziekte met meer dan 200 miljoen nieuwe ziektegevallen en 435.000 doden per jaar. De ziekte komt vooral voor in sub-Sahara Afrika en het zijn met name kinderen jonger dan 5 jaar die aan malaria overlijden. Malaria wordt veroorzaakt door *Plasmodium* parasieten welke worden overgedragen op de mens tijdens een beet van besmette vrouwelijke *Anopheles* muggen. Van de verschillende *Plasmodium* soorten die de mens kunnen infecteren, veroorzaakt *P. falciparum* het ernstigste ziektebeeld. Door de implementatie van effectieve maatregelen zoals het gebruik van geïmpregneerde klamboes en insecticiden daalde de malaria gerelateerde mortaliteit en morbiditeit in de periode 2000 tot 2015. Echter, door de opkomst van resistente *Plasmodium* parasieten en *Anopheles* muggen wordt er sinds 2015 weer een stijging in de incidentie van malaria geobserveerd. Er zijn nieuwe interventies nodig om malaria te bestrijden en een effectief malaria vaccin zou hierbij een belangrijke rol kunnen spelen. Voor de ontwikkeling van een malaria vaccin is goed begrip nodig van de gastheer respons van de mens op de *P. falciparum* parasiet.

Dit proefschrift bestaat uit twee delen: in deel I staat centraal het ontwikkelen van een malaria vaccin en het optimaliseren voor een testmodel voor transmissie blokkerende vaccins. Hierbij werd gebruik gemaakt van gecontroleerde humane malaria infectie studies (CHMI), waarbij gezonde vrijwilligers met malaria worden geïnfecteerd d.m.v. muggenbeten of door het intraveneus toedienen van *P. falciparum* geïnfecteerde erytrocyten. In deel II wordt ingegaan op hoe en waarom het immuunsysteem van vergelijkbare gezonde individuen zeer verschillend kan reageren op een malaria infectie. Om deze vragen te beantwoorden, is gebruik gemaakt van bloedsamples verzameld van verschillende CHMI studies die eerder waren verricht.

Deel I - Malaria vaccins

Deel I van dit proefschrift start met de beschrijving van een CHMI waarbij vrijwilligers geïmmuniseerd worden met de *P. berghei* parasiet, een knaagdieren *Plasmodium* soort welke niet pathogeen is voor mensen (**hoofdstuk 2**). Deze *P. berghei* parasieten zijn genetisch gemodificeerd waarbij 'repeats' van het geconserveerde membraaneiwit *circumsporozoite protein* (CPS) van *P. falciparum* zijn toegevoegd aan *P. berghei*. Op deze manier is er een genetisch gemodificeerde parasiet gemaakt die humane levercellen kan infecteren en immunogeen is maar niet kan resulteren in een infectie van humane erytrocyten. Tijdens het eerste deel van deze klinische studie is de veiligheid van het product (PbVac) aangetoond. Het tweede deel van deze studie laat zien dat een immunisatie met PbVac leidt tot ontwikkeling van antilichamen die resulteren in een daling van 90%-

99% van de *P. falciparum* leverinfectie, waardoor veel minder parasieten zich in de lever ontwikkelen en een vertraging in het ontwikkelen van de malaria bloedfase optreedt.

Met het huidige CHMI-model kunnen pre-erytrocytaire malaria vaccins getest worden. Echter, voor het testen van vaccins die de transmissie van *P. falciparum* parasieten van mens naar de mug blokkeren is echter nog geen goed model beschikbaar. Voor infectie van muggen tijdens een CHMI moeten de geïnfecteerde vrijwilligers hoge aantallen geslachtelijke parasieten (gametocyten) ontwikkelen. Deze gametocyten worden vervolgens opgenomen door nog niet-geïnfecteerde muggen tijdens een bloedmaal dat zij nemen op de huid van de vrijwilliger. Voor het bereiken van een hoog aantal gametocyten, is een hoge concentratie van parasieten in het bloed van de vrijwilligers nodig wat leidt tot significante klinische symptomen. In **hoofdstuk 3** is een eerder voorgesteld CHMI-transmissie model geoptimaliseerd door een groep vrijwilligers te infecteren d.m.v. het intraveneus toedienen van geïnfecteerde erytrocyten (bloed inoculum). Infectie met een bloed inoculum blijkt te resulteren in alleen milde klinische symptomatologie en in een hoger aantal gametocyten en daarmee geïnfecteerde muggen dan na een infectie d.m.v. muggenbeten. Het huidig voorgestelde model kan gebruikt worden om de effectiviteit van transmissie blokkerende malaria vaccins te testen.

Deel II - Heterogeniteit in de immuunrespons na een malaria infectie

In een literatuurbespreking in **hoofdstuk 4** wordt ingegaan op het gebruik van angiopoietine-1 en angiopoietine-2 om het ziektebeloop van malaria te kunnen voorspellen en daar therapeutische consequenties aan te verbinden. De markers laten grote verschillen zien in sensitiviteit en specificiteit in verschillende populaties m.b.t. het voorspellen van mortaliteit. Mogelijk liggen het verschil in malaria transmissie (e.g. hoog vs. laag endemische gebieden) in de studie populaties en daarmee de verschillen in de malaria immuniteit hieraan ten grondslag. Het lijkt niet mogelijk om de complexe pathogenese en immuunrespons tijdens malaria te voorspellen met één enkele marker.

Ook bij individuen zonder immuniteit voor malaria verloopt de gastheerrespons zeer wisselend blijkt uit de **hoofdstukken 5, 6 en 7**. In **hoofdstuk 5** wordt beschreven hoe een subgroep van vrijwilligers significante leverenzymstoornissen ontwikkelt als de behandeling met anti-malaria medicatie gestart wordt. Deze afwijkingen correleren met een inflammatoire gastheer response. Eenzelfde patroon wordt gezien in malaria patiënten. In zowel de CHMI-vrijwilligers als in de patiënten herstellen deze leverenzymstoornissen volledig. Deze bevindingen zijn belangrijk omdat zij laten zien dat leverenzymstoornissen niet per definitie een reden zijn tot het staken van een CHMI of stoppen met de ontwikkeling van anti-malaria medicatie waarbij leverenzymstoornissen worden geobserveerd.

Tijdens een CHMI kunnen twee groepen vrijwilligers worden onderscheiden (**hoofdstuk 6**): vrijwilligers die pro-inflammatoir reageren met een stijging van IFN-γ, en klinische symptomen zoals koorts en activatie van de stollingscascade. In de andere groep vrijwilligers wordt een vroege stijging van het anti-inflammatoire cytokine TGF-β geobserveerd, zij hebben minder klinische symptomen en geen evidente activatie van de stollingscascade. TGF-β zou in deze CHMI-setting kunnen functioneren als een predictor voor de individuele gastheerreactie op een malaria infectie. In **hoofdstuk 7** wordt onderzocht wat de mogelijke oorzaak is voor de verschillen in de gastheer reactie tussen de vrijwilligers na een malaria infectie. Er wordt aangetoond dat de vrijwilligers voor infectie al verschillend zijn in de mate van de activiteit van het immuunsysteem (de mate en de samenstelling van de geproduceerde cytokines bij een gelijke prikkel). Een initieel hoge pre-infectie TGF-β concentratie correleert met een dalend TGF-β tijdens infectie en vice versa. Ook de snelheid waarmee malaria immuniteit verworven wordt hangt samen met de initiële TGF-β reactie. Concluderend lijkt de initiële gastheerreactie samen te hangen met de pre-infectie statusvan het immuunsysteem.

Samenvattend presenteert dit proefschrift een nieuwe innovatieve manier van malaria immunisatie waarbij een knaagdierenmalaria wordt gebruikt als vector, waaraan een belangrijk antigeen van *P. falciparum* was toegevoegd. Ook al heeft het gebruik van deze huidige vector niet geresulteerd in steriele protectie, de mogelijkheid van het toevoegen van meer geconserveerde *P. falciparum* genen maakt dit een veelbelovende immunisatiestrategie. Ook laat dit proefschrift een aangepast CHMI-model zien waarin transmissie blokkerende vaccins of hun effectiviteit getest kunnen worden.

Het tweede deel van dit proefschrift laat zeer wisselende gastheerreacties zien bij CHMI-vrijwilligers na een malaria infectie welke correleren met de activiteit van het immuunsysteem voor infectie. Toekomstige studies zullen zich richten op het meten van deze activiteit d.m.v. functionele testen. Op deze manier kan de invloed van de activiteit van het immuunsysteem op het verwerven van immuniteit gemeten en mogelijk beïnvloed worden opdat de effectiviteit van malaria vaccinatie verbeterd kan worden.

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PhD Portfolio

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PhD period:	2015-2019
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	Prof. dr. R.W. Sauerwein
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	Dr. P.J.J. van Genderen

Courses	**Year**
Basic Statistical Methods (NIHES) (CC02)	2015
Second Summer course Research Master Infection and Immunity	2015
Journal clubs Research Master Infection and Immunity	2015, 2016
Second Winter course Research Master Infection and Immunity	2016
BROK course	2016
English Biomedical Writing and Communication	2017
Workshop Presenting skills	2017

Conferences and seminars	**Year**
Spring meeting 'Netherlands Society for Parasitology' (oral presentation)	2017
EMBL conference Heidelberg, BioMalPar XIII: Biology and Pathology of the Malaria Parasite (poster presentation)	2017
Science day- Erasmus MC University Medical Center (poster presentation)	2017
Medical Microbiology and Infectious Diseases ASTMH conference (2018), New Orleans (poster presentation)	2018
Science day – Erasmus University Medical Center (oral presentation)	2019
Medical Microbiology and Infectious Diseases Dutch malaria day, Radboudumc, Nijmegen (oral presentation)	2019

Teaching	Year
Supervision Bachelor internship	2015

Grants	Year
Travel grant, Netherlands Society for Parasitology	2018

Curriculum Vitae

Gerda (Gerdie) Maria de Jong (1989) was born on October 23th in Lekkerkerk, The Netherlands. She followed her secondary education (VWO) at the Driestar College in Lekkerkerk and at the Wartburg College in Rotterdam, which she successfully completed in 2008. She continued her education at the Erasmus MC University Medical Center where she studied 'Medicine' (2008-2015) and completed the research master 'Infection and Immunity' (2017). While finishing the research master, she started with her PhD project (2015) at the Harbour hospital and Erasmus MC University Medical Center in close collaboration with the Radboudumc were she enjoyed working for a year. She was supervised by prof. dr. A. Verbon and prof. dr. R.W. Sauerwein (promotors) and dr. J.J. van Hellemond and P.J.J. van Genderen (co-promotors). The results of the research she performed during her PhD-project are presented in this thesis. Gerdie is currently working as a clinician at the Intensive Care Unit of the Erasmus MC University Medical Center after a period of 6 month of clinical work at the Franciscus Gasthuis, Internal Medicine.

List of publications

de Jong, G.M., Slager, J.J., Verbon, A., van Hellemond, J.J**.** and van Genderen, P.J.J. (2016). *Systematic review of the role of angiopoietin-1 and angiopoietin-2 in Plasmodium species infections: Biomarkers or therapeutic targets?* Malaria Journal, 2016.

Reuling, I.J., **de Jong, G.M.,** Yap, X.Z., Asghar, M., Walk, J., van de Schans, L.A., Koelewijn, R., Färnert, A., de Mast, Q., van der Ven, A.J., Bousema T., van Hellemond, J.J., van Genderen, P.J.J., Sauerwein., R.W. (2018). *Liver injury in uncomplicated malaria is an overlooked phenomenon: An observational study.* eBioMedicine, 2018.

de Jong, G.M., A. Dik, W.A., Urbanus, R.T., McCall, M., Wammes, L.J., Koelewijn, R., Sauerwein, R.W., Verbon, A., van Hellemond, J.J., van Genderen, P.J.J. (2018). *Transforming Growth Factor-beta profiles correlate with clinical symptoms and parameters of haemostasis and inflammation in a controlled human malaria infection.* Cytokine, 2019.

Reuling, I.J. ‡, Mendes, A.M. ‡, **de Jong, G.M.**‡, Fabra-García, A. ‡, Nunes-Cabaco, H. ‡, Coffeng, L., de Vlas, L., Lee, C., Wu, Y., King, C.R., Ockenhous, C., Asley, J., Koelewijn, R., Yang, A., van Hellemond, J.J., van Genderen, P.J.J.*, Sauwerewin, R.W.*, Prudencio, M.A.* *Safety and efficacy of a genetically modified rodent malaria parasite against Plasmodium falciparum malaria: an open-label randomized phase 1/2a trial.* (Submitted).

Alkema, M.‡, Reuling, I.J.‡, **de Jong, G.M.**‡, Lanke, K., Coffeng Luc E., van Gemert, G., van de Vegte-Bolmer, M., de Mast, Q., van Crevel, R., Ivinson, K., Ockenhouse, G. F., McCarthy, J.S., Sauerwein, R.W.*, Collins, K.A.*, Bousema, T.*. *Induction of transmissible P. falciparum gametocyte densities by controlled human malaria infection with blood-stage or mosquito bite inoculation.* (Submitted*).*

de Jong, G.M., Yap, Z. ‡, Walk, J‡., Dik, W.A., McCall, M., Verbon, A., van Hellemond, J.J., van Genderen, P.J.J., Sauerwein, R.W. *Baseline immune activation status is associated with differential activation patterns and induction of protective immunity in the Controlled Human Malaria Infection model.* (Manuscript in preparation).

‡, *: contributed equally