Safety and efficacy after immunization with Plasmodium falciparum sporozoites in the controlled human malaria infection model

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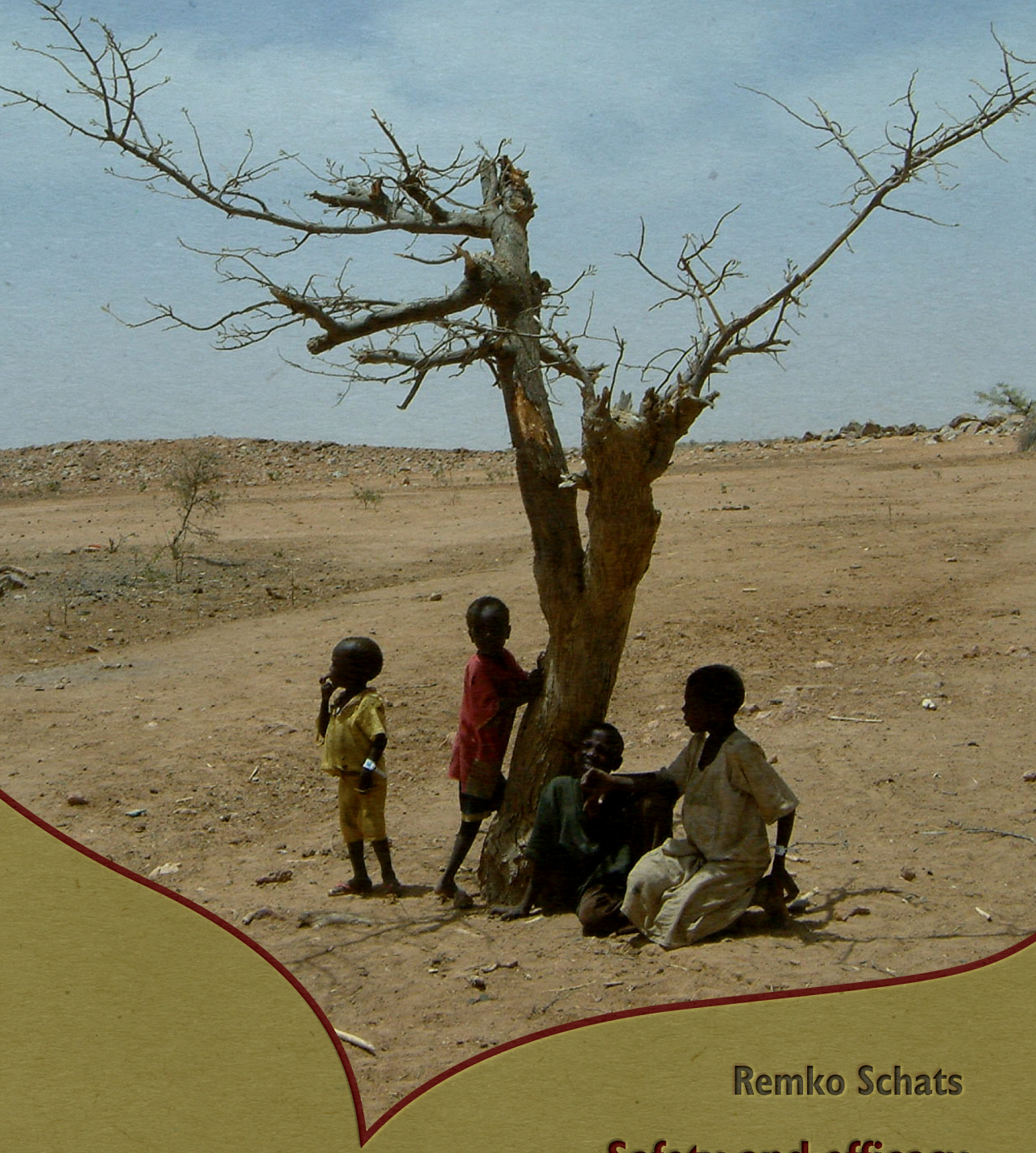

Remko Schats

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COLOFON

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Prof. dr. T.H.M. Ottenhoff
Prof. dr. A.J.A.M. van der Ven, Infectieziekten en International Health, Radboudumc
Dr. P.J.J. van Genderen, Infectieziekten en Medische Microbiologie, Erasmus MC

CONTENTS

Ghana. July 2004. Hospital Staff

CHAPTER 1

Introduction

1 INTRODUCTION

Despite recent successes, malaria remains a serious public health problem affecting approximately 40% of the world's population. Children and pregnant woman are the most vulnerable groups for severe disease. In 2015 the global incidence of malaria was estimated to be around 214 million clinical cases resulting in 438,000 deaths annually [1]. Africa is the most affected continent with more than 88% of all deaths globally, and among children it is the fourth highest cause of death. Ten percent of all child deaths in sub-Saharan Africa are attributable to malaria [1].

The development of effective field-applicable vaccines against malaria has proven to be extremely difficult. Firstly, this is due to the fact that it is still unknown which *Plasmodium* antigens and host immunological pathways are involved in the acquisition of sterile protection. Secondly, *Plasmodium* has evolved under continuous immunological selective pressure which resulted in a huge genetic diversity with subsequent high levels of antigenic variation. These ever changing antigens resemble a continuously moving target for the host immune system, and to cover these antigens by vaccines remains therefore a true challenge. To eradicate malaria from the face of the earth, a multitude of anti-malaria tools will be needed of which a vaccine will be of utmost importance. In this thesis we tried to answer several questions central in the development of a whole sporozoite malaria vaccine.

Biology of the malaria parasite

The malaria parasite belongs to the taxum Apicomplexa, a large phylum of parasitic protists. Apicomplexan parasites are eukaryotic unicellular endoparasites and many of them are important pathogens for invertebrates and vertebrates, including humans [2]. In all hosts malaria is caused by *Plasmodium* and in humans, five species of *Plasmodium* exist: *falciparum*, *vivax*, *ovale*, *malariae* and *knowlesi*.

After inoculation in the skin by an *Anopheles* mosquito, the parasites travel within 10-15 minutes [3] via the bloodstream or lymphatic system to the liver. Upon arrival in the liver, sporozoites invade and transverse several liver cells before each parasitizing a single liver cell to proliferate and differentiate. This stage in the life-cycle of the parasite is called the pre-erythrocytic stage. Within the liver cells, parasites reside clinically silent inside parasitophorous vacuoles (PV) for 5-6 days while transforming from sporozoites via schizonts into merosomes. Directly after release from liver cells and entering the blood-

stream, these merosomes release thousands of merozoites that rapidly enter (less than 30 seconds) erythrocytes [4]. Each merozoite transforms and divides via the trophozoite and schizont stage into merozoites by clonal multiplication. These merozoites are released by bursting of the erythrocyte and this cycle takes one to three days depending on the *Plasmodium* species. Simultaneously with the release of merozoites in the bloodstream, symptoms of malaria start to occur in the infected individual. Symptoms of uncomplicated malaria include flu-like symptoms like headache, fever and myalgia. Newly released merozoites again infect erythrocytes, perpetuating the cycle of infections and billions of parasites are formed. When left untreated, disease can worsen to complicated malaria and can include coma, shock, severe anaemia and can lead to death. High mortality rates can occur, especially in *Plasmodium falciparum* infections, in young infants and immune-naïve adults like travellers, pregnant women and people living in endemic areas with unstable transmission. Gametocytes are the sexual forms and are formed after several cycles of erythrocytic asexual multiplication. These gametocytes can be taken up by mosquitoes through bites allowing transmission of the disease. Only few circulating gametocytes are necessary for transmission and even if the gametocyte density in the bloodstream is as low as 1 parasite per µL, transmission remains fully possible [5]. Currently only few drugs are able to effectively kill gametocytes [6] and developing a vaccine against these sexual stages is important to further optimize vaccine effectivity of malaria control programs.

Combat against Malaria

The incidence of the individual species varies, but *P. falciparum* and *P. vivax* are primarily responsible for most of the morbidity and mortality, and most deaths are attributable to *P. falciparum* [1]. In the 1990s, the incidence of malaria increased dramatically, which was largely due to a rise in chloroquine-resistant parasites after decades of massive (mono-therapy) drug use across Asia and Africa. This changed after 1998 when the Director General Gro Harlem Brundtland called to "Roll Back Malaria" in his speech at the 51st World Health Assembly in Geneva [http://www.malaria.org/SPEECH.HTM].

Effective introduction and distribution of artemisinin-combination therapy (ACT), long-lasting Insecticide Treated Nets (ITN), Indoor Residual Spraying (IRS) and other tools to prevent malaria infection have resulted in a 30% reduction in malaria cases and a 47% reduction in deaths since 2000 [1]. Despite the implementation of ACT in many affected countries, artemisinine-resistant parasites are currently rapidly spreading across South East Asia. This is mainly due

to the use of artemisinin mono-therapy [7-9] and counterfeit poor quality anti-malarials [10]. Additionally, mosquitoes are becoming increasingly resistant to insecticides such as pyrethroids making the use of ITN and IRS less effective [11].

To reduce the incidence of (drug-resistant) malaria, a sustainable implementation of several effective anti-malaria tools is needed. These tools should minimally include adequate diagnosis and treatment, use of ITN, IRS, and vaccine development [1]. Although all elements might be equally important in the fight against malaria, the development of an effective vaccine, is not only essential but probably also the most cost-effective tool to combat malaria especially when integrated in existing expanded immunization programmes (EPI) for children [12].

Malaria vaccines

Up to this day, effective vaccines against parasites do not exist in humans [13]. The combination of the highly complex biology and the high degree of stage specific variation of surface antigens of the parasite makes vaccine development extremely challenging. Despite the fact that acquisition of natural immunity to malaria is possible, it requires years of repeated infections before an individual acquires protective IgG antibody responses against blood-stage *Plasmodium* [14, 15].

These antibody responses are able to control the number of parasites in the body, can prevent clinical malaria and reduce the risk of death. However, sterile protection is usually not accomplished under these circumstances [16] and people living in endemic areas often carry low-density parasitaemia generating symptomatic clinical episodes throughout their lives. The parasite benefits from this intricate (immunological) relationship. This interaction results in a state of chronic infection in the host without (excessive) clinical symptoms or death, and thereby facilitates continuous transmission of parasites.

In 2006 the PATH Malaria Vaccine Technology Roadmap initiative set out two goals for future vaccine development: a vaccine by 2015 that is 50% effective against severe disease and death and by 2025 a vaccine that reduces clinical malaria episodes by 80% [17]. Unfortunately, the first goal has not been realised yet. Ideally, vaccines against malaria should induce sterile protection and prevent both disease in infected individuals as well as block transmission to others. This can be achieved at several stages of the parasite life cycle in the human host; vaccines could target the parasite at the skin, liver or blood stage, or a combination of these stages (**Figure 1.1**). A vaccine blocking sporozoites at the skin or liver stage would prevent disease in an individual by killing or arrest-

ing the parasite in the earliest stages of infection. At the blood stage, vaccines could target blood-stage antigens that in its turn could eliminate blood-stage parasites and prevent disease, or target sexual stages that block transmission.

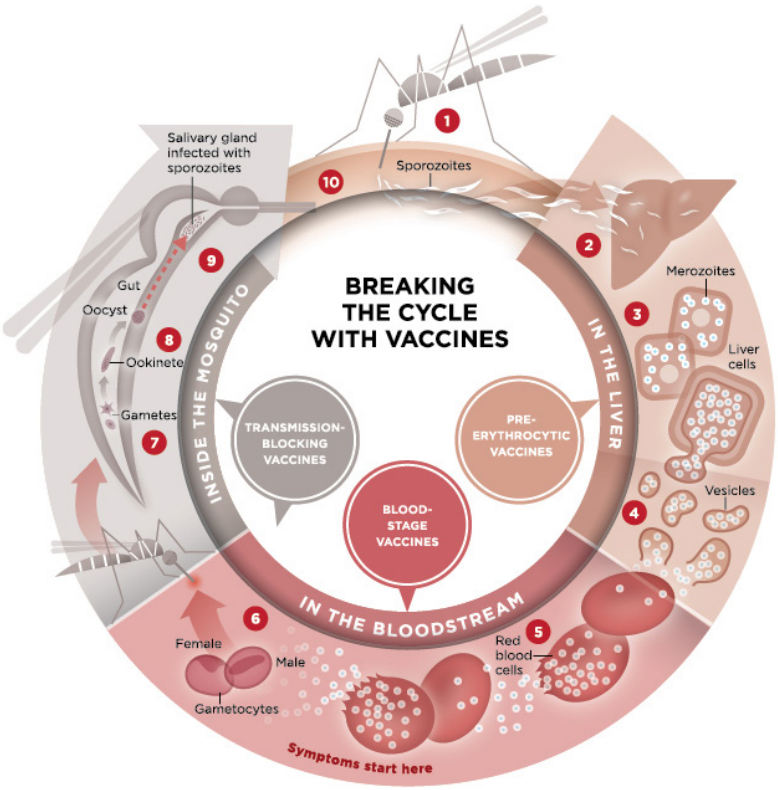

Figure 1.1 *Breaking the cycle with vaccines. Malaria Vaccine Initiative (MVI) PATH http://www.malariavaccine.org/malvac-lifecycle.php*

Although a wide range of vaccine initiatives are currently tested in clinical trials, only the RTS,S/AS01E subunit vaccine is currently being deployed in Africa [WHO Rainbow tables 2018]. RTS,S/AS01E consists of a Circumsporozoite Protein (CSP) antigen linked to the viral envelope surface protein of hepatitis B, and is administered together with the adjuvant AS01E to boost immune responses. Although RTS,S is the first licensed and distributed vaccine against malaria [18], data show a relatively low vaccine efficacy of 27%, especially under field conditions in young children [19, 20].

In addition to using subunit vaccine antigens to induce sterile protection, also whole, live, *P. falciparum* parasites can be used for vaccination. Already in the late sixties, sterile protection was established in a murine model using irradiated *P. berghei* parasites for immunisation [21]. The overall protective efficacy against a challenge with 1000 viable sporozoites was 59% between 12 and 19 days after immunization with 75.000 irradiated sporozoites.

In 1973 similar results were demonstrated with *P. falciparum* in humans [22]. However, to induce 100% protective immunity in humans, more than 1000 bites of irradiated *P. falciparum*-infected mosquitoes were needed [23]. More recently, similar results were obtained by intravenous injection of 5 times (four week interval) 1.35 x 10^5 radiation-attenuated aseptic, purified, cryopreserved sporozoites (*PfSPZ*) [24]. A challenge infection one year after immunizations conferred full homologous protection in 5 out of 5 subjects [25].

The Chemoprophylaxis Sporozoites model (CPS)

When whole sporozoites are used for vaccination, parasite development typically needs to be arrested during the parasite life cycle before symptoms or disease occur. Beside irradiation (RAS) [21], other modes of attenuation or inactivation of parasites before, during or shortly after the liver stage are possible: chemical attenuation (CAP) [26], heat [27], and genetic modification (GAP) [28].

Chemical attenuation or inactivation of blood-stage parasites can be achieved by administering blood-stage antimalarial drugs to subjects during or after inoculation with whole sporozoites. This allows the immune system to be exposed to a sufficient level and diversity of liver stage antigens for acquisition of protective immunity. This principle was first demonstrated in the murine model in 2004 using two intravenous injections of each 20.000 *P. yoellii* sporozoites under chloroquine chemoprophylaxis, and resulted in 100% protection against experimental infection [29]. Similar results were obtained in humans with the so-called Chemoprophylaxis Sporozoites model (CPS). CPS involves repeated exposure to *P. falciparum*-sporozoites infected mosquito bites under malaria chemoprophylaxis [30]. CPS-has proven to be highly effective and reproducible: three immunizations with 15 *Plasmodium*-infected mosquito bites each under chloroquine cover resulted in 100% sterile homologous protection against infection with *P. falciparum* NF54 strain [31]. Moreover, re-challenge of a subset of these subjects 48 months later showed long-lasting homologous sterile protection in 4 out of 6 subjects [32].

Controlled Human Malaria Infection model (CHMI)

Malaria vaccines candidates can be evaluated using a Controlled Human Malaria Infection model (CHMI) where small groups of malaria-naïve volunteers are immunized and subsequently challenged with a *P. falciparum* strain to assess efficacy and to evaluate reactogenicity and immunogenicity. Worldwide more than 1,300 volunteers have participated in the CHMI [33].

Besides from comparing the number of protected to unprotected individuals after challenge, vaccine efficacy in CHMI can also assessed by measuring the prepatent period in unprotected individuals. The prepatent period is the time between the challenge infection and the detection of parasites in the blood stream. Blood stream parasites can be detected in several ways, and traditionally microscopic examination of blood smears is used. A significant but incomplete elimination of the liver stage parasites will result in a prolonged prepatent period [34].

Aims of this thesis

In this thesis we evaluated efficacy, safety, and parasitological and immunological aspects of CPS using the Controlled Human Malaria Infection model.

CPS has proven to be highly effective and reproducible: three immunizations with 15 *Plasmodium*-infected mosquito bites each under chloroquine cover resulted in 100% sterile homologous protection against *P. falciparum* malaria [23]. In **Chapter 2** we determine the minimal number of infectious bites required to confer full sterile protection in a dose de-escalation immunization scheme. In CPS sterile immunity is acquired during the liver stage of the life-cycle of the parasite [35]. Although the exact mechanism how the induction of sterile protection is mediated is unknown, it is known that cytotoxic CD8+ T-cells, in association with IFNγ, IL2, TNF, granzymes and other cytotoxic mediators, play an important role in acquisition of pre-erythrocytic protection in mice [36], primates [37] and in humans [38]. However, the exact mechanism of T-cell mediated cytotoxic killing and related immunological mechanisms of protection remain to be elucidated further. In **Chapter 2** we compare cellular immune responses in protected and unprotected individuals to elucidate these T-cell mediated cytotoxic immune response associated with protection.

Chloroquine (CQ) possesses immune-modulatory properties and is able to enhance $CD8^+$ T cell responses by induction of cross-presentation [39]. Because of these properties, CQ could have boosted immune responses and may have aided in the acquisition of sterile protection in CPS. However, due to the current worldwide CQ resistance of *P. falciparum*, the use of CQ in CPS

may be limited, and the efficacy of other *P. falciparum* blood-stage chemoprophylaxis for future field vaccinations with immunizing strains resistant to CQ needs to be assessed. Therefore, we compare in **Chapter 3** the ability of CQ and mefloquine (MQ) to induce sterile protection in CPS. MQ is one of few other blood-stage anti-malarial drugs that theoretically could replace CQ in CPS. MQ, a quinine-related schizonticidal antimalarial drug, was developed during the Vietnam War in order to counteract the rapid and widespread emergence of resistance to CQ. MQ has been widely used as chemoprophylaxis in travellers and businessmen to allow travel to areas with CQ-resistant *falciparum* malaria [40] [41]. MQ has similar mode of action as CQ, but it lacks the immune modulatory properties. MQ targets blood stage malaria parasites without affecting proliferation of liver stage parasite.

Worldwide, the *P. falciparum* NF54 strain has been most often used to immunize and challenge volunteers [42]. The *P. falciparum* NF54 strain is a laboratory strain, obtained from a case of airport malaria in het Netherlands, and originates most probably from West-Africa. The NF54 strain is sensitive to chloroquine, mefloquine, atovaquone/proguanil and arthemeter/lumefantrine.

However, in malaria-endemic areas there is a large genetic and antigenic diversity between *P. falciparum* strains. It is unclear to what extent diversity in immunizing strains is required for the development of a sufficient heterologously protective malaria vaccine [43]. Previously, heterologous protection has only been reported in 4 out of 6 RAS-immunized volunteers [44], but this required large numbers of mosquito bites. Assessing heterologous protection is essential for future deployment of these vaccines in the field. In **Chapter 4**, we assess heterologous protection against a *P. falciparum* NF135 strain, originating from Cambodia [42]. A subset of volunteers who had previously participated in the dose de-escalation NF54 CPS-immunization and homologous challenge trial described in Chapter 1 were re-challenged with the NF135 strain to assess heterologous protection after more than one year.

During CHMI the presence of blood stage parasites is traditionally detected by microscopic examination of thick blood smears. A more accurate and sensitive tool is PCR. Real-time quantitative PCR (qPCR) can detect parasite DNA before being detectable by microscopic examination, and this is called the sub-microscopic period. Parasite DNA can be detected as early as 6 days after challenge. The length of the pre-patent period is associated with the level of relative protection. In addition, the use of qPCR allows for studying the kinetics of parasite multiplication by statistical modeling.

The introduction of the more sensitive qPCR instead of thick smear for the determination of the pre-patent period will also result in earlier treatment of volunteers in CHMI, with less blood-stage parasites and fewer adverse events

(AE). In **Chapter 5** we explore the dynamics of parasitaemia and adverse events during immunizations and after challenge and the consequences if qPCR was used to initiate treatment using the two clinical trials described in **Chapters 2 and 3**. In **Chapter 6** we assess the use of qPCR as a primary diagnostic test and provide directions on how to operate and to collect parasitological and immunological data in CHMIs in the future.

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Chad. April 2005. Team refugee camp Forchana

CHAPTER 2

Cytotoxic markers associate with protection against malaria in human volunteers immunized with *Plasmodium falciparum* sporozoites

Else M. Bijker*, Anne C. Teirlinck*, Remko Schats, Geert-Jan van Gemert, Marga van de Vegte-Bolmer, Lisette van Lieshout, Joanna IntHout, Cornelus C. Hermsen, Anja Scholzen, Leo G. Visser, Robert W. Sauerwein.

*Contributed equally

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ABSTRACT

Background

Immunization of healthy volunteers under chloroquine chemoprophylaxis by bites from *Plasmodium falciparum (Pf)*-infected mosquitoes (CPS immunization) induces sterile protection against malaria. CPS-induced protection is mediated by immunity against pre-erythrocytic stages, presumably at least partially by cytotoxic cellular responses. We therefore aimed to investigate the association of CPS-induced cytotoxic T cell markers with protection.

Methods

In a double-blind randomized controlled trial (NCT01218893), we performed dose titration of CPS immunization followed by homologous challenge infection in 29 subjects. Immune responses were assessed by *in vitro* restimulation of PBMCs and flow cytometry.

Results

Dose-dependent complete protection was obtained in 4/5 volunteers after immunization with bites from a total of 45, in 8/9 volunteers with 30, and in 5/10 volunteers with 15 *Pf*-infected mosquitoes respectively (OR=5.0; 95% CI 1.5-17). Proportions of CD4 T cells expressing the degranulation marker CD107a and CD8 cells producing granzyme B after *Pf*-restimulation were significantly higher in completely protected subjects (OR=8.4; 95% CI 1.5-123; p=0.011 and OR=11; 95% CI 1.9-212; p=0.004 respectively).

Conclusions

These data underline the efficiency of CPS immunization to induce sterile protection, and support a possible role for cytotoxic CD4 and CD8 T cell responses in pre-erythrocytic immunity.

INTRODUCTION

Malaria remains a major public health problem, with an estimated incidence of 207 million clinical cases leading to approximately 627,000 deaths every year (1). *Plasmodium falciparum (Pf)* is the most severe and lethal of five species that can cause malaria in humans. Availability of an effective vaccine will be critical to fight this disease, but currently there is no licensed vaccine available, despite decades of research. Most efforts have focused on the development of subunit vaccines, unfortunately showing only limited protective efficacy (2, 3). Immunization strategies based on whole parasites, however, have repeatedly induced high levels of protection in experimental settings (4-7). Previously we showed that immunization of healthy, malaria-naive subjects, while taking chloroquine chemoprophylaxis, with live sporozoites delivered by 36-45 mosquito bites (ChemoProphylaxis and Sporozoites (CPS) immunization) induces robust, long-lasting sterile protection against *Pf* malaria (8, 9). CPS immunization is about 20 times more efficient than the only alternative approach for complete sterile protection against malaria in humans i.e. immunization with radiation-attenuated *Pf* sporozoites (RAS), requiring bites from >1000 infected and irradiated mosquitoes (4), or intravenous administration of 675,000 sporozoites (10).

CPS-induced protective immunity targets the earliest stages of the parasite lifecycle, i.e. sporozoites and/or liver stages, rather than the subsequently developing asexual blood stages (11). The immune pathways responsible for this pre-erythrocytic protection, however, remain unknown. In murine malaria models, cytotoxic killing of *Plasmodium*-infected hepatocytes appears to play a role in protection, but the exact contribution and mechanism of cytotoxicity remain elusive (12, 13). Also in humans, a role for both cytotoxic CD4 T cells and CD8 T cells has been suggested, but evidence is scarce and largely circumstantial (reviewed by Tsuji et al. (14)). We conducted a double-blind randomized controlled CPS immunization dose titration and challenge study. Subjects, while taking chloroquine prophylaxis, were immunized by bites from a total of 45 (3x15), 30 (3x10) or 15 (3x5) infected mosquitoes followed by a challenge infection, resulting in dose-dependent protection. Next, we explored markers of cytotoxic T cell responses induced by CPS immunization and identified two cytotoxic markers associated with protection.

MATERIALS AND METHODS

Human ethics statement

All subjects provided written informed consent before screening. The study was approved by the Central Committee for Research Involving Human Subjects of The Netherlands (NL33904.091.10) and complied with the Declaration of Helsinki and Good Clinical Practice including monitoring of data. ClinicalTrials.gov Identifier: NCT01218893.

Clinical trial design and procedures

A single centre, double-blind study was conducted at the Leiden University Medical Center from April 2011 until April 2012. Healthy subjects between 18 and 35 years of age with no history of malaria were screened as described previously (11). Thirty subjects were randomly divided into four groups using a computer-generated random-number table. Subjects, investigators and primary outcome assessors were blinded to the allocation. All subjects received CPS immunization as described previously (8, 11), but the number of NF54 *Pf* infected versus uninfected mosquitoes varied per group: five subjects received three times bites from 15 infected mosquitoes (Group 1), ten subjects received three times bites from 10 infected and 5 uninfected mosquitoes (Group 2), ten subjects received three times bites from 5 infected and 10 uninfected mosquitoes (Group 3) and five control subjects received three times bites from 15 uninfected mosquitoes (Group 4). Nineteen weeks after the last immunization (fifteen weeks after the last chloroquine dose), all subjects were challenged by the bites of five mosquitoes infected with the homologous NF54 *Pf* strain, according to previous protocols (8, 15). The primary outcome was prepatent period, defined as the time between challenge and first positive thick blood smear. Thick blood smears were prepared and read as described previously (11). For more details about the immunization and challenge procedures and follow-up, see supplementary information.

Immunological methods

Peripheral blood mononuclear cells (PBMCs) were collected on the following time points: before initiation of chloroquine prophylaxis (baseline; B), 27 days after each immunization; I1, I2 and I3 (I1 and I2 are one day before the second and third immunization respectively), the day before and twenty weeks after the challenge infection (C-1 and C+140). For the assessment of *Pf* specific immune responses, PBMCs were restimulated *in vitro* with *Pf* infected red blood cells (*Pf*RBC) as described before (16). Expression of the degranulation marker CD107a, the cytotoxic molecule granzyme B and the cytokine IFNγ by CD4, CD8 and γδ T cells was assessed by flow cytometry. For a detailed description, see supplementary information.

Statistical analysis

The dose-dependent induction of protection was tested by logistic regression using SPSS 20. Comparison of CD107a expression and granzyme B and IFNγ production by T cell subsets between immunized unprotected and protected volunteers after CPS immunization was done per selected cellular response by means of Firth's penalized logistic regression (17, 18), resulting in p-values, odds ratios (OR) related to a change of one interquartile range, and 95% profile likelihood Confidence Intervals (95% CI) for the OR, using R software version 3.0.1 (19), with R packages logistf version 1.21 (20), rms version 4.1-3 (21) and penalized version 0.9-42 (22, 23). The ability of (a combination of) markers to discriminate between protected and unprotected volunteers was assessed with the Area under the Receiver Operator Curve (ROC), based on leave-one-out cross-validation (LOOCV), using the R-software and pROC package version 1.7.1 (24). For further details, see supplementary information.

RESULTS

CPS immunization

Thirty volunteers were included (median age 21 years, range 19–31), out of sixty-three subjects screened for eligibility (**Figure 2.S1**). Volunteers were randomly assigned to four groups and received CPS immunization by bites from 3x15 (Group 1, n=5), 3x10 (Group 2, n=10) or 3x5 (Group 3, n=10) mosquitoes infected with strain NF54 sporozoites. Control subjects (Group 4, n=5) received chloroquine prophylaxis and bites from 3x15 uninfected mosquitoes. After each consecutive immunization the number of subjects with parasitemia, as retrospectively detected by qPCR, steadily decreased in Group 1 and 2. In Group 3, however, five volunteers still showed parasitemia after the second and third immunization (**Figure 2.1**). Remarkably, in four immunized subjects, parasitemia was never detectable by qPCR at any time point (three subjects in Group 2, one in Group 3). One subject from Group 2 withdrew consent after the first immunization for reasons unrelated to the trial, and was excluded from the analysis.

Challenge infection

Nineteen weeks after the last immunization, volunteers were challenged by standard exposure to bites from five homologous strain NF54-infected mosquitoes (5). Protection by CPS immunization was dose-dependently induced in four out of five subjects in Group 1, eight out of nine subjects in Group 2 and five out of ten subjects in Group 3, while all control subjects became thick smear positive (OR=5.0; 95% CI 1.5-17; p=0.01). The median prepatent period was 2.5 days longer in CPS-immunized unprotected subjects compared to controls, both by thick smear and qPCR. Although not statistically significant (p=0.22 and 0.31 respectively), this delay is suggestive for the presence of partial protection at least in some of the unprotected CPS-immunized subjects (**Figure 2.2** and **Table 2.1**). In retrospect, all six volunteers with detectable parasitemia by qPCR after the third immunization were not completely protected from challenge infection, while 17 out of 18 subjects with a negative qPCR after the third immunization were fully protected.

Platelets decreased below reference value ($150x10^9$/L) in eight out of twelve thick smear positive (TS+; i.e. both controls and CPS-unprotected) subjects at any point after challenge (median for all TS+: $134x10^9$/L, range 79 - $213x10^9$/L). D-dimer was elevated in all TS+ subjects after challenge (median

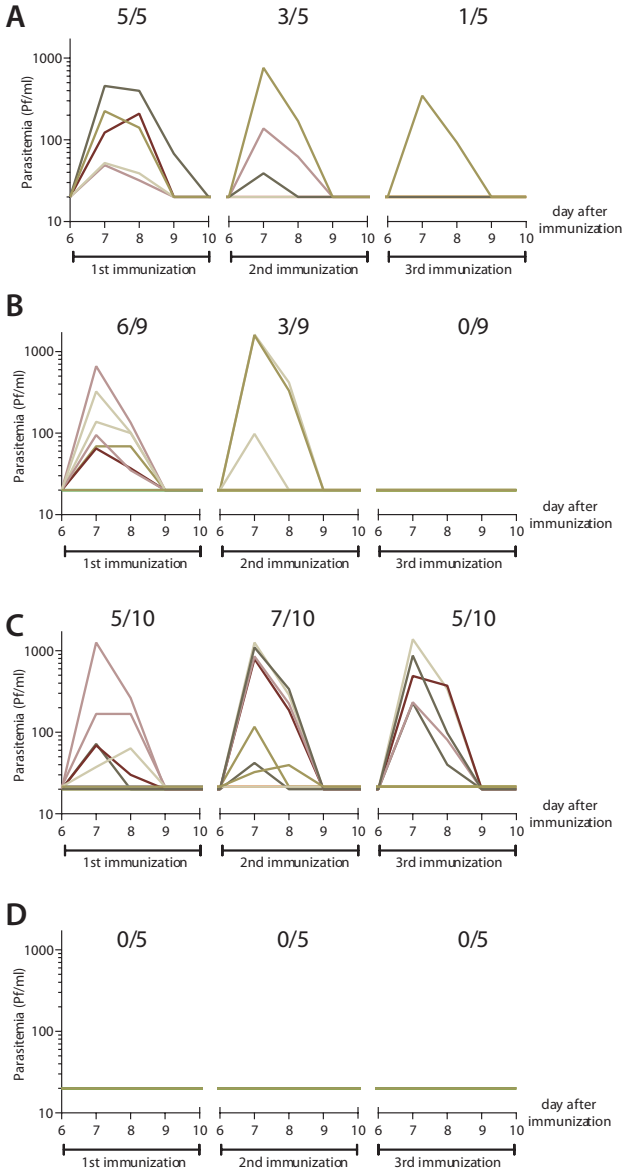

Figure 2.1 Parasitemia after the first, second and third CPS immunization.
*Parasitemia was determined once daily by qPCR from day 6 until day 10 after each immunization. Each line represents an individual subject. Panels show data for volunteers from (**A**) Group 1 (3x15), (**B**) Group 2 (3x10), (**C**) Group 3 (3x5) and (**D**) Group 4 (controls). Values shown as 10 on the log-scale were negative (i.e. half the detection limit of the qPCR: 20 parasites/ml). The number of subjects with a positive qPCR/total number of volunteers after each immunization are shown below the graphs.*

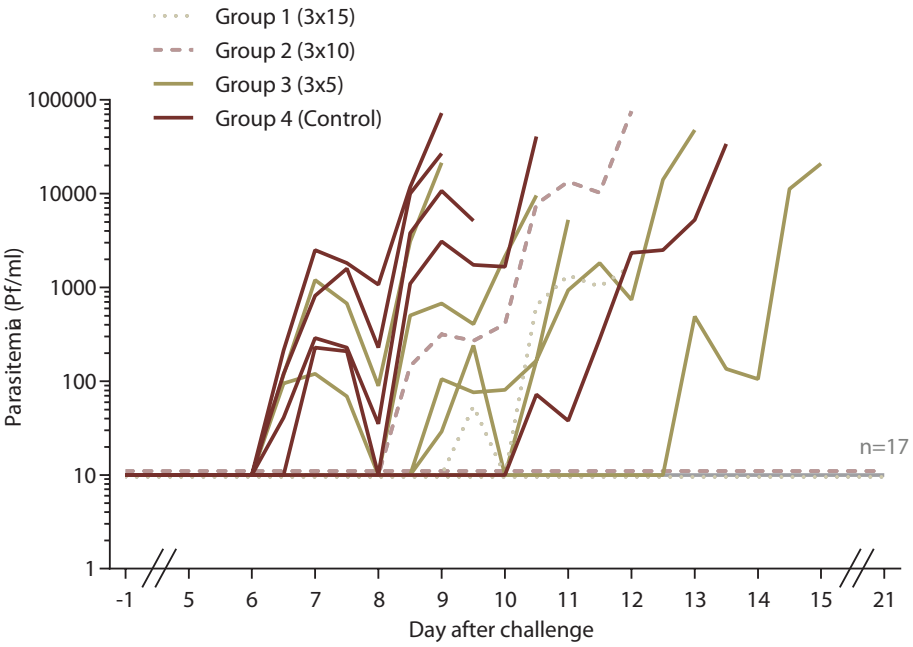

Figure 2.2 Parasitemia after challenge infection.
*Parasitemia was assessed retrospectively by real-time quantitative PCR (qPCR) from day 5 after challenge onwards, up until day 21, at two time points per day for TS+ volunteers, and one time point per day for protected volunteers. Each line represents an individual subject. **Brown** dotted lines show CPS-immunized volunteers from group 1 (3x15; n=5), **red** dashed lines subjects from group 2 (3x10; n=9), **brown** solid lines subjects from group 3 (3x5; n=10) and **red** lines represent malaria-naive control subjects (n=5). Values shown as 10 on the log-scale were negative. The two TS+ subjects from Group 1 and 2 became qPCR positive on day 8.5 and 9.5 respectively, both became thick smear positive on day 12.0.*

Table 2.1 Protection against challenge infection after CPS immunization.

Group (# of Pf-infected mosquitoes used for immunization)	Protection		Day of positivity after challenge (TS+ subjects)[c]	
	Number[a]	Percentage[b]	Thick smear	qPCR
Group 1 (3x15)	4/5	80 (36.0 – 98.0)	12.0	9.5
Group 2 (3x10)	8/9	89 (54.3 – >99.9)	12.0	8.5
Group 3 (3x5)	5/10	50 (23.7 – 76.3)	11.0 (9.0-15.0)	9.0 (6.5-13.0)
Group 4 (Control)	0/5	0 (0.0 – 48.9)	9.5 (9.0-13.5)	6.5 (6.5-10.5)

[a] *Presented as protected/total number of subjects*
[b] *Presented as % protected (95% CI by modified Wald Method)*
[c] *Presented as median (min-max).*

peak concentration 2431 ng/mL, range 1014-5000 ng/ml). Parameters normalized in all subjects after treatment without complications. All TS+ subjects experienced solicited adverse events (AEs) during challenge infection consistent with uncomplicated malaria (median number of AEs per subject 9.5 (range 4-14), median duration of each AE 1.1 days (range 0.0-12.3)). As expected, protected subjects presented with less AEs: 15 out of 17 subjects experienced solicited AEs possibly or probably related to the challenge (median number of AEs per subject: 2 (range 0-15), median duration 0.7 days (range 0.00-15.9)). One subject from Group 2 was preliminarily treated with atovaquone/proguanil at day 10.5 after challenge because of unrelated exertional rhabdomyolysis after extensive sports activity (weightlifting) followed by sauna visits. No other severe adverse events (SAE) occurred. One volunteer from Group 1 was treated for reasons unrelated to the trial at day 19. Both these volunteers remained parasite negative by qPCR analysis after the third immunization and at any time point after challenge and were considered protected in further analysis.

Analysis of cytotoxic T cell markers after in vitro Pf-stimulation

Next, we tested a panel of representative cytotoxic T cell markers including surface expression of degranulation marker CD107a, and granzyme B and IFNγ production in CD4, CD8 and γδ-T cells after *in vitro* restimulation with *Pf*-infected red blood cells (*Pf*RBC) in all immunized subjects (**Table 2.2**). CPS-immunization induced a significant increase in both the percentage and iMFI of CD107a positive CD4 and γδ-T cells, already after the first immunization up until challenge. Similarly CD8 T cells expressed a significantly higher CD107a iMFI after the second immunization. The proportion of granzyme B positive cells did not change after immunization, but granzyme B iMFI was significantly increased in both CD8 and γδ-T cells, returning to baseline at C-1. Production of IFNγ was induced in all T cell subsets, but most pronouncedly in CD4 and γδ-T cells. There were only weak correlations between cellular responses on C-1 and total blood-stage parasite exposure, as calculated by the sum of parasites/ml after all three immunizations (data not shown, Spearman's rho for all <0.5). None of the responses in the control group changed significantly from baseline at any point of time (**Table 2.2**), suggesting that chloroquine alone did not affect *P. falciparum* specific T cell responses.

We next assessed the association of these markers with protection after challenge (**Figure 2.3**). Indeed, complete protection associated with the proportion of CD107a positive CD4 T cells (OR=8.4; 95% CI 1.5-123; p=0.011, **Figure 2.3A**), the iMFI of CD107a on CD4 T cells (OR=11; 95% CI 1.6-188; p=0.011, data not shown) and granzyme B by CD8 T cells (OR=11; 95% CI 1.9-212; p=0.004,

Table 2.2 Cytotoxic T cell markers induced by CPS immunization[a,b,c]

		CD4 T cells									CD8 T cells					γδ T cells				
Marker		B	I1		I2		I3		C-1		B	I1	I2	I3	C-1	B	I1	I2	I3	C-1
CD107a	%	0,26	0,53	***	0,64	***	0,59	***	0,53	***	0,08	0,09	0,15	0,14	0,19	26,6	36,5 ***	41,2 ***	40,3 ***	33,8 **
CD107a	iMFI	15,7	38,5	***	46,0	***	41,0	***	37,0	***	7,6	14,5	19,2 *	17,5 *	20,9 **	4472	6925 ***	7925 ***	7594 ***	6262 **
Granzyme B	%	0,25	0,49		0,41		0,38		0,43		0,50	0,89	0,92	1,03	0,94	6,06	7,35	8,27	8,96	8,79
Granzyme B	iMFI	1,40	9,80		11,3		7,50		3,24		5,11	71,3 *	40,5	26,7	19,9	2,54	15,3 ***	15,0 ***	12,4 ***	8,18
IFNγ	%	0,08	0,43	***	0,54	***	0,42	***	0,47	***	0,04	0,11	0,13 *	0,08	0,13 **	6,01	13,6 ***	16,3 ***	14,9 ***	12,0 **
IFNγ	iMFI	5,16	32,2	***	37,7	***	25,7	*	37,2	***	2,96	9,8	9,3	6,4	12,5 **	407	1020 **	1173 ***	1029 **	982 *

[a] *PfRBC-specific responses were corrected for uRBC background, mean responses for all immunized volunteers (n=24) are shown. B= baseline; I= 27 days after indicated immunization; C-1=one day before challenge.*

[b] * *=p<0.05, **=p<0.01, ***=p<0.001 (one-way ANOVA; Dunnett's post hoc test, with B as control column)*

[c] *iMFI = integrated geometric mean fluorescence intensity (percentage positive cells multiplied by the MFI of this positive population).*

Figure 2.3E) at C-1. A subgroup analysis of data from Group 3 only confirmed these findings: the proportion of both CD107a positive CD4 T cells and granzyme B positive CD8 T cells were the only markers higher in protected subjects (OR=4.2; 95% CI 0.9-140; p=0.081 and OR=27; 95% CI 1.5-27687; p= 0.019 respectively). While expression of CD107 on CD4 T cells and granzyme B in CD8 T cells predicted protection with an Area Under the ROC Curve (AUC) of 0.73 (95% CI 0.48-0.98) and 0.81 (95% CI 0.63-0.99) respectively, combining both markers resulted in only a slight improvement of the AUC (0.82, 95% CI 0.61-1).

Pf-specific IFNγ production by CD4, CD8 or γδ-T cells could not distinguish protected volunteers (**Figure 2.3G, 3H** and **3I**). Also pluripotent (IFNγ+IL-2+) effector memory T cell (CD4+ CD62L- CD45RO+) responses, previously shown to be significantly increased by CPS immunization (8), were again induced (p=0.013), but did not differentiate between protected and unprotected volunteers (OR=1.6; 95% CI 0.5-4.9; p=0.41; data not shown).

CD107a expressing CD4 T cells presented as the clearest marker associated with protection, consistently higher in fully protected subjects from I1 onwards (**Figure 2.4A**), and independent of immunization dose (**Figure 2.4B**). A significant correlation was found between CD107a expression by CD4 T cells after one immunization and prepatent period after challenge-infection in all TS+ (Spearman's rho=0.69; p=0.013, **Figure 2.4C**). The proportion CD107a+ CD4 T cells in the control subject who developed parasitemia significantly later than the other controls (i.e. day 13.5 versus day 9-10.5), was at baseline on average 2.8 fold higher than in the other subjects. Possibly, the inherently higher response in this volunteer contributed to delayed pre-patency after challenge.

CD107a+ CD4 T cells expressed proportionally more granzyme B (7.4% versus 0.39% on C-1; p<0.0008) in protected subjects, indicative for their cytotoxic phenotype, and IFNγ (13.3% versus 0.39% on C-1; p<0.0001) than CD4 T cells negative for CD107a (**Figure 2.4D** and **Figure 2.4E**). CD8 T cells, traditionally considered the cytotoxic subclass of T cells, indeed contained a larger proportion of CD107a positive cells at baseline than CD4 T cells when unstimulated (uRBC); 0.39% versus 0.19% respectively; p<0.0001 (all volunteers). However, the proportion of *Pf*-specific degranulation of CD8 T cells was not notably increased by CPS immunization (p=0.44), in contrast to CD4 T cells (p<0.0001, **Figure 2.S2A&B**).

Both CD107a expression by CD4 T cells and granzyme B production by CD8 T cells remained significantly elevated up to twenty weeks after the challenge-infection (C+140) (p<0.05 and p<0.01; **Figure 2.5A** and **2.5B**), demonstrating longevity of the CPS-induced T cell response.

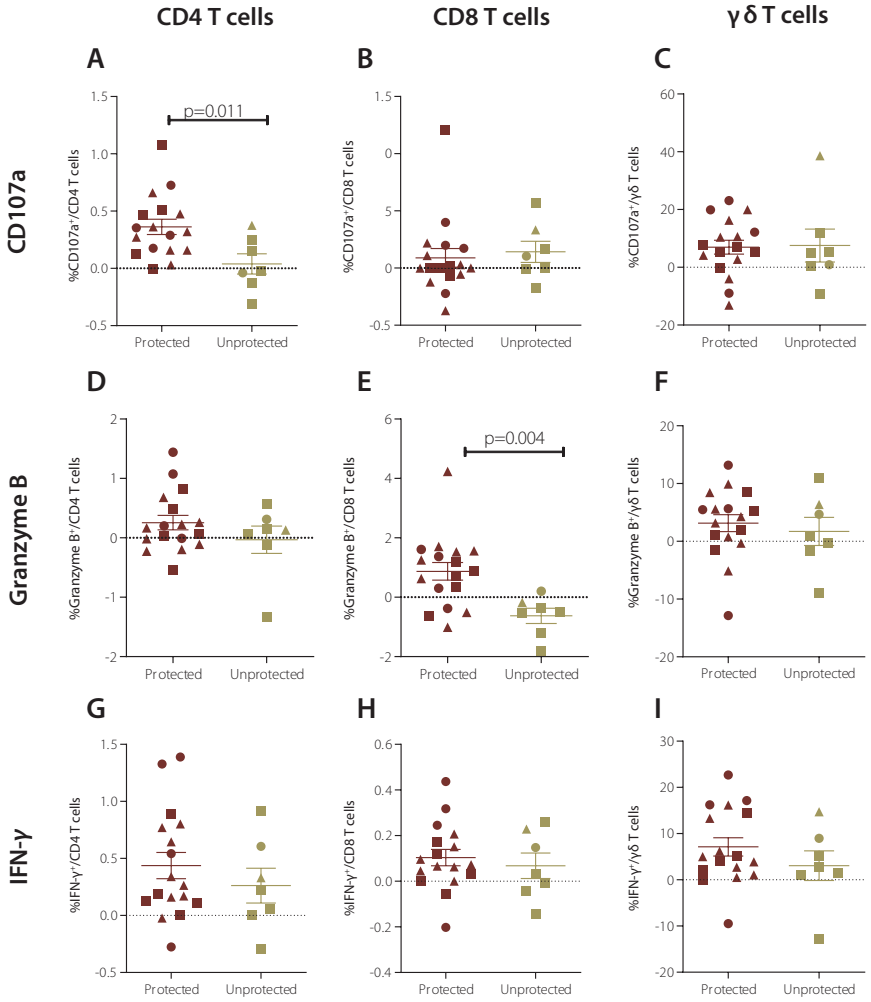

Figure 2.3 Cytotoxic immune responses upon in vitro PfRBC stimulation at one day before challenge infection (C-1).

*Each symbol represents a single protected (**red symbols**) or CPS-immunized unprotected (**brown symbols**) individual from group 1 (dots), group 2 (triangles) or group 3 (squares). Horizontal bars and whiskers represent means and SEMs. Panels show CD107a+ CD4 (**A**), CD8 (**B**) and γδ (**C**) T cells, granzyme B expression on CD4 (**D**), CD8 (**E**) and γδ (**F**) T cells and IFNγ+ CD4 (**G**), CD8 (**H**) and γδ (**I**) T cells. Values are corrected for uRBC background and for baseline-response before immunization. Background responses to uRBC stimulation were 0.19±0.01, 0.41±0.02 and 0.61±0.05 for CD107a, 1.65±0.50, 15.34±1.46 and 64.56±1.74 for granzyme B and 0.09±0.00, 0.07±0.00 and 0.14±0.01 for IFNγ, on CD4, CD8 and γδT cells respectively (mean ±SEM, calculated for all volunteers on both baseline and C-1). High uRBC granzyme B responses in CD8 and γδ T cells indicate that a significant percentage of these cells contains granzyme B even in a resting situation. uRBC responses did not change significantly from baseline for any of the readouts. The differences between responses of protected and unprotected volunteers in the graphs without a p-value are non-significant. The differences between protected and unprotected volunteers are calculated using logistic regression.*

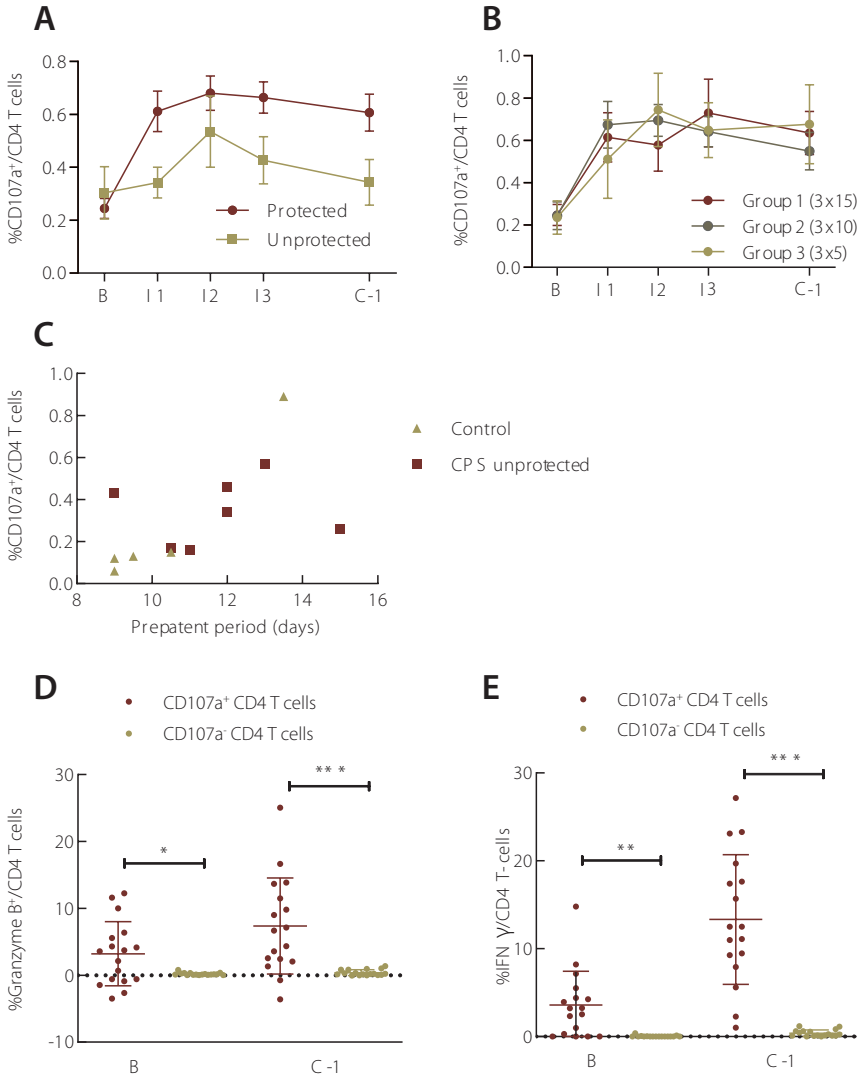

Figure 2.4 Cytotoxic profile of CPS-induced CD4 T cells.

*(**A+B**) Induction of Pf-specific CD107a positive CD4 T cells was determined (**A**) in protected and unprotected CPS-immunized subjects over the course of immunization and (**B**) in protected subjects separated for each immunization dose. Horizontal bars and whiskers represent mean responses and SEM. (**C**) The relationship between Pf-specific CD107a CD4 T cells on I1 and the prepatent period after challenge for all TS+ volunteers (CPS-immunized and controls). Within protected CPS-immunized subjects, (**D**) granzyme B and (**E**) IFNγ production by CD107a+ (**red dots**) and CD107a- (**brown dots**) CD4 T cells was analyzed at baseline (B on x-axis) and after CPS immunization (C-1) in all protected subjects. Horizontal bars show the mean response. All data were corrected for uRBC background for every volunteer at each time point. Abbreviations on the x-axis: B= baseline; I= 27 days after indicated immunization; C-1=one day before challenge. *=p<0.05 **p<0.01 ***p<0.001*

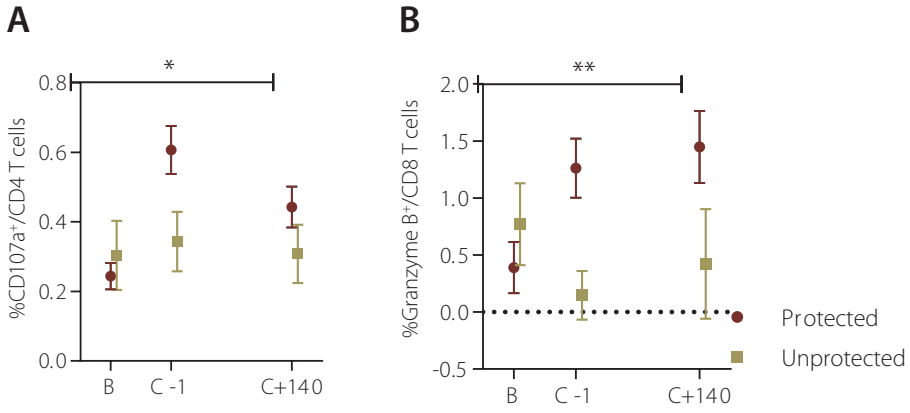

Figure 2.5 Longevity of cellular immune responses after CPS immunization.
*Pf-specific cellular immune responses (corrected for uRBC background) were assessed in protected (**red dots**) and unprotected (**brown squares**) CPS-immunized volunteers before CPS immunization (**B**), and before (C-1) and 20 weeks after challenge infection (C+140). Data are shown as mean ±SEM for (**A**) CD107a expression on CD4 T cells and (**B**) granzyme B production by CD8 T cells. Tests are performed separately for protected and immunized unprotected volunteers, by the repeated measures ANOVA (including all time points before and after immunizations) and the Dunnett's Multiple comparison post test, using B as control column. Only the test results of C+140 compared to baseline for protected volunteers are displayed. For immunized unprotected volunteers, all results were non-significant. *=p<0.05 **p<0.01*

DISCUSSION

We show that CPS immunization reproducibly and dose-dependently induces protection against a homologous challenge infection. With exposure to a total number of *Pf* infected mosquito bites as low as 30, CPS immunization still induces 89% protection in healthy volunteers. We furthermore demonstrate that markers of cytotoxic T cell responses are associated with protection against malaria after whole sporozoite immunization.

This study provides further support for the remarkable potency of the CPS-protocol to induce complete protection by using even lower numbers of *Pf*-infected mosquitoes than before (8). The observed dose-dependent protection is in line with results from RAS immunization trials with sporozoites administered either intravenously by needle and syringe (10) or by bites from irradiated infected mosquitoes (4). Although the delay of patency in unprotected CPS-immunized subjects was not statistically significant, the patterns of parasitemia indicate partial protection in some subjects. The unexpectedly delayed control subject hampered statistical significance but could be considered an outlier, possibly because of the inherently high baseline immune response. The establishment of a sub-optimal CPS immunization regimen inducing protection in 50% of the immunized volunteers with 3x5 mosquito bites will facilitate further studies of protective immune mechanisms against *Pf* malaria.

Our data provide evidence for a role of cytotoxic T cell responses in pre-erythrocytic immunity in humans. Due to obvious practical limitations, we only assessed immune cells in the peripheral blood, which may not necessarily reflect responses in the liver but rather represent a surrogate. The results of this exploratory analysis will have to be confirmed in future trials, and the functional relevance remains to be investigated.

'Classical' cytotoxic CD8 T cells can be activated by malaria antigen on infected hepatocytes via major histocompatibility complex (MHC) class I (25) and are associated with protection in a number of (animal) models (13, 14, 26). CD8 T cells are involved in protection in the murine CPS and RAS models (27-29), but their precise effector mechanisms remain subject of debate. They might either require direct contact with infected hepatocytes (13), or in fact be independent of granzyme B and/or other cytotoxic molecules, suggestive for a more indirect cytokine mediated effect by CD8 T cells (12) or other hepatic immune cells (30). In addition, a functional role for cytotoxic CD4 T cells is also conceivable as these cells can use cytolytic pathways such as granulysin, perforin and granzymes and FAS-L, as shown mostly in viral infections (31, 32). The protective role of CD4 T cells in murine malaria has been suggested, using *in vitro* experiments (33), and *in vivo* depletion (12) or passive transfer (34). Fur-

thermore, functional cytotoxic CD4 T cells, derived from RAS- or synthetic peptide immunized volunteers, are able to lyse autologous B cells pulsed with a peptide from the circumsporozoite protein (35-37). We used surface expression of CD107a (LAMP-1), a marker for cytotoxic degranulation, to phenotypically identify cytotoxic CD4 T cells (31). In order to directly kill a *Pf*- infected hepatocyte, parasite antigens should be presented in the context of MHC class II (MHCII) to the cytotoxic CD4 T cells. Although hepatocytes do not express MHCII in non-inflammatory circumstances, the presence of MHCII on human hepatocytes has been shown in a small number of patients with chronic hepatitis (38) and immune mediated liver disorders (39, 40). Functionally, over-expression of MHCII on hepatocytes in a transgenic mice model showed their capacity for co-stimulation, antigen-presentation and CD4 T cell activation (41). Only indirect evidence suggests that MHCII expression on mice hepatocytes may play a role in murine malaria (33, 42), and the presence of MHCII on hepatocytes in human malaria has never been studied. Here, we show for the first time that degranulating CD4 T cells are associated with protection in human malaria and already significantly induced after one immunization.

The observed lack of boosting by the second and third immunization may reflect a saturated response of antigen specific memory cells. This raises the possibility that fewer immunizations may be sufficient to induce protection, supported by the increased proportion of volunteers without parasitemia after the second and third immunization in Group 1 and 2. Moreover, the observed longevity of the immune response is in line with long-term protection after CPS immunization in a previous study (9).

The T_H1 cytokine IFNγ has been repeatedly shown to be an important effector molecule in protection against the malaria parasite (43), and the clear induction of T_H1 responses in our study corroborates earlier findings in both animals and humans after whole sporozoite immunization (8, 10, 12, 26, 27). We previously showed that a broad range of both innate and adaptive cellular subsets contribute to CPS-induced *Pf*-specific IFNγ production (16), which is sustained at least up to 2.5 years after immunization (9). IFNγ production alone, however, does not correlate with protection in neither RAS (10) nor our CPS model. Also production of both IFNγ and IL-2 by effector memory CD4 T cells, and IFNγ production by γδ-T cells, although clearly increased in immunized volunteers (8, 16), did not differentiate between protected and unprotected volunteers.

During CPS immunization, four protected subjects did not show parasitemia by qPCR at any measured time point, not even after the first immunization. A possible explanation is that the number of merozoites released from the liver is too low for qPCR detection. A strong primary innate immune response

may be responsible for clearing sporozoites and/or killing infected hepatocytes upon first encounter. Previous studies in mice indeed showed that inflammatory cytokines IL-1 and IL-6 block pre-erythrocytic development in mice (16, 44). Alternatively, chloroquine may have contributed to the decreased, i.e. undetectable number of parasites released from the liver either by direct killing, or indirectly by stimulating the immune system.

Antigen recognition and immune cell activation are essential for an effective response. To investigate pre-erythrocytic cellular immune responses, stimulation with cultured *Pf* liver stages would be preferred, but this is currently impossible. We therefore used asexual blood stage parasites for our experiments and although responses to purely pre-erythrocytic antigens may be missed, the majority of potential memory responses are likely detected upon *Pf*RBC stimulation, given the large overlap between liver and blood stage antigens (45). Future antigen screening by stimulation with a comprehensive library of pre-erythrocytic and cross-stage proteins or peptides, and subsequent functional studies focussing on cytotoxic T cells will further identify and delineate the specificity of protective responses (33, 46).

In conclusion, we identified two *in vitro* cellular cytotoxic immune markers that are associated with protection against malaria in a controlled clinical setting. Furthermore, this study confirms the robustness of CPS immunization as a highly efficient and reproducible immunization strategy for complete homologous protection. Further exploration of immune responses induced by CPS immunization will make important contributions to pre-erythrocytic malaria vaccine development and clinical testing.

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SUPPLEMENTARY INFORMATION

CPS immunization and challenge

All subjects received a standard prophylactic regimen of chloroquine consisting of a loading dose of 300 mg on each of the first two days and then 300 mg once a week for a total duration of 14 weeks. During this period, all subjects were exposed three times to the bites of *Anopheles stephensi* mosquitoes at monthly intervals starting eight days after the first chloroquine dose as described previously (11). All volunteers were exposed to bites from exactly 15 mosquitoes at each session, but the number of NF54 *Pf* infected versus uninfected mosquitoes varied per group: five subjects received three times bites from 15 infected mosquitoes (Group 1), ten subjects received three times bites from 10 infected and 5 uninfected mosquitoes (Group 2), ten subjects received three times bites from 5 infected and 10 uninfected mosquitoes (Group 3) and five control subjects received three times bites from 15 uninfected mosquitoes (Group 4). From day 6 to 10 after each immunization, subjects were checked daily on an outpatient basis and blood was drawn for peripheral blood smears, standard haematological measurements and cardiovascular safety markers and stored for retrospective analysis of parasitemia by quantitative real-time PCR (qPCR) (47).

After the challenge-infection, volunteers were checked twice daily on an outpatient basis from day 5-21 for (un)solicited symptoms and signs. As soon as parasites were detected by thick smear, subjects were treated with a standard curative regimen of 1000 mg atovaquone and 400 mg proguanil once daily for three days, according to Dutch national guidelines. If subjects remained thick smear negative, they were presumptively treated with the same curative regimen on day 21 after challenge infection. Chloroquine levels one day before challenge were measured in EDTA-plasma by liquid chromatography and were below detection limit (5 µg/L) in all volunteers one day before challenge (48).

Retrospectively, parasitemia was quantified on day six until day ten after each immunization and from day five until day 21 after challenge by qPCR using *Pf* standard curves prepared by DNA extraction from titrated samples of ring-infected cells (47). Adverse events (AEs) were recorded as described previously (11).

Platelet counts were determined in EDTA-anticoagulated blood with the Sysmex XE-2100 (Sysmex Europe GmbH, Norderstedt, Germany). D-dimer concentrations were assessed in citrate plasma by STA-R Evolution (Roche Diagnostics, Almere, The Netherlands).

PBMC isolation and cryopreservation

Venous whole blood was collected into citrated vacutainer cell preparation tubes (CPT; Becton and Dickinson) and stored at room temperature for a maximum of 4 hours; PBMCs were isolated by centrifugation and washed four times in ice-cold phosphate-buffered saline (PBS). Cells were counted and cryopreserved at a concentration of 10^7 cells/ml in ice-cold foetal-calf serum (Gibco) containing 10% dimethylsulfoxide (Merck, Germany) using Mr. Frosty freezing containers (Nalgene). Samples were stored in vapour-phase nitrogen.

In vitro *Pf*- infected erythrocyte re-stimulation assay

PBMC were thawed, washed twice in Dutch-modified RPMI 1640 (Gibco/ Invitrogen) and counted in 1% trypan blue containing 5% zap-oglobin II Lytic Reagent (Beckman Coulter) using a Neubauer improved bright line counting chamber (Marienfield, Germany); median cell recovery was 80%. PBMCs were *in vitro* re-stimulated with cryopreserved NF54 *Pf*-infected erythrocytes (*Pf*RBC) as described previously (16). Cells were re-suspended in complete culture medium (Dutch-modified RPMI 1640 containing 2 mM glutamine, 1mM pyruvate, 0.05 mM gentamycine and 10% human A+ serum, (Sanquin, Nijmegen) at a final concentration of $2.5x10^6$/ml. PBMC were transferred into polystyrene 96-well round-bottom plates and stimulated in duplicate wells with either $5x10^6$/ml (final concentration) cryopreserved *Pf*RBC or uRBC (uninfected erythrocytes) in a total volume of 110 µl/well for 24 hours at 37°C/ 5%CO_2. For the last four hours, 10 µg/ml Brefeldin A (Sigma-Aldrich) and 2µM monensin (eBioscience) were added, based on pilot experiments. In positive control wells, PMA (50 ng/ml, Sigma-Aldrich) and ionomycin (1 mg/ml, Sigma-Aldrich) were added the last four hours. After a total of 24 hours, cells were harvested and stained.

Flow cytometry analysis

PBMCs were co-incubated during the 24 hour-stimulation with CD107a Pacific Blue (Biolegend, clone H4A3). All cells were transferred to a polystyrene V-bottom plate and washed twice with 200µl PBS. Next, cells were stained with Live/Dead fixable dead cell stain dye aqua (Invitrogen) in 50 µl PBS for 30 minutes at 4°C. After washing with PBS containing 0.5% bovine serum albumin (BSA, Sigma-Aldrich) cells were stained with antibodies against the surface markers CD3 PerCP (Biolegend, clone UCHT1), CD4 ECD (Beckman-Coulter, clone SF-

CI12T4D11) CD8 APC-H7 (BD Biosciences, clone SK1), γδ-T cell receptor PE (Beckman-Coulter, clone IMMU510) and CD56 APC (eBioscience, clone MEM188) in 50 µl PBS containing 0.5% BSA for 30 minutes at 4°C. Cells were washed again and fixed in Foxp3 fixation/permeabilization buffer (eBioscience). Following a wash step with Foxp3 permeabilization buffer (eBioscience), cells were stained in permeabilization buffer containing granzyme B FITC (Biolegend, clone GB11) and IFNγ PeCy7 (Biolegend, clone 4S.B3). Cells were washed again in permeabilization buffer and kept cold and dark in fixation buffer (1% paraformaldehyde in PBS) until measured by flow cytometry on the same day. For every individual volunteer, all time points were thawed, stimulated and stained within the same experimental round. In a separate experiment, cells from the time points B and C-1 were *in vitro* re-exposed to *Pf* infected erythrocytes and stained for viability, γδ-T cell receptor PE, CD56 PE, CD3 PerCP, CD45RO ECD (Beckman-Coulter, clone mIgG2a), CD62L PeCy7 (Biolegend, clone DREG-56) CD4 Pacific Blue (eBioscience, clone OKT-4) CD8 AF700 (Biolegend, clone HIT8A), IFNγ FITC and IL-2 APC (eBioscience, clone MQ1-17H12) using the same protocol as described for the other staining panel.

Samples were acquired using a 9-color Cyan ADP (Beckman Coulter), each round using single stained cells for compensation. Per sample, a median of $93.8x10^3$ (range $12.5x10^3$ - $221x10^3$) singlet living lymphocytes were acquired. Data analysis was performed using FlowJo software (version 9.6; Tree Star). A representative example showing the gating strategy is shown in S3. The definition of cell positivity (for cytokines and cytotoxic molecules) was performed automatically, based on the MFI of unresponding PBMCs for each sample separately. Responses to uRBC were subtracted from the response to *Pf*RBC for every volunteer on every time point.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5 unless mentioned otherwise. Differences between immunized unprotected and control volunteers in prepatent periods by thick smear and qPCR were tested by Mann-Whitney U test. Induction of cytotoxic immune responses on the time points I1, I2, I3 and C-1 were tested by the repeated measures ANOVA and the Dunnett's Multiple comparison post test, with baseline as control column. Induction of immune responses on 140 days after challenge (C+140) was tested separately for protected and immunized unprotected volunteers, by the repeated measures ANOVA (including all previous time points mentioned above) and the Dunnett's Multiple comparison post test, with baseline as control column.

The correlation of CD107a expression by CD4 T cells with the prepatent period, and the correlation of cellular immune responses with cumulative parasitemia during CPS immunization were assessed by non-parametric Spearman correlation. The proportion of CD107a+ CD4 vs CD8 T cells and the production of granzyme B and IFNγ on CD107a^{+} vs CD107a- CD4 T cells were tested by the paired Student's t-test. For the correlation of CD107a CD4 T cells with prepatent period after challenge, immune re-call responses to *Pf*RBC (corrected for uRBC stimulation background) were tested on the different time points, while for all other tests we assessed the change from baseline (B).

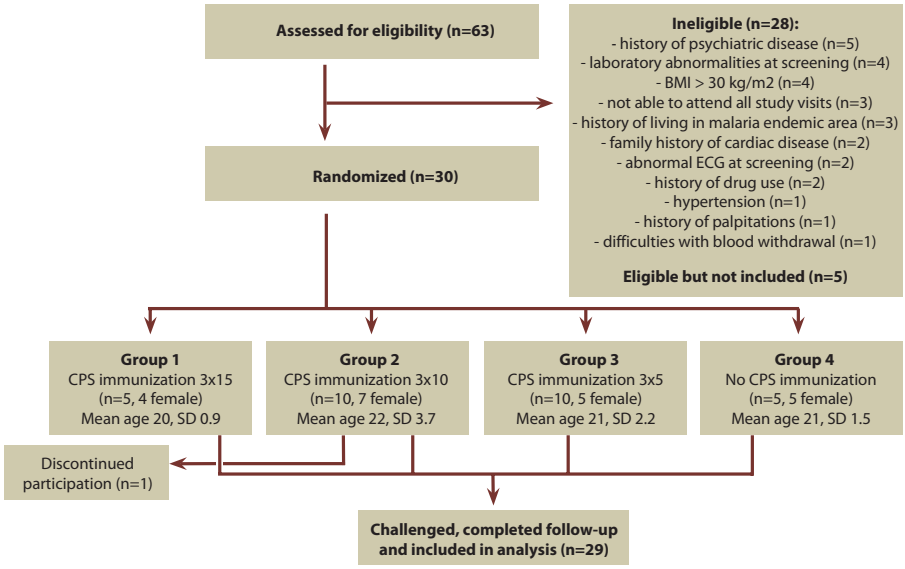

Figure 2.S1 Study flow diagram.
Twenty-five subjects were randomly assigned to receive different doses of CPS immunization in a double-blind fashion; five control subjects received bites from uninfected mosquitoes. One subject withdrew informed consent after the first immunization for reasons unrelated to the trial. Twenty-nine subjects received a challenge infection by the bites of five infected mosquitoes fifteen weeks after discontinuation of chloroquine chemoprophylaxis.

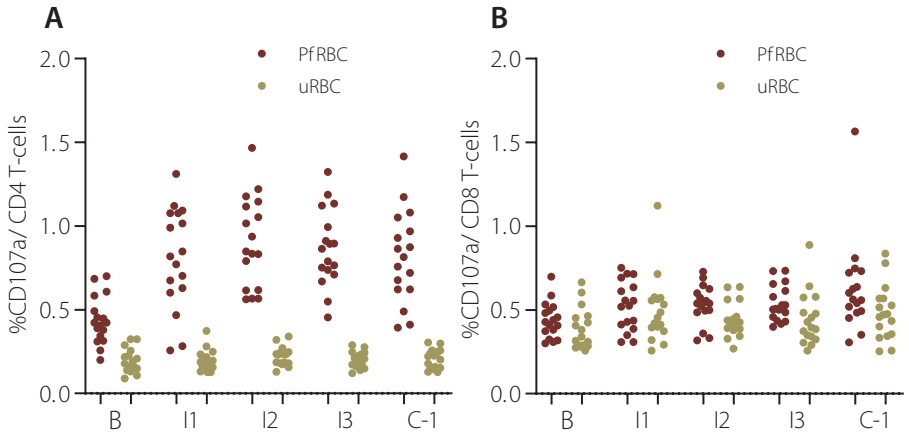

Figure 2.S2 Induction of cytotoxic CD4 and CD8 T cell responses by CPS immunization. *CD107a expression was assessed on (**A**) CD4 T cells and (**B**) CD8 T cells after stimulation with PfRBC (**red dots**) and uRBC (**brown dots**) before, during and after CPS immunization (protected subjects only). B= baseline; I= 27 days after indicated immunization; C-1=one day before challenge.*

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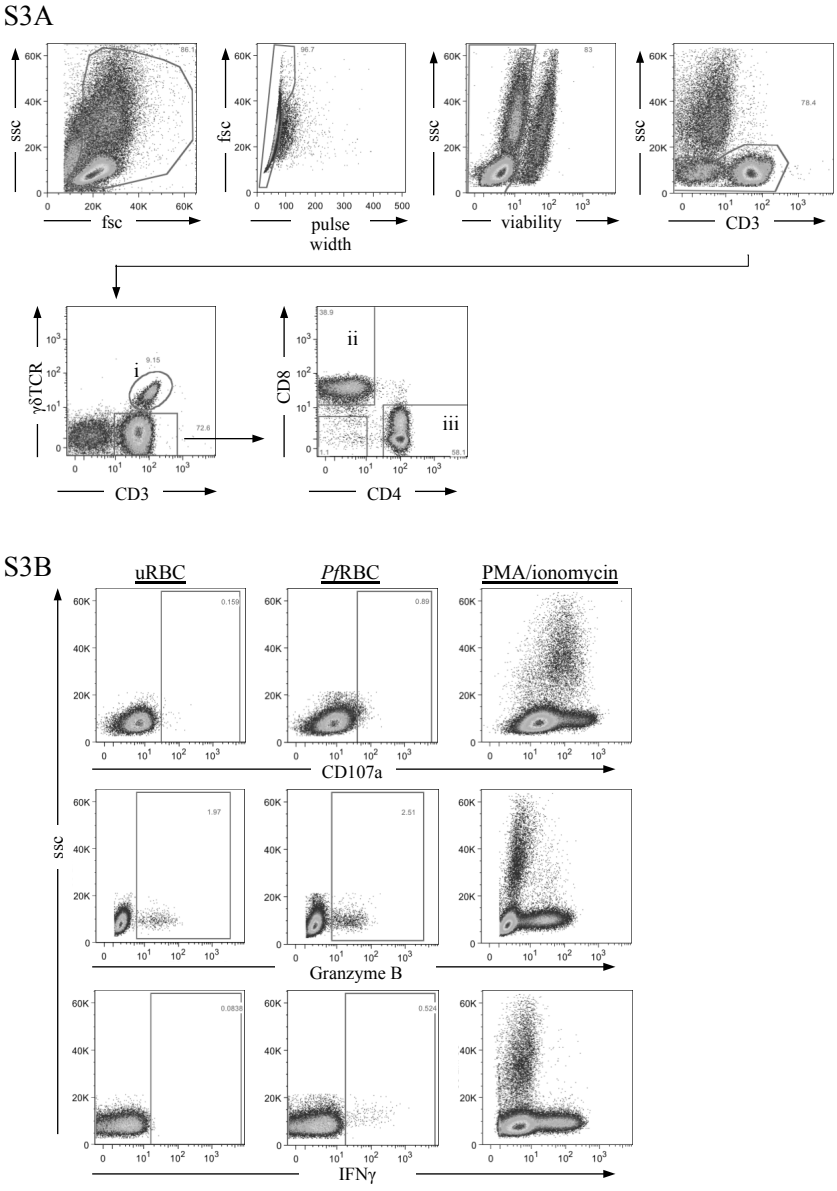

Figure S2.3 Flow cytometry gating strategy.
*(**A**) Representative flow cytometry plots for a uRBC stimulated sample from one volunteer at baseline (before immunization). Singlet viable CD3+ PBMC were subdivided into (i) γδT cells, (ii) CD8 T cells and (iii) CD4 T cells; No additional dump channel for CD14, CD19 and CD20 was used. (**B**) Gating of CD107a, granzyme B and IFNγ positive cells for uRBC, PfRBC and PMA/ionomycin re-stimulated cells at baseline. For uRBC and PfRBC stimulation CD4 T cells are shown, for PMA/ionomycin total viable PBMCs. Within each sample, gating of marker positive cells was performed automatically, based on the MFI of marker negative cells.*

Chad. March 2005: Forchana refugee camp on the border of Chad and Sudan; accommodating 17.000 refugees from Darfur.

CHAPTER 3

Sporozoite immunization of human volunteers under mefloquine prophylaxis is safe, immunogenic and protective: a double-blind randomized controlled clinical trial

Else M. Bijker*, Remko Schats*, Joshua M. Obiero, Marije C. Behet, Geert-Jan van Gemert, Marga van de Vegte-Bolmer, Wouter Graumans, Lisette van Lieshout, Guido J.H. Bastiaens, Karina Teelen, Cornelus C. Hermsen, Anja Scholzen, Leo G. Visser, Robert W. Sauerwein.

*Contributed equally

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ABSTRACT

Immunization of healthy volunteers with chloroquine ChemoProphylaxis and Sporozoites (CPS-CQ) efficiently and reproducibly induces dose-dependent and long-lasting protection against homologous *Plasmodium falciparum* challenge. Here, we studied whether chloroquine can be replaced by mefloquine, which is the only other licensed anti-malarial chemoprophylactic drug that does not affect pre-erythrocytic stages, exposure to which is considered essential for induction of protection by CPS immunization. In a double blind randomized controlled clinical trial, volunteers under either chloroquine prophylaxis (CPS-CQ, n = 5) or mefloquine prophylaxis (CPS-MQ, n = 10) received three sub-optimal CPS immunizations by bites from eight *P. falciparum* infected mosquitoes each, at monthly intervals. Four control volunteers received mefloquine prophylaxis and bites from uninfected mosquitoes. CPS-MQ immunization is safe and equally potent compared to CPS-CQ inducing protection in 7/10 (70%) versus 3/5 (60%) volunteers, respectively. Furthermore, specific antibody levels and cellular immune memory responses were comparable between both groups. We therefore conclude that mefloquine and chloroquine are equally effective in CPS-induced immune responses and protection.

INTRODUCTION

Malaria remains one of the most important infectious diseases worldwide and still causes approximately 207 million cases and 627,000 deaths every year (1). Anti-disease immunity against malaria is not easily induced: in endemic areas this takes many years of repeated exposure to develop (2), and sterile protection against infection does not seem to be induced at all (3). Also candidate vaccines have shown only limited protective efficacy so far (4, 5). Novel vaccines and drugs can be tested for efficacy at an early stage of clinical development in Controlled Human Malaria Infection (CHMI) studies, exposing a small number of healthy volunteers to *Plasmodium falciparum* by bites from infected *Anopheles* mosquitoes. Immunization of healthy volunteers under chloroquine ChemoProphylaxis with Sporozoites (CPS-CQ immunization) efficiently, reproducibly and dose-dependently induces protection against homologous CHMI (6, 7), shown in a subset of volunteers to last for more than 2 years (8). CPS-CQ immunization requires exposure to bites from only a total of 30-45 *P. falciparum* infected mosquitoes to induce 89–95% protection (6, 7, 9). In contrast, protection by immunization with radiation-attenuated sporozoites (RAS) requires a minimum of 1000 infected mosquito bites (10), or intravenous injection of five times 135,000 cryopreserved sporozoites (11).

The unprecedented efficiency of the CPS immunization regime may relate to its design: in contrast to RAS, CPS immunization allows full liver stage development and exposure to early blood-stages. Moreover, chloroquine is known for its immunomodulatory capacities (12-14) that may play a role in induction of protection, which is mediated by pre-erythrocytic immunity (9) including antibodies directed against sporozoites (15-17), and likely T cells targeting liver-stages (7). Next to chloroquine, mefloquine (MQ) is the only licensed drug for chemoprophylaxis that does not affect pre-erythrocytic stage development (18). We therefore aimed to assess whether chloroquine could be replaced by mefloquine for CPS immunization. In a double blind randomized controlled clinical trial we assessed safety, immunogenicity and protection against challenge for CPS-MQ compared to CPS-CQ.

METHODS

Study subjects

Healthy subjects between 18 and 35 years old with no history of malaria were screened for eligibility based on medical and family history, physical examination and standard hematological and biochemical measurements. Urine toxicology screening was negative in all included subjects; none of the subjects were pregnant or lactating. Serological analysis for HIV, hepatitis B, hepatitis C and *P. falciparum* asexual blood-stages was negative in all subjects. All subjects had an estimated 10-year risk smaller than 5% of developing a cardiac event as estimated by the Systematic Coronary Evaluation System adjusted for the Dutch population (19). None of the subjects had travelled to a malaria-endemic area during or within 6 months prior to the start of the study. All subjects provided written informed consent before screening. The Central Committee for Research Involving Human Subjects of The Netherlands approved the study (NL 37563.058.11). Investigators complied with the Declaration of Helsinki and Good Clinical Practice including monitoring of data. This trial is registered at ClinicalTrials.gov, identifier NCT01422954.

Study design and procedures

This single center, double blind randomized controlled trial was conducted at Leiden University Medical Center (Leiden, the Netherlands) from April 2012 until April 2013 (**Figure 3.1**). Twenty subjects were randomly divided into three groups by an independent investigator using a computer-generated random-number table. Subjects, investigators and primary outcome assessors were blinded to the allocation. Subjects in the CPS-CQ group (n = 5) received a standard prophylactic regimen of chloroquine consisting of a loading dose of 300 mg on the first and fourth day and subsequently 300 mg once a week for 12 weeks. Subjects in the CPS-MQ group (n = 10) and the control group (n = 5) received mefloquine prophylaxis starting with a loading split dose regimen to limit potential side-effects: 125 mg twice per week for a duration of 3 weeks and subsequently 250 mg once a week for 12 weeks. Chloroquine and mefloquine were administered as capsules, indistinguishable from each other. During this period all subjects were exposed to the bites of 8 *Anopheles* mosquitoes three times at monthly intervals, starting 22 days after start of mefloquine prophylaxis and 8 days after start of chloroquine prophylaxis.

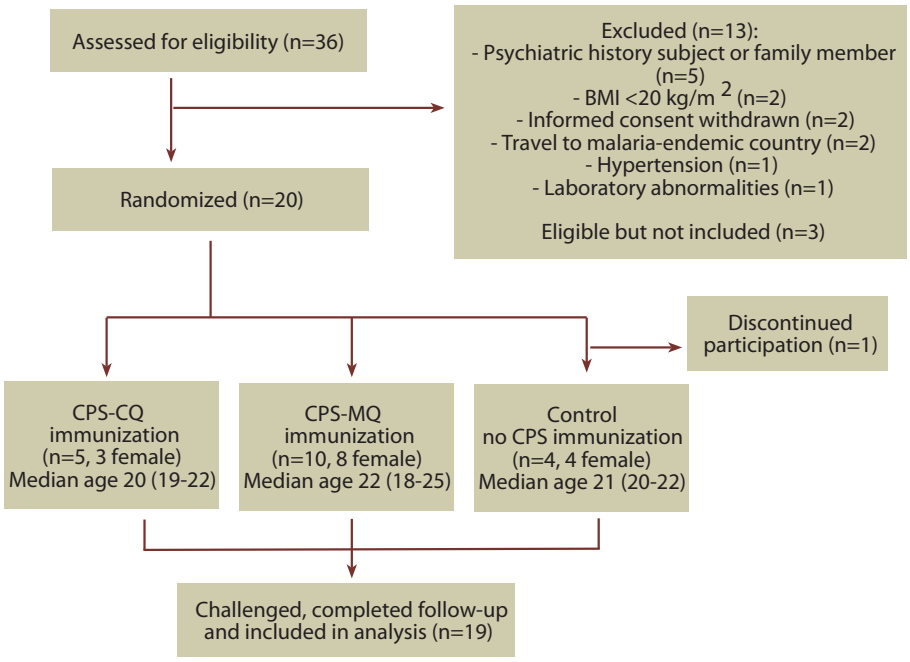

Figure 3.1 Study flow diagram.
Thirty-six subjects were screened for eligibility, of whom twenty were included in the trial and randomized over three groups. One control subject was excluded after initiation of chemoprophylaxis but before the first immunization because of an unexpected visit to a malaria-endemic area during the study period. In a double-blind fashion, fifteen subjects received either CPS-CQ or CPS-MQ immunization and four control subjects received bites from uninfected mosquitoes and mefloquine prophylaxis. Subjects received a challenge infection by bites of five infected mosquitoes sixteen weeks after discontinuation of prophylaxis.

Volunteers in the CPS-CQ and CPS-MQ groups received bites from mosquitoes infected with the *P. falciparum* NF54 strain, control subjects received bites from uninfected mosquitoes. The immunization dose was based on our previous dose-de-escalation trial (7) and aimed to establish partial protection in the CPS-CQ group in order to enable detection of either improved or reduced protection in the CPS-MQ group. Sample sizes were calculated based on the expected difference of 4 days in prepatent period between the CPS-CQ and CPS-MQ groups, a standard deviation of 1.6 and 2.3 days respectively, an α of 5% and a power of 0.90. This calculation resulted in a CPS-CQ group of 4 and a CPS-MQ group of 8 subjects. To account for possible dropouts based on (perceived) side effects we included one and two extra volunteers in the CPS-CQ and CPS-MQ groups respectively. The control group was included as infectivity control for the challenge infection.

On days 6 to 10 after each immunization by mosquito exposure, all subjects were followed on an outpatient basis and peripheral blood was drawn for blood smears, standard hematological measurements, cardiovascular markers and retrospective qPCR.

Twenty weeks after the last immunization, sixteen weeks after discontinuation of prophylaxis, all subjects were challenged by the bites of five mosquitoes infected with the homologous NF54 *P. falciparum* strain, according to previous protocols (20). After this challenge-infection, all subjects were checked twice daily on an outpatient basis from day 5 up until day 15 and once daily from day 16 up until day 21 for symptoms and signs of malaria. Thick blood smears for parasite detection were made during each of these visits after challenge, hematological and cardiovascular markers were assessed daily. As soon as parasites were detected by thick smear, subjects were treated with a standard curative regimen of 1000 mg atovaquone and 400 mg proguanil once daily for three days according to Dutch national malaria treatment guidelines. If subjects remained thick smear negative, they were presumptively treated with the same curative regimen on day 21 after challenge infection. All subjects were followed closely for 3 days after initiation of treatment and complete cure was confirmed by two negative blood smears after the last treatment dose. Chloroquine and mefloquine levels were measured retrospectively in citrate-plasma from the day before challenge by liquid chromatography (detection limit for both chloroquine and mefloquine: 5 µg/L) (21).

Anopheles stephensi mosquitoes for immunizations and challenge-infection were reared according to standard procedures at the insectary of the Radboud university medical center. Infected mosquitoes were obtained by feeding on NF54 gametocytes, a chloroquine- and mefloquine-sensitive *P. falciparum* strain, as described previously (22). After exposure of volunteers, all blood-engorged mosquitoes were dissected to confirm the presence of sporozoites. If necessary, feeding sessions were repeated until the predefined number of infected or uninfected mosquitoes had fed.

Endpoints

The primary endpoint was prepatent period, defined as the time between challenge and first positive thick blood smear. Secondary endpoints were parasitemia and kinetics of parasitemia as measured by qPCR, adverse events and immune responses.

Detection of parasites by thick smear

Blood was sampled twice daily from day 5 until day 15 and once daily from day 16 up until day 21 after challenge and thick smears were prepared and read as described previously (9). In short, approximately 0.5 µl of blood were assessed by microscopy and the smear was considered positive if two unambiguous parasites were seen.

Quantification of parasitemia by qPCR

Retrospectively, parasitemia was quantified by real-time quantitative PCR (qPCR) on samples from day 6 until day 10 after each immunization and from day 5 until day 21 after challenge as described previously (23), with some modifications. Briefly, 5 µl Zap-Oglobin II Lytic Reagent (Beckman Coulter) was added to 0.5 ml of EDTA blood, after which the samples were mixed and stored at –80°C. After thawing, samples were spiked with the extraction control Phocine Herpes Virus (PhHV) and DNA was extracted with a MagnaPure LC isolation instrument. Isolated DNA was resuspended in 50 µl H_2O, and 5 µl was used as template. For the detection of *P. falciparum*, the primers as described earlier (23) and the TaqMan MGB probe AAC AAT TGG AGG GCA AG-FAM were used. For quantification of PhHV the primers GGGCGAATCACAGATTGAATC, GCGGTTCCAAACGTACCAA and the probe Cy5-TTTTTATGTGTCCGCCACCATCTGGATC were used. The sensitivity of qPCR was 35 parasites/ml of whole blood.

Adverse events and safety lab

Adverse events (AEs) were recorded as following: mild events (easily tolerated), moderate events (interfering with normal activity), or severe events (preventing normal activity). Fever was recorded as grade 1 (>37·5°C–38·0°C), grade 2 (>38·0°C–39·0°C) or grade 3 (>39·0°C). Platelet and lymphocyte counts were determined in EDTA-anti-coagulated blood with the Sysmex XE-2100 (Sysmex Europe GmbH, Norderstedt, Germany). D-dimer concentrations were assessed in citrate plasma by STA-R Evolution (Roche Diagnostics, Almere, The Netherlands).

Immunological analyses

In order to assess cellular immune memory responses, peripheral blood mononuclear cell (PBMC) re-stimulation assays were performed as described previously (7). PBMCs were collected, frozen in fetal calf serum containing 10% dimethylsulfoxide, and stored in vapor phase nitrogen before initiation of prophylaxis (baseline; B) and one day before the challenge infection (C-1).

After thawing, PBMCs were re-exposed *in vitro* to *P. falciparum*-infected red blood cells (*Pf*RBC) and incubated for 24 hours at 37°C in the presence of a fluorochrome-labeled antibody against CD107a. Uninfected red blood cells (uRBCs) were used as a negative control. During the last 4 hours of incubation, 10 µg/ml Brefeldin A and 2 µM Monensin were added, allowing cytokines to accumulate within the cells. As a positive control, 50 ng/ml PMA and 1 mg/ml ionomycin were added for the last four hours of incubation. After 24h stimulation, cells were further stained with a viability marker and fluorochrome-labeled antibodies against CD3, CD4, CD8, CD56, γδ-T cell receptor, IFNγ and granzyme B (**Table 3.S1** (7)). For each volunteer, cells from all time points were tested in a single experiment: thawed and stimulated on the same day and stained the following day. Samples were acquired on a 9-color Cyan ADP (Beckman Coulter) and data analysis was performed using FlowJo software (version 9.6.4; Tree Star). A representative example showing the full gating strategy is shown in **Figure 3.S1.** Gating of cytokine-positive cells was performed in a standardized way by multiplying a fixed factor with the 75 percentile of the geometric Mean Fluorescent Intensity (MFI) of cytokine negative PBMCs for each volunteer, time point and stimulus. Responses to uRBC were subtracted from the response to *Pf*RBC for each volunteer on every time point.

Plasma for the assessment of malaria-specific antibodies was collected and stored at baseline (B), 27 days after the first immunization (I1; one day before the second immunization), 27 days after the second immunization (I2; one day before the third immunization), and one day before the challenge infection (C-1). Antibody titers were assessed as described previously (17). In summary, serially diluted citrate plasma was used to perform standardized enzyme-linked immunosorbent assay (ELISA) in NUNC™ Maxisorp plates (Thermo Scientific) coated with 1 µg/ml circumsporozoite protein (CSP), liver-stage antigen-1 (LSA-1) or merozoite surface protein-1 (MSP-1) antigen, diluted in PBS. Bound IgG was detected using horseradish peroxidase (HRP) conjugated anti-human IgG) (Thermo Scientific, 1/60000) and Tetramethylbenzidine (all Mabtech). Spectrophotometrical absorbance was measured at 450 nm. OD values were converted into AUs by four-parameter logistic curve fit using Auditable Data Analysis and Management System for ELISA (ADAMSEL-v1.1, http://

www.malariaresearch.eu/content/software; accessed 27 October 2014). Levels of antibodies were calculated in relation to a pool of 100 sera from adults living in a highly endemic area in Tanzania (HIT serum (24)), which was defined to contain 100 arbitrary units (AU) of IgG directed against each antigen.

Statistical analyses

The proportion of protected subjects in the CPS-CQ versus CPS-MQ group was tested with the Fisher's exact test using Graphpad Quickcalcs online and the 95% confidence interval (CI) of protection for each group was calculated by modified Wald Method (25). Further statistical analyses were performed with GraphPad Prism 5. Differences in prepatent period and time from qPCR positivity until thick smear positivity were tested by Mann Whitney test. Antibody levels are shown as individual titers with medians and differences between time points were analyzed by Friedman test with Dunn's multiple comparison post-hoc test. Induction of cellular immune responses was tested for CPS-CQ and CPS-MQ groups separately by Wilcoxon matched-pairs signed rank test (B versus C-1). A p-value of <0.05 was considered statistically significant. Analyses of parasitemia were performed on log transformed data, the geometric mean peak parasitemia after each immunization was calculated using the maximum parasitemia for each subject.

RESULTS

Safety of CPS-CQ and CPS-MQ immunization

Twenty out of 36 screened subjects (median age 21 years; range 18–25) were included in the study (**Figure 3.1**). One control subject was excluded between start of prophylaxis and the first immunization because of an unexpected intermittent visit to a malaria-endemic area. Thick blood smears performed from day 6 up until day 10 after each immunization remained negative in all volunteers. As determined retrospectively by qPCR, 2/5 subjects in the CPS-CQ group and 7/10 subjects in the CPS-MQ group showed sub-microscopic parasitemia after the first immunization (geometric mean peak parasitemia for positive subjects: 948 parasites/ml [range 228–3938] and 256 parasites/ml [range 48–1559] respectively, **Figure 3.2**). After the second immunization, four CPS-MQ subjects showed sub-microscopic parasitemia (geometric mean peak parasitemia for positive subjects 104 parasites/ml [range 48–223]), while none of the CPS-CQ subjects showed parasitemia. After the third immunization, only one CPS-MQ subject showed parasitemia by qPCR (peak parasitemia 1059 Pf/ml).

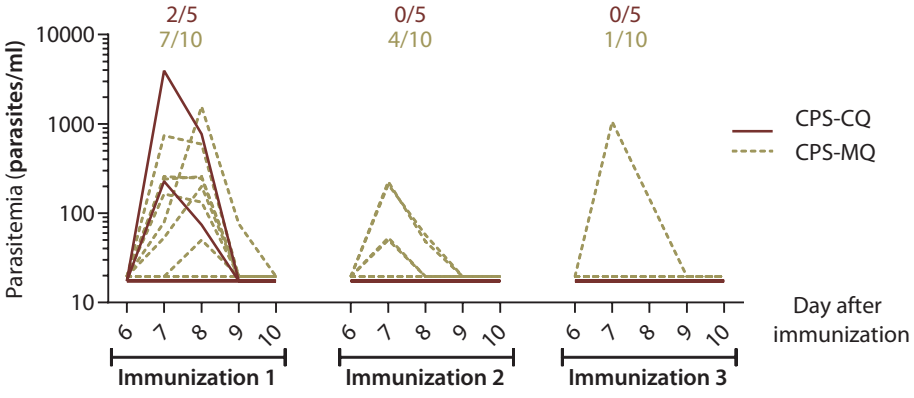

Figure 3.2 Parasitemia during CPS immunization.
*Parasitemia was determined retrospectively, once daily from day 6 until day 10 after each immunization, by real-time quantitative PCR (qPCR). Each line represents an individual subject from the CPS-MQ (**dashed brown lines**) or CPS-CQ group (**red lines**). The number of subjects with a positive qPCR/total number of volunteers in the CPS-MQ (**brown**) and CPS-CQ (**red**) groups after each immunization are shown above the graph. Values shown as 17.5 on the log-scale were negative (i.e. half the detection limit of the qPCR: 35 parasites/ml).*

After the first immunization, all subjects (5/5) in the CPS-CQ group and almost all CPS-MQ subjects (8/9) experienced possibly or probably related AEs. One subject in each group had a grade 3 AE (headache and vomiting, respectively). Two control volunteers reported mild AEs (**Figure 3.3** and **Table 3.S2**). After the second immunization, two CPS-CQ volunteers and six volunteers in the CPS-MQ group had mild AEs. Two control subjects experienced moderate and severe headache, respectively. After the third immunization, one volunteer in the CPS-CQ group and four CPS-MQ volunteers had AEs; one control subject experienced mild AEs (**Figure 3.3** and **Table 3.S2**). One CPS-CQ subject reported moderate sleeping problems while taking chloroquine prophylaxis. One control subject had moderate problems with initiation of sleep and another control subject experienced vivid dreams under mefloquine prophylaxis. Other than mild to moderate dizziness and sleep related AEs, which all resolved after chemoprophylaxis was stopped, no neuropsychiatric AEs occurred. No serious adverse events occurred.

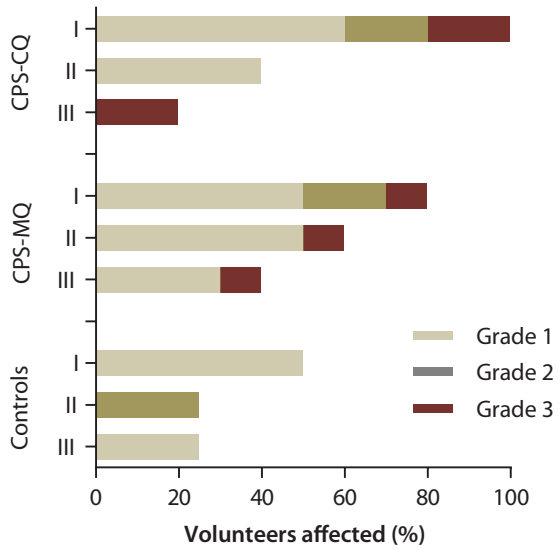

Figure 3.3 Adverse events during CPS immunization.
*Percentage of volunteers in each group experiencing possibly or probably related AE after the first (I), second (II) and third (III) immunization. AEs were evaluated at each visit and graded for severity as described in the methods paragraph: mild (**light brown**), moderate (**dark brown**) and severe (**red**). Only the highest intensity per subject is listed. No Serious Adverse Events occurred.*

During immunization, one subject each in the CPS-CQ, CPS-MQ and control groups showed platelet counts below the lower limit of normal ($150x10^9$/L); lowest values $105x10^9$/L, $116x10^9$/L and $131x10^9$/L, respectively. Three, five and two subjects from the CPS-CQ, CPS-MQ and control groups respectively, showed leukocyte counts below the lower limit of normal ($4x10^9$/L); mean lowest value during immunization period: $3.8x10^9$/L [SD 1.2], $4.0x10^9$/L [SD 1.1] and $4.2x10^9$/L [SD 0.7] respectively. No subject developed leukocyte counts lower than $2.0x10^9$/L. One volunteer in each group showed leukocyte counts above the upper limit of normal ($10x10^9$/L; highest values $10.8x10^9$/L, $13.8x10^9$/L and $10.1x10^9$/L respectively). After the first immunization, 3/5 CPS-CQ subjects, 7/10 in the CPS-MQ group and none in the control group developed elevated d-dimer levels (>500 ng/ml). After the second immunization, six CPS-MQ subjects but none in the CPS-CQ or control groups showed elevated d-dimer levels. After the third immunization, three CPS-MQ subjects showed elevated d-dimer levels, while none of the subjects in the other groups did.

Protection against challenge infection

In the CPS-CQ group 3/5 subjects and in the CPS-MQ group 7/10 volunteers were protected against challenge infection (Fisher's exact test $p = 1.0$). All control subjects became thick smear positive (median day 8.5, range 7–12, $p = 0.03$ versus CPS-immunized subjects; **Table 3.1**). None of the protected subjects showed parasitemia by qPCR at any time point during follow-up (**Figure 3.4**). The median prepatent period was not significantly different between the CPS-CQ and CPS-MQ groups, neither when protected subjects were arbitrarily set at a prepatent period of 21 days ($p = 1.00$), nor when comparing unprotected subjects only ($p = 0.1$). The median chloroquine plasma concentration on the day before challenge infection was 9 µg/L (range 7–10) in the CPS-CQ group, and the median mefloquine concentration was 24 µg/L (range 5–116) in the mefloquine groups.

Table 3.1 Protection against challenge infection after CPS-CQ and CPS-MQ immunization

Group	Protection			Unprotected volunteers					
				Day of positivity after challenge[c]					
	n[a]	%[b]	p	Thick smear	p	qPCR	p	ΔTS+qPCR+[c]	p
CPS-CQ	3/5	60 (23-88)		14.0 (14.0-14.0)		11.3 (10.5-12.0)		2.8 (2.0-3.5)	
CPS-MQ	7/10	70 (39-90)	1.0[d]	12.0 (11.0-12.0)	0.10[f]	10.0 (9.0-10.0)	0.10[f]	2.0 (2.0-2.0)	0.40[f]
Control	0/4	0% (0-55)	0.03[e]	8.5 (7.0-12.0)	0.048[g]	6.3 (5.0-9.5)	0.056[g]	2.5 (1.5-2.5)	0.70[g]

[a] *Presented as protected/total number of subjects*
[b] *Presented as % protected (95% CI by modified Wald Method)*
[c] *Presented as median (range) days.*
[d,e] *p-value calculated by Fisher's exact test comparing [d]CPS-MQ versus CPS-CQ or [e]control versus all CPS-immunized subjects*
[f,g] *p-value calculated by Mann Whitney test comparing [f]CPS-MQ versus CPS-CQ or [g]control versus all CPS-immunized subjects (both excluding protected subjects)*

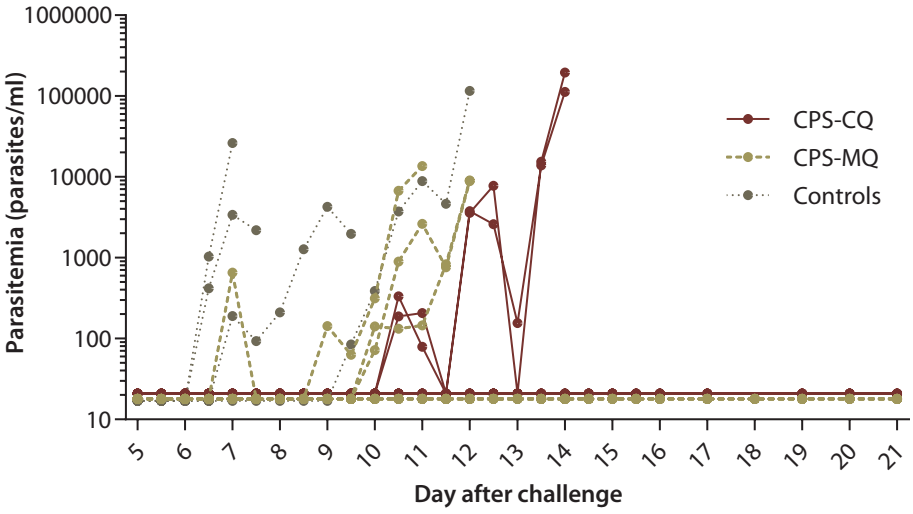

Figure 3.4 Parasitemia after challenge infection.
*Parasitemia was assessed retrospectively by real-time quantitative PCR (qPCR) twice daily from day 5 until day 15 and once daily up until day 21 after challenge. Each line represents an individual subject. **Red** lines represent CPS-CQ immunized volunteers (n = 5), dashed **brown** lines CPS-MQ immunized subjects (n = 10) and dotted **grey** lines malaria-naive control subjects (n = 4). Values shown as 17.5 on the log-scale were negative (i.e. half the detection limit of the qPCR: 35 parasites/ml).*

Immunogenicity of CPS-CQ and CPS-MQ

Antibodies against the pre-erythrocytic antigens CSP and LSA-1 and the cross-stage antigen MSP-1 were assessed by ELISA. Antibodies against CSP were induced in both CPS-CQ and CPS-MQ immunized volunteers ($p<0.05$ and $p<0.01$ respectively, on C-1; **Figure 3.5A and 3.5B**), but not significantly higher in protected compared to unprotected subjects ($p = 0.88$ and $p = 0.48$ respectively). Antibodies against LSA-1 were only significantly induced in CPS-MQ immunized volunteers on I2 ($p<0.001$; **Figure 3.5C and 3.5D**), although not higher in protected subjects ($p = 0.39$). Anti-MSP-1 antibodies by CPS immunization were not statistically significant increased in either group (**Figure 3.5E and 3.5F**).

IFNγ production by both adaptive and innate cell subsets in response to *in vitro P. falciparum* re-stimulation was induced by both CPS-CQ and CPS-MQ (**Figure 3.S2**), without a clear quantitative or qualitative difference between the study groups. Next, CD107a expression by CD4 T cells and granzyme B production by CD8 T cells, both associated with protection in a previous CPS-CQ trial (7), were assessed by flow cytometry. Four out of 5 CPS-CQ and 8/10 CPS-MQ immunized subjects showed induction of CD107a expression by CD4 T cells upon *in vitro* re-stimulation after immunization (**Figure 3.6A** and **3.6B**). Although volunteer numbers were too low to reach statistical significance, the magnitude of this response appeared to be associated with protection for CPS-CQ (**Figure 3.6A**), while for CPS-MQ it was not (**Figure 3.6B)**. Granzyme B production by CD8 T cells was not significantly induced in either CPS-CQ or CPS-MQ group, nor was it associated with protection (**Figure 3.6C** and **3.6D**).

After challenge, MSP-1 specific antibodies were boosted in all unprotected volunteers (fold change median 20.4 (range 7.1–33.6), 76.0 (5.7–06.3) and 7.7 (2.9–15.3) for CPS-CQ, CPS-MQ and control groups respectively). None of the protected subjects showed an increase in MSP-1 antibody levels on C+35 compared to C-1 (median fold change 1.0 (range 1.0–1.3) and 1.0 (0.6–2.4) for CPS-CQ and CPS-MQ groups, respectively).

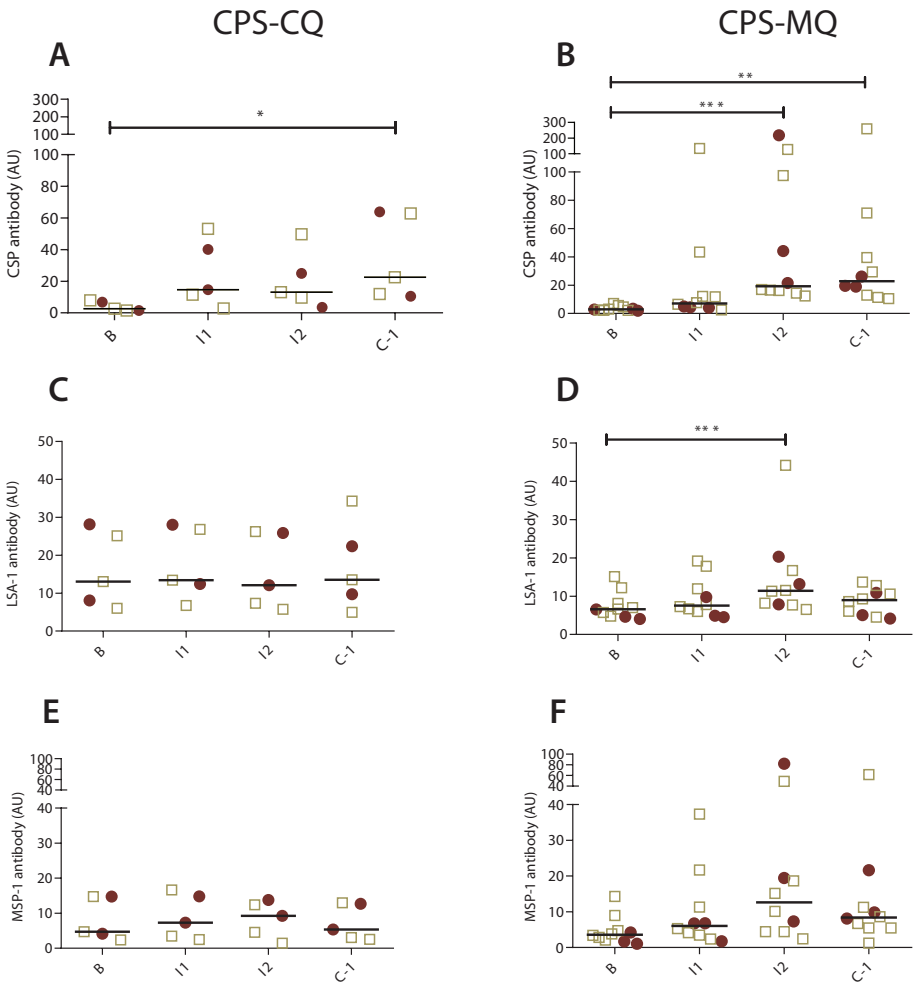

Figure 3.5 Antibody responses induced by CPS-CQ and CPS-MQ immunization.
*Antibodies against CSP (**A** and **B**; in AU), LSA-1 (**C** and **D**), and MSP-1 (**E** and **F**) were analyzed at baseline (**B**), 28 days after the first (I1) and second (I2) immunization and one day before challenge (C-1; 20 weeks after the last immunization) for all CPS-CQ (**A**, **C** and **E**, n = 5) and CPS-MQ (**B**, **D** and **F**, n = 10) immunized volunteers. Data are shown as individual titers with medians. Open squares indicate protected subjects, filled circles indicate unprotected subjects. Differences between the time points were analyzed by Friedman test with Dunn's multiple comparison post-hoc test. Significant differences are indicated by asterices with * (p<0.05), ** (p<0.01), *** (p<0.001).*

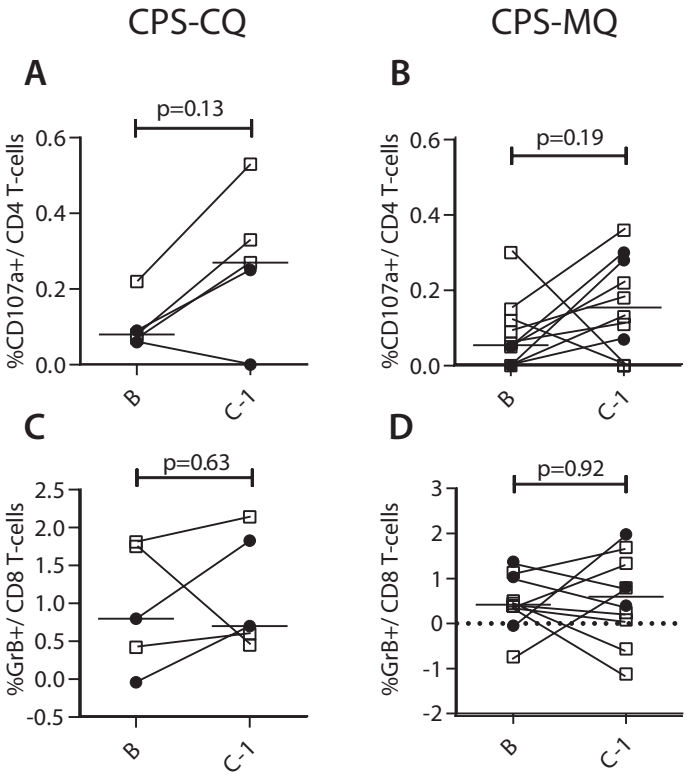

Figure 3.6 Cellular immune responses: CD107a expression by CD4 T cells and granzyme B production by CD8 T cells.

*CD107a expression by CD4 T cells after PfRBC re-stimulation, corrected for uRBC background in CPS-CQ (**A**) and CPS-MQ (**B**) groups; granzyme B production by CD8 T cells after PfRBC re-stimulation, corrected for uRBC background in CPS-CQ (**C**) and CPS-MQ (**D**) groups. Symbols and lines represent individual subjects before immunization (**B**) and one day before challenge (C-1). Open squares indicate protected subjects, filled circles indicate unprotected subjects. Differences between B and C-1 for all subjects were tested by Wilcoxon matched-pairs signed rank test.*

DISCUSSION

Immunization of healthy volunteers with *P. falciparum* sporozoites while taking mefloquine prophylaxis is safe, induces both humoral and cellular immune responses and protects against homologous malaria challenge.

Although most volunteers experienced AEs after the first immunization, their frequency declined after subsequent immunizations in line with a reducing number of volunteers developing parasitemia. The majority of AEs was mild, with only 10–20% of subjects experiencing a grade 3 AEs after each immunization. In general, the reported neurologic and psychiatric side effects of mefloquine are a major concern limiting its acceptability and clinical application. In this study, mild to moderate dizziness and sleep-related complaints occurred in a small number of subjects in both chloroquine and mefloquine groups. Although this study was not powered to detect differences in AEs, frequency of neuropsychiatric AEs did not appear to differ between both drugs. This is in line with most reports in literature comparing AEs of mefloquine or chloroquine (with or without proguanil) for chemo-prophylactic use (26-29) although one study found more neuropsychiatric AEs in subjects taking mefloquine by retrospective questionnaire (30). Taking the small sample size into consideration, both CPS-CQ and CPS-MQ immunization regimens appear to be reasonably well tolerated and safe. In 2013, however, after completion of this study, the U.S. Food and Drug Administration (FDA) issued a boxed warning for mefloquine, stating that neurologic side effects might be permanent. This might lead to adjustment of prophylaxis guidelines and limitation of mefloquine use where alternatives are available, as for now it remains a recommended antimalarial prophylactic for several target groups (31).

In previous studies we showed that 19/20 subjects (95%) were protected after bites from 45 infected mosquitoes, 8/9 (89%) after bites from 30 and 5/10 (50%) after bites from 15 infected mosquitoes during chloroquine prophylaxis (6, 7, 9). The 60–70% protection observed in the current CPS-CQ and CPQ-MQ groups, immunized with bites from 24 mosquitoes, demonstrates the reproducibility of CPS immunization and indicates a linear relationship between immunization dose and protection. This confirms the consistency of the CPS approach and is remarkable, given the assumed variation in the number of sporozoites injected by mosquitoes (32). This study further establishes CPS immunization as a worthwhile immunization protocol to relatively easily induce protection and create differentially protected cohorts to study target antigens and correlates of protection, both of which would be highly valuable tools in the search for *P. falciparum* vaccines and biomarkers of protection (33).

Although the study was not powered to detect these differences, there are hints suggestive of more efficient induction of protection by CPS-CQ compared to CPS-MQ: i) the two unprotected CPS-CQ volunteers showed a longer prepatent period than the CPS-MQ subjects (14 versus 12 days, Mann-Whitney test $p = 0.13$); ii) induction of immunity required less immunizations in the CPS-CQ group i.e. none of these subjects showed blood-stage parasites after the second immunization while subjects in the CPS-MQ group still developed parasitemia after the second and third immunization. If there is a difference between CPS-CQ and CPS-MQ in protective efficacy, it is small, but possibly detectable in larger cohorts or when the immunization dose is further reduced.

Induction of anti-circumsporozoite antibodies by CPS-CQ is consistent with previous work, but neither anti-LSA-1, nor MSP-1 antibodies were induced by CPS-CQ in the current study (17). Antibodies against the latter antigens are dose-dependently induced (17), and the current immunization regime using bites from 3×8 *P. falciparum*-infected mosquitoes might have been insufficient (7). The induction of cellular *P. falciparum*-specific memory responses, as reflected by IFNγ production, is in line with previous CPS-CQ studies, even though limited sample size hampered statistical significance for some cell types. Interestingly, CD107a expression by CD4 T cells upon *in vitro* re-stimulation, associated with protection in a previous CPS-CQ study (7), appeared again to be associated with protection in the CPS-CQ group, but not the CPS-MQ group. Granzyme B production by CD8 T cells upon *in vitro* re-stimulation did not appear to be a reproducible marker of protection in this second CPS study (7). Whether this might be related to immunization dose remains to be investigated in future CPS trials.

The striking efficiency of CPS immunization might at least be partly due to the established immune modulating properties of the 4-amino-quinoline chloroquine (12), possibly reflected by the more efficient induction of degranulating CD4 T cells. Chloroquine has been shown to increase cross-presentation in hepatitis B vaccination and influenza (13, 14), and thus may enhance cellular immune responses considered essential for protection against liver-stages (12). For mefloquine, a 4-methanolquinoline, this immune-modulating property has, to our knowledge, not been reported. A possible strategy to assess whether chloroquine and/or mefloquine indeed have immune enhancing effects on whole sporozoite immunization would be to compare immunization with RAS in the presence or absence of these drugs.

Mefloquine or chloroquine plasma concentrations were still detectable in all volunteers one day before the challenge infection. Possible contributing effects of these remaining drug levels to the protective efficacy outcome were considered in several ways; i) The interval between first qPCR and thick smear

positivity, as proxy for parasite multiplication, was 2.8 in the CPS-CQ group, 2.0 in the CPS-MQ group and 2.5 in the control group. This interval is similar to previous CHMI studies with the NF54 *P. falciparum* strain in the absence of prophylactic drug levels (7, 34); ii) the two volunteers with the highest mefloquine levels (116 and 77 µg/L) were control subjects who became thick smear positive with only a minimal delay in patency within the time-frame of historical controls (35); iii) plasma chloroquine and mefloquine levels at C-1 were in all volunteers well below the minimum therapeutic concentration (CQ: 30 µg/L (36)) or the concentration at which breakthrough infections are observed in non-immune people (MQ <406 – 603 µg/L (37)). iv) We cannot rule out that protected subjects experienced transient parasitemia after challenge, which was cleared in the first blood-stage cycle by remaining drug levels. But because parasitemia was not detected by qPCR in any of the protected subjects at any time point after challenge potential parasitemia must have been below the qPCR detection limit of 35 parasites/ml, indicating a reduction of at least 92% in liver load, given a geometric mean height of the first peak or parasitemia in non-immune historical controls of 456 parasites/ml (35); v) None of the protected subjects showed a boost in anti-MSP-1 antibodies after challenge while all unprotected subjects did, suggesting that protected subjects did not experience blood-stage parasitemia after challenge. (9). From these combined data we believe that remaining drug concentrations are unlikely to have contributed to the observed protection, although this cannot be formally excluded.

A review of rodent studies using different attenuation methods for whole sporozoite immunization shows that increased development of the parasite in the liver, but absence of blood-stage parasitemia during immunization is associated with the highest protective efficacy (38). It would therefore be interesting to investigate CPS immunization with alternative antimalarials with varying targets in the parasite life cycle. CPS immunization with causal prophylactic drugs affecting liver-stages, e.g. primaquine, will likely results in a reduction of AEs because of reduced or absent blood-stage exposure. Whether antigen-exposure is sufficient to induce protection when the liver-stage is abrogated, remains to be answered.

In conclusion, we show that immunization of healthy volunteers under mefloquine prophylaxis with *P. falciparum* sporozoites is safe, immunogenic and protective. These findings could have important implications for malaria vaccine development and further development of CPS approaches.

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CHAPTER 4

Heterologous protection against malaria after immunization with *Plasmodium falciparum* sporozoites

Authors: Remko Schats*, Else M. Bijker*, Geert-Jan van Gemert, Wouter Graumans, Marga van de Vegte-Bolmer, Lisette van Lieshout, Mariëlle C. Haks, Cornelus C Hermsen, Anja Scholzen, Leo G. Visser, Robert W. Sauerwein

*contributed equally

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ABSTRACT

Background

Sterile protection in >90% of volunteers against homologous *Plasmodium falciparum* infection has been achieved only using the controlled human malaria infection (CHMI) model. This efficient model involves whole parasite immunizations under chloroquine prophylaxis (CPS-immunization), requiring only 30-45 mosquitoes bites infected with *P. falciparum*-sporozoites. Given the large diversity of *P. falciparum* parasites, it is essential to assess protection against heterologous parasite strains.

Methods

In an open-label follow-up study, 16 volunteers previously CPS-immunized and challenged with *P. falciparum* NF54 (West-Africa) in a dose de-escalation and challenge trial were re-challenged with clone NF135.C10 (Cambodia) at 14 months after the last immunization (NCT01660854).

Results

Two out of thirteen NF54 protected volunteers previously fully protected against NF54 were also fully protected against NF135.C10, while 11/13 showed a delayed patency (median prepatent period of 10.5 days (range 9.0-15.5) versus 8.5 days in 5 malaria-naïve controls (p=0.0005). Analysis of patency by qPCR indicated a 91 to >99% estimated reduction of liver parasite load in 7/11 partially protected subjects. Three volunteers previously not protected against NF54, were also not protected against NF135.C10.

Conclusion

This study shows that CPS-immunization can induce heterologous protection for a period of more than one year, which is a further impetus for clinical development of whole parasite vaccines.

INTRODUCTION

Malaria remains a tremendous public health problem affecting approximately 40% of the world's population. The global incidence of malaria is estimated to be around 198 million clinical cases resulting in 584.000 deaths [1] most of which are caused by *P. falciparum*. Since current interventions fail to reduce malaria incidence sufficiently, a vaccine is urgently needed to combat this disease.

Sterile protection against *P. falciparum* malaria can efficiently and reproducibly be achieved in the Controlled Human Malaria Infection (CHMI) setting by repeated inoculation of live sporozoites by bites of laboratory-reared *Anopheles* mosquitoes to healthy malaria-naïve volunteers under chemoprophylaxis: ChemoProphylaxis and Sporozoites (CPS-) immunization [2,3]. CPS-induced protection is dose-dependent [3] and was shown in a subset of volunteers to last for more than two years [4]. Furthermore, bites from only 30-45 *P. falciparum*-infected mosquitoes are sufficient to induce sterile protection in >90% of subjects, while immunization with radiation-attenuated sporozoites (RAS) requires a minimum of 1,000 *P. falciparum*-infected mosquitoes, or intravenous injection of 675,000 cryopreserved sporozoites [5,6]. So far CPS-immunizations and challenges have been performed with the homologous NF54 strain only, while in malaria-endemic areas there is a large genetic and antigenic diversity of *P. falciparum* strains. This diversity is considered an important reason why naturally acquired immunity is obtained slowly, only after several years of repeated exposure [7]. Previously, heterologous protection has been reported in 4/6 RAS-immunized volunteers [5].

Next to the widely used *P. falciparum* strain NF54 and its clone 3D7, NF135.C10 originating from Cambodia has become available for CHMI studies [7]. In this study, volunteers who had previously participated in a NF54 dose de-escalation CPS-immunization and challenge trial were re-challenged with NF135.C10 after more than one year.

MATERIALS AND METHODS

The protocol for this trial and supporting TREND checklist are available as supporting information; see S1 Checklist and S1 Protocol.

Study design

A single centre open label clinical trial was conducted at the Leiden University Medical Center (LUMC) from July 2012 until February 2013. The study was approved by the Central Committee for Research Involving Human Subjects of The Netherlands (NL39414.000.12) and complied with the Declaration of Helsinki and Good Clinical Practice including monitoring of data. ClinicalTrials.gov Identifier: NCT01660854.

Study participants

Eighteen volunteers from a NF54 CPS dose-de-escalating study (ClinicalTrials.gov Identifier: NCT01218893;[8]) and 8 newly recruited malaria-naïve subjects aged 18-35 years were all screened in July 2012 for eligibility based on medical and family history, physical examination and standard haematological and biochemical measurements (**Figure 4.1**). Seventeen NF54 CPS-immunized volunteers and five controls were included. One volunteer had to be excluded because of a positive urine toxicology test for cannabis and was treated with atovaquone/proguanil two days after challenge. Two of the remaining included volunteers had previously received the highest dose of NF54 CPS (3x15 bites), 8 a medium dose (3x10 bites) and 6 the lowest dose (3x5 bites). Thirteen were NF54 protected, of which one volunteer was presumptively treated because of a non-malaria related SAE on day 10,5 after NF54 challenge but considered NF54 protected [8]. The sample size calculation for this study is described in detail in Supplemental Methods.

None of the female volunteers were pregnant or lactating. Serology for HIV, hepatitis B and hepatitis C was negative in all volunteers. Plasma samples tested by Enzyme-Linked ImmunoSorbent Assay (ELISA) against crude NF54 asexual blood stages were negative in all control volunteers. None of the volunteers had travelled to a malaria-endemic area within 6 months prior to the start of the study. All volunteers provided written informed consent before screening.

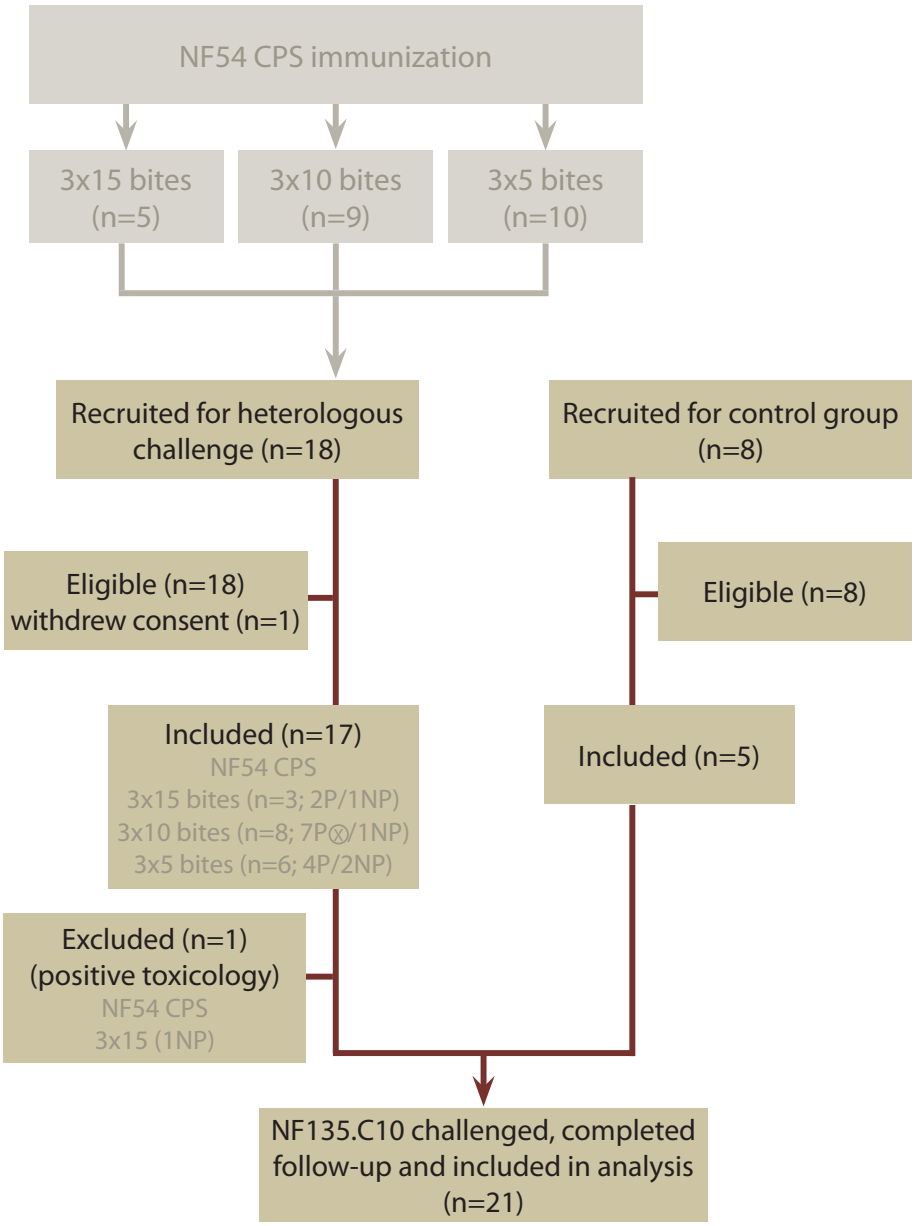

Figure 4.1 Study flow diagram.
*The previous NF54 CPS-immunization study is shown in **grey**.*
P=NF54 protected, NP=NF54 unprotected. ⊗=Volunteer presumptively treated on day 10.5 after NF54 challenge and considered NF54 protected

Study procedures

All volunteers were challenged simultaneously by exposure to five bites of *Anopheles stephensi* mosquitoes infected with the NF135.C10 *P. falciparum* clone in August 2012 [9]. This heterologous challenge was performed 14 months after the last NF54 CPS-immunization and 9.5 months after NF54 challenge. Volunteers were followed-up on an outpatient basis once daily on days 5-6 after challenge, twice daily between days 7-15 and once daily between days 16-21. During each visit, blood was drawn for parasite detection by thick smear. Volunteers were treated with 1000 mg atovaquone and 400 mg proguanil once daily for three days according to Dutch national malaria guidelines as soon as parasites were detected by thick smear, or on day 21 after challenge if they had remained thick smear negative. The last visit for volunteers was conducted in February 2013.

Safety parameters were determined daily: platelet counts were determined in EDTA blood with the Sysmex XE-2100 (Sysmex Europe GmbH. Norderstedt. Germany). D-dimer concentrations were assessed in citrate plasma by STA-R Evolution (Roche Diagnostics, Almere, The Netherlands; upper limit of detection 5000 ng/ml), Highly sensitive (Hs) Troponine T and Lactate Hydrogenase (LDH) were determined in serum by Modular E170 (Roche Diagnostics, Almere, The Netherlands).

Endpoints

The primary endpoint was time to parasitemia after challenge infection as assessed by thick smear. Blood was screened by microscopy for parasites as described before, and the thick smear was considered positive if two unambiguous parasites were detected in 0.5µL of blood, confirmed by a second independent reader. Volunteers were considered protected when thick smear remained negative up until 21 days after challenge.

Secondary endpoints were the kinetics of parasitemia and frequency of signs and symptoms. Parasitemia was retrospectively quantified by qPCR on samples collected up to twice daily from day 5 until day 21 after challenge as described previously [10] with some modifications. Briefly, 5µL Zap-oglobin II Lytic Reagent (Beckman Coulter) was added to 0.5ml of EDTA blood, after which the samples were mixed and stored at -80°C. After thawing, samples were spiked with the extraction control Phocine Herpes Virus (PhHV) and DNA was extracted with a MagnaPure LC isolation station. Isolated DNA was resuspended in 50µl H_2O and 5µl was used as template. For the detection of *P. falci-*

parum, the primers as described earlier [10] and the TaqMan MGB FAM-labelled probe 5'-AACAATTGGAGGGCAAG-3' were used. For quantification of PhHV the primers 5'-GGGCGAATCACAGATTGAATC-3', 5'-GCGGTTCCAAACGTACCAA-3' and the probe Cy5-5'-TTTTTATGTGTCCGCCACCATCTGGATC-3' were used.

Adverse events (AE) reported by volunteers or observed by the investigator were recorded according to the following scale: *mild* (grade 1; easily tolerated), *moderate* (grade 2; interferes with normal activity) or *severe* (grade 3; prevents normal activity). Fever was recorded as grade 1 (37.5-38.0°C), grade 2 (38.0-39.0°C) or grade 3 (>39.0°C).

Statistical analysis

All possibly and probably (both solicited and unsolicited) related AE were tabulated, grouped and analysed by calculating the average number of mild, moderate or severe AE per volunteer in each group. Statistical analyses were performed using GraphPad Prism 6.02. Differences in prepatent period and parasitemia at time of treatment between two groups (NF54 protected and controls) were tested by Mann Whitney test, and between the three dose groups by Kruskal-Wallis test with Dunn's multiple comparison post-hoc test. A p value of <0.05 was considered statistically significant.

RESULTS

Heterologous protection induced by CPS-immunization

Sterile heterologous protection against NF135.C10 was complete in 15% (2/13) of NF54 protected volunteers **(Figure 4.2A)**. Patency was significantly delayed in the other 11 volunteers, indicative of partial protection (median prepatent period determined by thick smear was 10.5 days [range 9.0-15.5] versus 8.5 days [range 8.5-8.5] in controls; p=0.0005 (**Table 4.1, Figure 4.2B**). Seven out of 11 partially protected subjects showed a delay in patency by qPCR of at least 48 hours, and thus more than one *P. falciparum* multiplication cycle.

The 3 volunteers previously not protected against NF54 were neither protected against NF135.C10 (**Table 4.1, Figure 4.1**). The prepatent period by thick smear did not differ significantly between NF54 CPS-immunization dose groups (**Figure 4.2C/2D**). Parasitemia at time of treatment was higher in controls compared to CPS immunized (p=0.047; **Figure 4.3**).

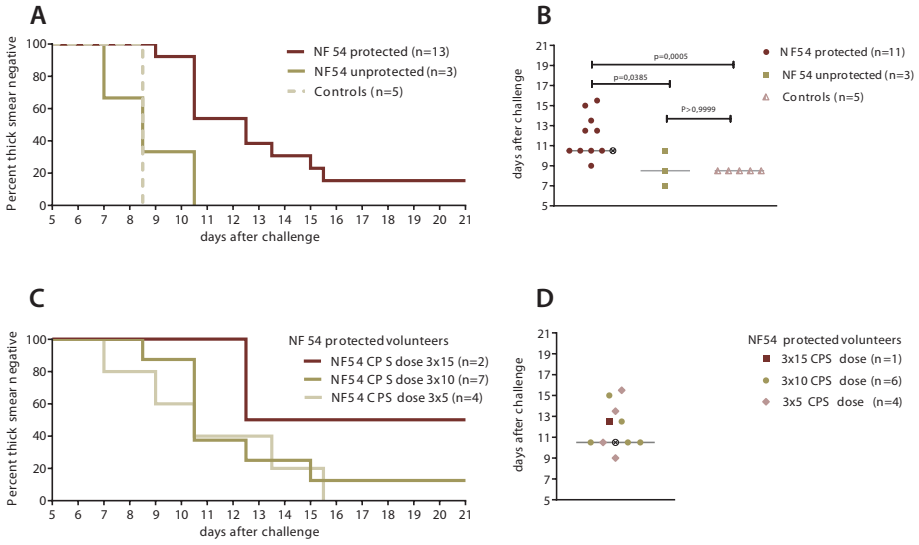

Figure 4.2 Protection and prepatent period after heterologous NF135.C10 challenge.
*Left panels: Kaplan-Meier curves showing percentage of thick smear negative volunteers after NF135.C10 challenge according to previous NF54 protection status (**A**) and NF54 CPS-immunization dose (**C**).*
*Right panels: The corresponding distribution of prepatent period of thick smear positive volunteers is shown in dot plots according to NF54 protection status (**B**) and NF54 CPS-immunization dose (**D**). Lines represent medians.*
⊗=Volunteer presumptively treated after NF54 challenge and considered NF54 protected.

Table 4.1 Protection against NF135.C10 challenge after NF54 CPS-immunization

	NF135.C10 Protected (n)	NF135.C10 TS+ (n)	Prepatent period[a]
NF54 protected			
3x15	1	1	12.5
3x10	1	6	10.5 (10.5-15.0)
3x5	0	4	12.0 (9.0-15.5)
all	2	11	10.5 (9.0-15.5)***
NF54 unprotected			
3x15	0	0	
3x10	0	1	8.5
3x5	0	2	8.8 (7.0-10.5)
all	0	3	8.5 (7.0-10.5)
Malaria-naive controls	0	5	8.5 (8.5-8.5)

Sixteen previously CPS-immunized and challenged with P. falciparum NF54 volunteers in a CPS dose de-escalation and challenge trial were re-challenged with clone NF135.C10.

[a] *Presented as median (range) N: number of volunteers. TS: Thick smear*

**** p=0.0005 compared to controls*

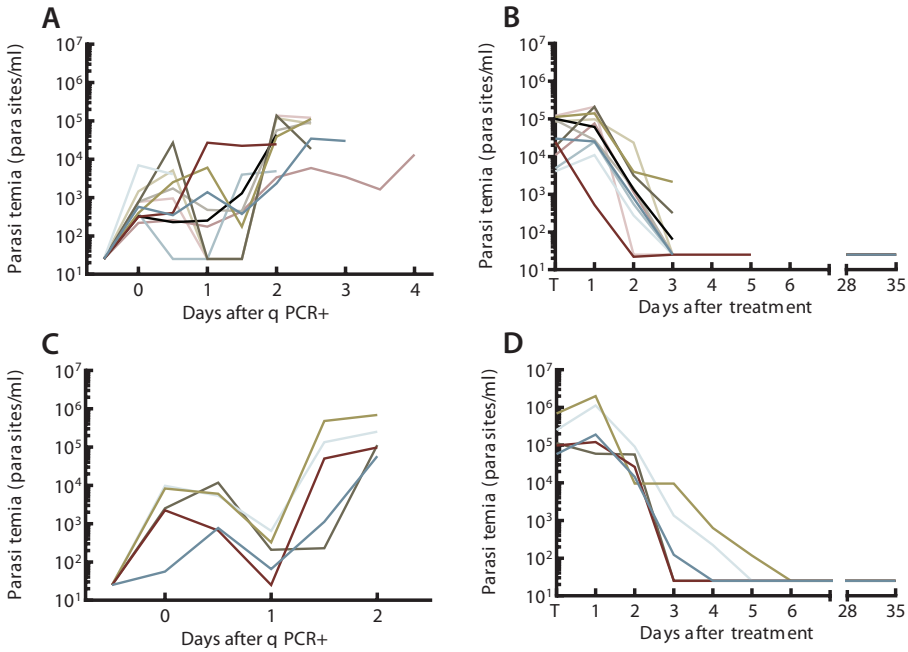

Figure 4.3 Parasitemia before and after treatment.

*Parasitemia measured by qPCR up until initiation of treatment (**A** and **C**) and from treatment onwards (**B** and **D**) in previously NF54 protected volunteers (**A** and **B**) and controls (**C** and **D**). Each line represents an individual subject with the same colour before and after treatment. Values shown as 25 Pf/ml were negative (i.e. half the detection limit of the qPCR: 50 parasites/ml).*

Adverse events

We next analysed adverse events in relation to the day of treatment to determine any early blood stage immune recognition to the parasite reflected in AE. Adverse events experienced by volunteers represent clinical manifestations of a malaria infection and can be possibly and probably related (both solicited and unsolicited) to the infection.

All volunteers reported possibly or probably related AE after challenge. Partially protected volunteers and controls showed a peak of AE on the first day after start of treatment (**Figure 4.4**). Fourteen volunteers experienced related grade 3 AE, which were more frequently reported in partially protected than in control volunteers (8/10 versus 2/5 respectively). There were no serious AE. In partially protected volunteers, delayed patency concurred with earlier onset of AE in relation to detection of parasites by thick smear. While control volunteers did not experience any AE up until one day before detection of parasites by thick smear, partially protected volunteers experienced AE as early as three days before initiation of treatment.

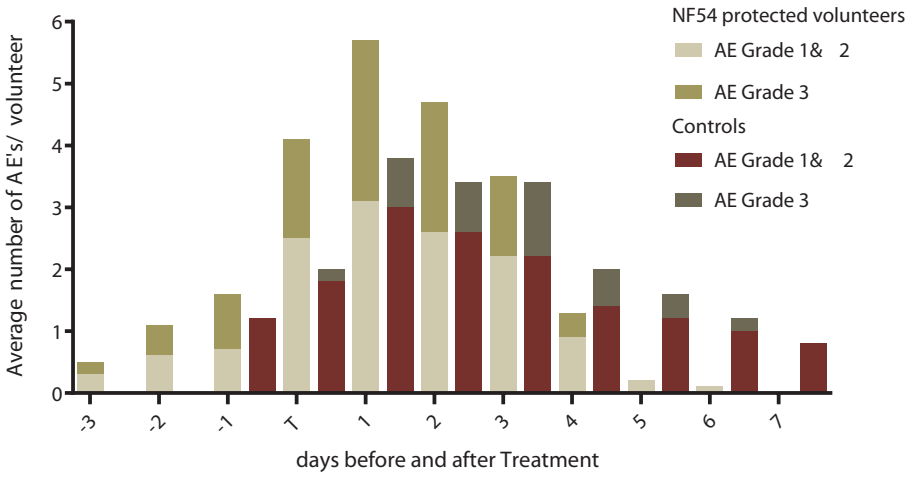

Figure 4.4 Adverse events before and after initiation of treatment.
Average number of possibly and probably related (both solicited and unsolicited) AE per previously NF54 protected or control volunteer in relation to the time of positive thick smear (day of treatment). Time points are plotted towards day of treatment, depicted as 'T', from 3 days before until 7 days after start of treatment.

All controls and one partially protected volunteer showed persisting fever (maximum 39.0 °C) and/or mild to moderate complaints in the evening of day 3 after start of treatment. Resolution of the AE took longer (up to 7 days) in controls compared to partially protected volunteers, and to historical controls [11]. Additional thick smears performed in these volunteers on day 4, 5 and 6 after start of treatment were negative. All volunteers recovered fully without requiring additional antimalarial treatment.

Safety parameters

Hs troponin T concentrations remained within normal range (<0.03 µg/L) in all volunteers. LDH was elevated in ten volunteers after initiation of treatment (median maximum value 242 U/L, range 182-718 U/L) and returned within normal range (0-248 U/L) during follow-up. D-dimer levels were elevated in all volunteers (median maximum value 1748 ng/ml, range 524 – <5000 ng/ml) and returned within normal range (0-220 ng/ml) during follow-up. The number of platelets decreased below lower reference value ($150x10^9$/L) in 13 volunteers (median lowest value $127x10^9$/L, range $51-275x10^9$/L) without apparent clinical manifestations of bleeding or thrombotic complications. Safety parameters returned within normal range in all volunteers after treatment.

DISCUSSION

Our principle finding is that protection against a heterologous challenge infection with NF135.C10 is present in NF54 CPS-immunized and protected volunteers challenged more than one year before. Heterologous protection against NF135.C10 was complete in 15% (2/13) of volunteers while there was a delayed patency of more than 48 hours in 54% (7/13) of subjects. Taking into account a mean multiplication factor of 11.1 [11] and the presumed absence of functional blood stage immunity at this low parasitemia [3], this delay indicates that liver parasite load was reduced by approximately 91%. In three out of these seven volunteers a delay of more than two or three cycles was observed, indicating an estimated reduction of >99%. Three volunteers with no protection in the earlier homologous NF54 challenge study were also fully susceptible to NF135.C10.

Previous CPS studies showed that protection is mediated by immunity against pre-erythrocytic stages rather than asexual blood stages [3]. NF135.C10 originates from Cambodia, while NF54, isolated near Schiphol Amsterdam airport, likely originates from West Africa [9]. Both isolates show distinct differences in genes encoding three well-established antigens (MSP-1, MSP-2 and GLURP) as well as in the rif repetitive elements [9]. The target antigens of CPS-mediated protection remain to be elucidated in further studies including possible differences in antigen-specific responses to NF54 and NF135.C10.

Heterologous protection was incomplete in the majority of NF135.C10 re-challenged volunteers demonstrated by a delayed patency compared to controls. Apart from the genetic/antigenic variation between NF135.C10 and NF54, and thus insufficient breadth of the induced immune response, this incomplete heterologous protection may relate to a number of alternative explanations: i) Waning immunity: the heterologous challenge was performed at 14 months, rather than the usual 2 to 5 months post CPS-immunization; ii) Suboptimal sporozoite immunization dose received by the majority (14/16) of volunteers, indicating an antigen threshold for complete protection [8]. The minimally required immunization dose may increase for longevity of homologous protection and may be even higher for (long-lasting) heterologous protection. This trial was not powered to detect any dose-response relationships, but the two fully protected volunteers had indeed been immunized with the medium and high dose. iii) A possible difference between NF54 and NF135C.10 in sporozoite infectivity for liver cell invasion and/or maturation. This is supported by the higher first peak of NF135.C10 parasitemia was higher compared to historical NF54 controls (2871 *Pf*/ml versus 456 *Pf*/ml respectively [11].

In partially protected volunteers, delayed patency concurred with earlier onset of AEs . This might be due to the longer time-frame before parasitemia reaches the thick smear detection limit. Alternatively, early immune recognition of blood stage parasites by the host may result in an increased inflammatory response and subsequent increase in AEs. A comparable effect was observed in a previous trial, where CPS-immunized subjects who received a blood-stage challenge developed inflammatory markers and fever earlier than naïve controls [3].

Compared to partially protected volunteers, control volunteers showed prolonged AEs after treatment. This continuation of AEs until day 7 after treatment has not been observed in previous CHMI trials with either strain NF54 or NF135.C10, neither in the CPS studies nor in RAS studies [5]. Whether this represents an incidental finding or strain-specific characteristics needs to be investigated in future trials.

In conclusion, NF54 CPS-immunization induces heterologous protection against the geographically and genetically distinct *P. falciparum* NF135.C10 clone. Increasing the immunization dose, altering the immunizing strain, or even immunization with a combination of strains may further improve protection. These results and further optimization of CPS-immunization regimens will prove highly valuable for the clinical development of whole sporozoite vaccines.

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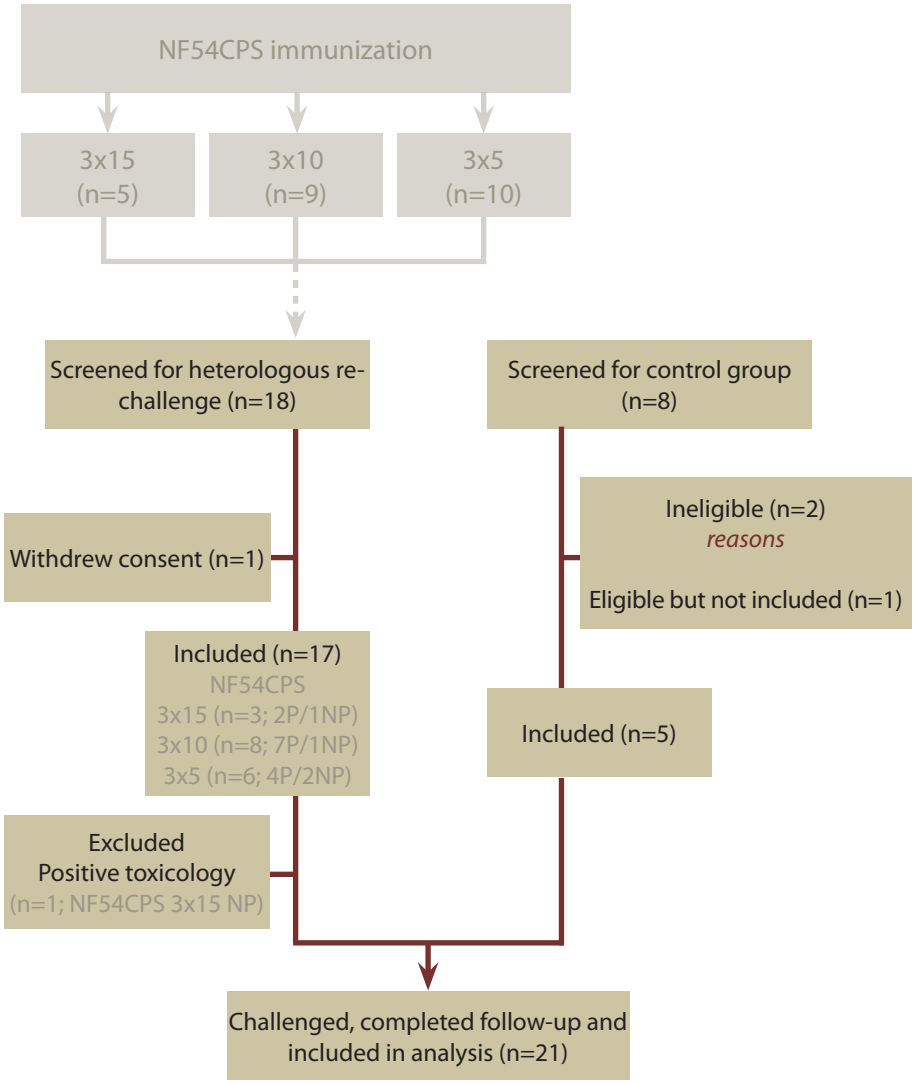
SUPPLEMENTARY INFORMATION

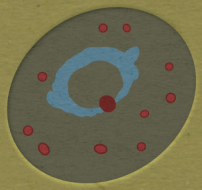
S4.I Flow Diagram



Chad. June 2005. Two refugee women from Darfur presenting a Teletubby.

CHAPTER 5

Relationship between parasitaemia and Adverse Events after sporozoite immunization and challenge in the Controlled Human Malaria Infection model



Authors: Remko Schats, Else M. Bijker, Mariëlle C. Haks, Meta Roestenberg, Cornelus C. Hermsen, Leo G. Visser, Robert W. Sauerwein.

ABSTRACT

Background

Controlled Human Malaria Infection (CHMI) with sporozoites of *Plasmodium falciparum* (*Pf*) is a powerful tool for selecting pre-erythrocytic vaccine candidates for further testing. Volunteers in these trials are intensely monitored and immediately treated for malaria upon detection of parasitaemia by thick smear.

In this study we compare adverse events, parasitaemia and parasitological endpoints of two previously published trials during immunization and challenge if treatment would be initiated based on qPCR rather than on positive thick smears.

Methods

Data from two single center double-blind clinical trials were used. In total 39 vaccinees received three immunizations with *Plasmodium falciparum* (*Pf)*-infected mosquito bites under chemoprophylaxis. All subjects were homologously challenged. Thick smears were made according to the visit schedule after each immunization and challenge. Parasitaemia was retrospectively quantified by qPCR on all samples. For the purpose of this study clinical symptoms and parasitaemia were determined at the time of the second consecutive positive qPCR result and at the time of positive thick smear.

Results

Vaccinees that were protected against challenge infection experienced less adverse events and lower parasite densities after each immunization compared to unprotected vaccines, indicating acquisition of protection. After challenge the cumulative parasite numbers from three days before treatment up to day of treatment in unprotected vaccinees and controls was reduced by 99% and 91%, respectively if treatment would be initiated based on qPCR result. In addition, cumulative numbers of AEs would be reduced with 92% in unprotected vaccinees and 75% in controls from three days before day of treatment up to day of treatment. Discrimination between unprotected vaccinees and controls remains possible using qPCR based initiation of treatment.

Conclusion

The use of qPCR for monitoring of subjects after challenge resulted in a one-and-half days earlier detection of malaria parasites at lower levels and with less accompanying adverse events in comparison to thick smear examination. Using parasite detection by qPCR for initiation of treatment would markedly reduce the burden for subjects with more than 90% reduction in parasites and 70% of AEs. Earlier treatment does not compromise the discrimination by pre-patent period between controls and vaccinees that were not fully protected.

INTRODUCTION

Malaria is a global health problem that affects almost half of the world's population. Recent achievements in reducing DALY's due to *falciparum* malaria are threatened by changing biting behavior of the vector, resistance to insecticides and failing artemisinine combination therapies [1, 2], and emphasize the urgent need for an effective malaria vaccine.

Controlled Human Malaria Infection (CHMI) with sporozoites of *Plasmodium falciparum* (*Pf*) is a powerful tool for selecting pre-erythrocytic vaccine candidates for further testing in the field [3]. The CHMI model was used to demonstrate that sterile protection against *Pf* malaria can be achieved through repeated inoculation of live *Pf* sporozoites delivered by bites of *Anopheles* mosquitoes to healthy malaria-naïve subjects under malaria chemoprophylaxis (Chemoprophylaxis and Sporozoites (CPS)-immunization protocol) [4-8] or by repeated intravenous injection of irradiated non-replicating *Pf* sporozoites [9].

Traditionally the efficacy of a vaccine is quantified by determining the difference in prepatent period between controls and vaccinees using blood smears: subjects are monitored daily by thick smears (TS) and treatment is started at the first positive blood slide. Thick smears become positive when approximately 4000 parasites are present in 1 mL of whole blood [3]. Quantitative polymerase chain reaction (qPCR) has a much higher sensitivity: 20-35 parasites per mL of blood. Treatment at the first positive qPCR instead of TS results would allow to start therapy at, very low, sub-microscopic levels of parasites and may improve the safety of volunteers. However, earlier treatment may also reduce the discriminative power of prepatent periods between vaccinees especially in vaccines that do not provide full protection.

In this study we compare adverse events, parasitaemia and parasitological endpoints of two previously published trials during immunization and challenge if treatment would be initiated based on qPCR rather than on positive thick smears.

MATERIALS AND METHODS

Study design and subjects

Data from two double blind clinical trials were used [7,8]. Both studies were conducted at the Leiden University Medical Center (LUMC) in a collaboration with Radboud UMC.

In short, study A is a dose de-escalation study in which 24 vaccinees were randomized between three CPS immunization schedules with in total 45, 30 or 15 *Pf*-infected mosquito bites. Five subjects were included as controls [7]. In study B 15 vaccinees received either chloroquine or mefloquine prophylaxis during three immunizations with in total 24 *Pf*-infected mosquito bites. Four subjects were included as controls [8]. As a challenge, a controlled infection with five NF54 *Pf*-infected mosquito bites was used.

Study allocation was concealed for subjects, investigators and primary outcome assessors. The primary outcome of both trials was the pre-patent period, defined as the time between challenge and first positive thick smear. Complete protection was defined as negative thick smears till day 21 after challenge infection.

Safety monitoring during immunizations and after challenge

In both studies subjects were monitored on an out-patient basis from day 6 till day 10 after each immunization. After challenge, subjects were also monitored daily for adverse events (AEs) as out-patients. AEs were defined as any undesirable experience occurring to a subject during the study, whether or not considered related to the trial.

In study A, subjects were monitored twice daily from day 5 after challenge until 3 days after treatment. In study B subjects were monitored twice daily from day 5 until day 15 and once daily from day 16 till day 21 after challenge and twice daily from the day of treatment up to 3 days later.

All AEs (solicited and unsolicited symptoms and signs) reported spontaneously by the subjects or observed by the investigators were recorded. All AEs except fever were judged for their intensity according to the following scale: mild (grade 1): awareness of symptoms that are easily tolerated and do not interfere with usual daily activity; moderate (grade 2): discomfort that interferes with or limits usual daily activity; severe (grade 3): disabling, with subsequent inability to perform usual daily activity, resulting in absence for example from work or study or required bed rest.

Abnormal laboratory findings (e.g., clinical chemistry, haematology, urinalysis) or other abnormal assessments that were judged by the investigator to be clinically significant, were recorded as an AE or SAE.

Parasitological monitoring

After each immunization thick smears were made once daily and after challenge once or twice daily according to the visit schedule for both studies. Blood (0.5µL) was screened by microscopy for the presence of parasites as described before [10]. The detection limit for thick smear is approximately 4000 parasites per mL [3].

Parasitaemia was retrospectively quantified by qPCR on samples as described previously [7, 8, 11]. The qPCR was considered positive if both in duplex performed samples were found positive for *Pf*. A cycle threshold (Ct) value of ≥40 was considered negative for *Pf*. The detection limit for qPCR was either 20 or 35 parasites per mL [11] depending on the study. If CT values were ≥40 the parasite density was set at half the detection threshold at respectively 10 or 17,5 parasites per mL.

In both studies, treatment with 1000 mg atovaquone and 400 mg proguanil once daily for three days was initiated when two unambiguously identifiable parasites were detected in the thick smear. If subjects remained thick smear negative following challenge, they were presumptively treated with the same curative regimen on day 21 after challenge infection.

For the purpose of this study clinical symptoms and parasitaemia were quantified at the time of the positive thick smear and at time of the second consecutive positive qPCR result. This time point was chosen to obtain additional information on the dynamics of the parasite density in time.

In 10 out of 12 unprotected vaccinees and all controls, the first positive qPCR was followed by the second positive qPCR at the next sampling visit, 12 hours later. In one vaccinee the second positive qPCR occurred at the third sampling visit (24 hours later), and in one at the fourth visit (48 hours later).

Statistical analysis

All AEs for each subject were tabulated and grouped according to intensity (grade 1, 2 or 3) starting from three days before (T-3) until the day of second consecutive positive qPCR or positive thick smear (T). Subjects did not experience significant numbers of AEs *before* T-3 (by thick smear) and therefore only

AEs from T-3 till T were included in the analysis to be compared with parasitaemia in a similar time-window. The proportion of subjects who reported mild, moderate or severe AEs was calculated for both the time point of second consecutive positive qPCR and thick smear.

Definitions

Mean AEs

The mean AEs per subject per time point by either thick smear or qPCR

Total number of AEs

The total number of AEs was calculated as the sum of the mean AEs per subject per time point from T-3 till day T by either thick smear or qPCR. The differences in AEs between qPCR and thick smear-based initiation of treatment were calculated by subtracting the total number of AEs of both techniques.

Cumulative number of parasites

The cumulative number of parasites up to T was calculated by adding the number of parasites per day from T-3 till T. It should be noted that in contrast to study A, subjects in study B were monitored for parasites once daily from day 16 after challenge onwards instead of twice daily. However, since no subjects from either study A or B became thick smear positive after day 15 after challenge, this difference in follow-up had no consequences for the analyses performed here. Differences between groups were analysed with the Mann-Whitney statistical test.

Comparisons between unprotected vaccinees and controls were analysed with the Mann-Whitney statistical test; comparisons of AEs between protected and unprotected vaccinees during immunizations were performed with the Wilcoxon rank-sum test.

RESULTS

Of the 39 vaccinees that were included, 25 were fully protected while 12 vaccinees and all 9 controls were not protected against a malaria challenge. Of the 39 included vaccinees, two subjects (from study A) were treated presumptively on day 10,5 and 19 and were considered protected [7] but excluded from further analysis in this study. One control subject was excluded from study B between start of prophylaxis and first immunization [8].

Both studies were highly comparable. Although different drugs and immunizing doses were used in both studies, there were no significant differences observed in pre-patent period by thick smear (Kruskal Wallis statistical test between all groups: p=0,168) and AEs profiles were similar (data not shown). Therefore, both studies were pooled for further analysis.

Adverse events and parasitaemia during immunization

After each subsequent immunization protected vaccinees experienced significantly less AEs with a concomitant reduction in mean parasite densities, reflecting an evident relationship between low-density asexual parasitaemia and adverse events **(Figures 5.1A/B).** The mean number of AEs per volunteer decreased with 38% between the first and second immunization and with 48% between the second and third. The mean number of grade 3 AEs per volunteer did not change during all three immunizations.

In contrast to protected vaccinees, unprotected vaccinees showed higher grade AEs after the third immunization **(Figures 5.1A/B)** while parasite densities remained similar after each immunization. The total (mean) number of AEs remained unchanged during all immunizations while grade 3 AEs increased more than 5-fold between the first and third immunization (Wilcoxon test p=0,042).

In addition, the total cumulative number of parasites, determined by qPCR during all three immunizations combined, was significantly higher in unprotected vaccinees (median 1930 parasites per mL) compared to protected vaccinees (median 315 parasites per mL) (Mann-Whitney test; p<0,0001) **(Figure 5.1B).** In protected vaccinees but not in unprotected vaccinees there was a reduction in parasites numbers during each subsequent immunization **(Figure 5.1B).** These results indicate that the reduction in parasite numbers after each subsequent immunization in protected vaccinees reflects acquisition of protection during immunizations. In contrast, although the parasitaemia after each immunization remains unchanged, the increase in intensity of AEs may reflect an increased inflammatory response to blood stage parasites in unprotected vaccinees.

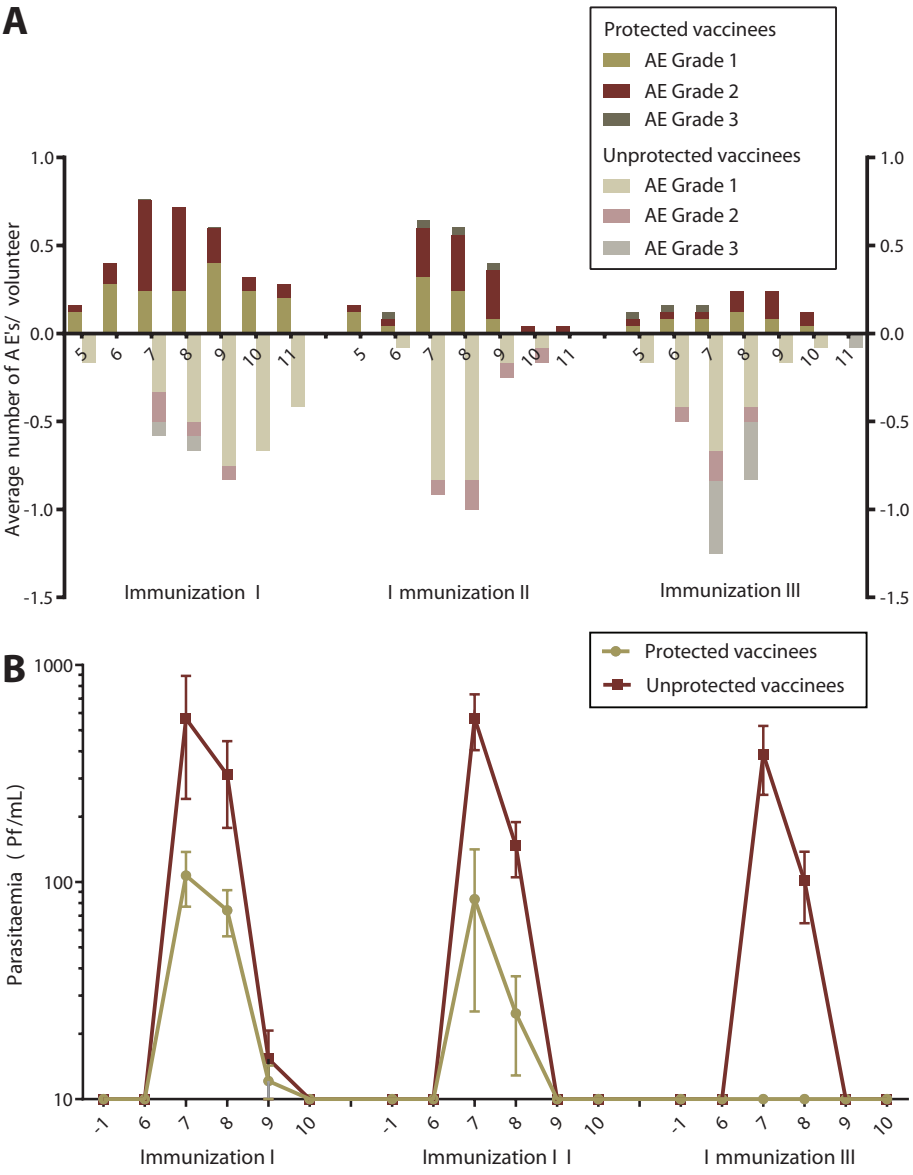


Figure 5.1 Mean number of adverse events and parasitaemia during immunization in protected and unprotected vaccinees

1(A) *Mean number of adverse events (AEs) per subject during immunization shown for protected and unprotected vaccinees per time point according to intensity (grade 1, 2 and 3).*

1(B) *Parasite density quantified by quantitative polymerase chain reaction (qPCR) in protected and unprotected vaccinees during immunization. The qPCR cycle threshold ≥40 was plotted as the assay cutoff as 10 Pf/mL. Graphs show means with SEM.*

Adverse events and parasitaemia after challenge

Using the first positive qPCR (median 8,5; range 6,5-13,0), as a cut-off for the prepatent period instead of using a positive thick smear (median 11; range 7,0-15,0) would advance patency by 2,5 days (unprotected and controls combined).

If treatment would have been initiated at the second consecutive positive qPCR this would advance the day of treatment by 1,5 days (median 9,5 days; range 7,0 - 13,5 days) compared to thick smear-based treatment (median 11 days; range 7,0 – 15,0 days). Using the second consecutive positive qPCR as initiation of treatment would reduce the total number of AEs (all grades) from T-3 until T by 92% (from a total of 105 to 18 AEs) in unprotected vaccinees and by 70% (from a total of 81 to 24 AEs) in controls. **(Figure 5.2A and 5.2B).** Grade 3 AEs were reduced by 100% in unprotected vaccinees and by 95% in controls.

It would also reduce the total cumulative numbers of blood-stage parasites from T-3 to T significantly: by 99% (from 656.588 to 8.629 parasites per µl; $p<0.001$) in unprotected vaccinees and by 91% (from 417.988 to 37.012 parasites per µl; $p<0.003$) in controls **(Figures 5.3A and 5.3B)**. The reduction in parasite numbers on day of treatment *only* was 99% (from 522.185 to 4.808 parasites per mL) for the unprotected subjects and 90% (from 326.056 to 34.119 parasites per mL) for controls.

The prepatent period determined by thick smear was significantly longer (2,5 days) in unprotected vaccinees (median 12, range 9,0-15,0 days) than controls (median 9,5; range 7,0-13,0) and reflects partial protection in unprotected vaccinees **(Figure 5.4)**. Despite earlier detection by the first positive qPCR in unprotected vaccinees (median 9,25; range 6,5-13,0) and controls (median 6,5; range 6,5-10,5) this difference in prepatent period (2,75 days) remained. This difference between unprotected vaccinees (median 10; range 7,0-13,5) and controls (median 7; range 7,0-11,0) was even larger (3 days) using the second consecutive positive qPCR as a cut-off **(Figure 5.4)**.

After challenge, using thick smear initiation of treatment, the total cumulative number of parasites during the entire challenge period did not differ between unprotected vaccinees and controls (median respectively 24156 vs 39702 parasites per mL; Mann-Whitney test p=0,69) or using 2 consecutive positive qPCR (median respectively 615 vs 656 parasites per mL; Mann-Whitney test p= 0,64). Also, the peak parasitaemia at treatment using two consecutive positive qPCR did not differ significantly (Mann-Whitney test p=0,21) between unprotected vaccinees (median 170; range 63-1349 parasites per mL) and controls (median 390; range 38-26381 parasites per mL µl). Neither did the peak parasitaemia differ by thick smear (Mann-Whitney test p=0,96) in unprotected vaccinees (median 17277; range 1698-195704) and controls (median 26915; range 1970- 116393).

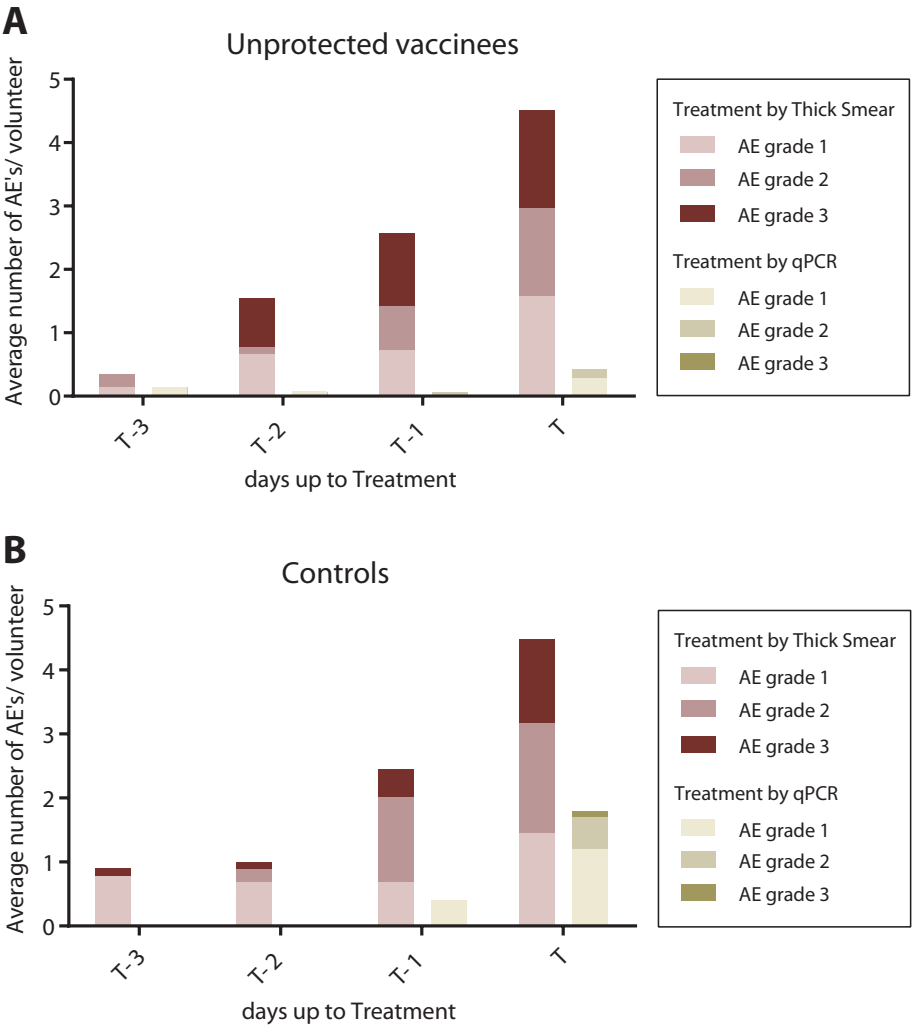


Figure 5.2 Reduction of AEs in unprotected vaccinees and controls following in silico application after second consecutive qPCR-based treatment.
2(A) *AEs in unprotected vaccinees by thick smear-based (**red bars**) and second consecutive qPCR-based (**brown bars**) initiation of treatment. Cumulative reduction of AEs/per subject of 92% from T-3 to T.*
2(B) *AEs in controls by thick smear-based (**red bars**) and second consecutive qPCR-based (**brown bars**) initiation of treatment. Cumulative reduction of AEs/per subject of 75% from T-3 to T. T=day of treatment, T-1= one day before day of treatment.*

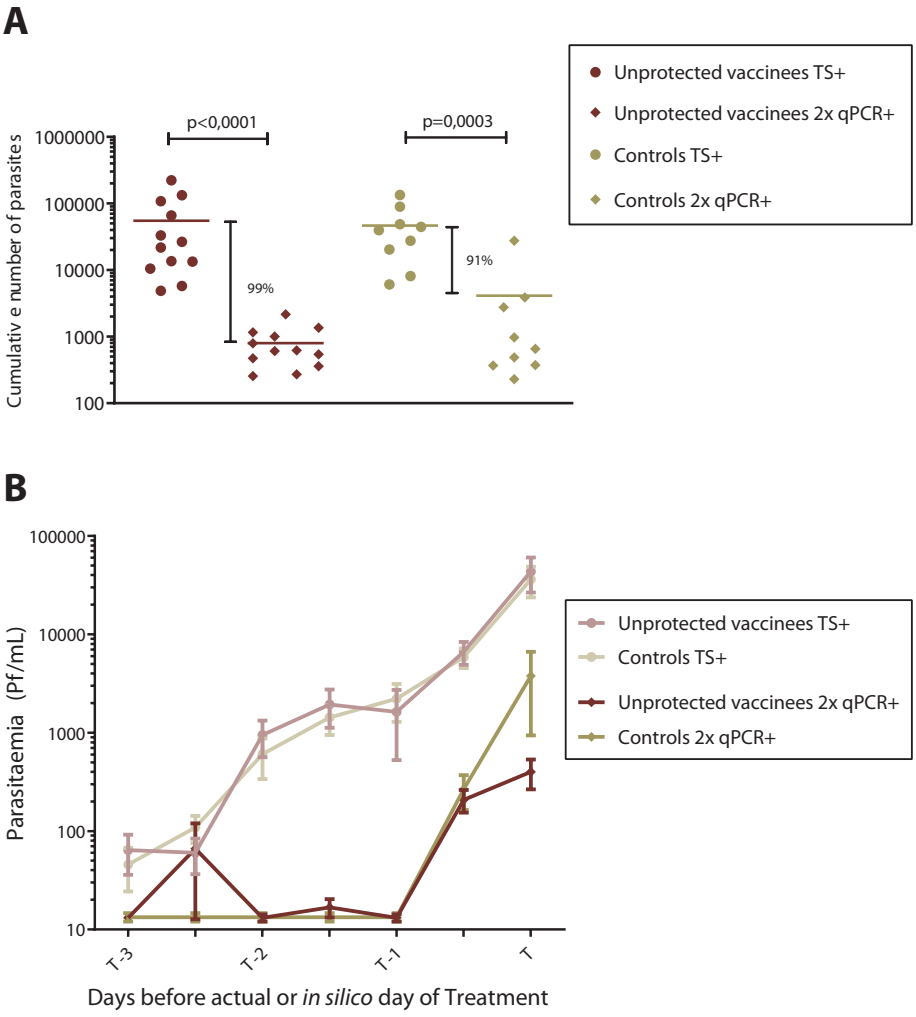


Figure 5.3 Significant reduction of cumulative number of parasites after challenge when applying the new treatment in unprotected vaccinees and controls

3(A) *Cumulative number of parasites after challenge in unprotected (**red circles and diamonds**) vaccinees and controls (**brown circles and diamonds**) when using thick smear or second consecutive qPCR for initiation of treatment. Mann-Whitney test for group comparison. Lines represent mean values.*

3(B) *Number of parasites calculated between three days before day of treatment till actual day of treatment by thick smear (circles) or in silico day of treatment by second consecutive qPCR (diamonds) in unprotected vaccinees (**red circles and diamonds**) and controls (**brown circles and diamonds**). Indicated are the mean values with SEM.*

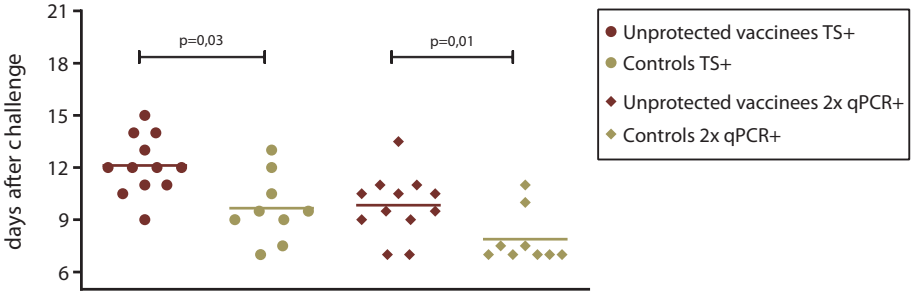


Figure 5.4 Discrimination between unprotected vaccinees and controls remains achievable using second consecutive qPCR-based initiation of treatment.
*Days after challenge are shown for individuals in unprotected (**red circles and diamonds**) vaccinees and controls (**brown circles and diamonds**) using thick smear-based (circles) and second consecutive qPCR-based (diamonds) initiation of treatment. The line represents the mean value. Mann-Whitney statistical test used.*

In addition, no difference was observed in the parasite multiplication rate (PMR) between unprotected vaccinees and controls using thick smear initiation of treatment (median respectively 24 vs 37; Mann-Whitney test p=0,47) and this was reflected by a comparable fold increase in number of parasites between two erythrocytic multiplication cycles in both groups. Calculating the PMR using two consecutive positive qPCR was not feasible due to lack of two erythrocytic multiplication cycles.

DISCUSSION

In a retrospective analysis, we show that the apparent use of the second consecutive positive qPCR for initiation of treatment after challenge would presumably result in a one-and-half days shorter pre-patent period accompanied by less adverse events when compared to standard thick smear examination as performed in these two clinical trials.

Obviously, this type of retrospective analysis performed after these two trials were completed, has its limitations [7, 8]. Since AE that may occur after treatment cannot be compared between the two diagnostic methods, we limited the analysis to AE occurring 3 days prior to (presumed) treatment. It is reasonable to assume, however, that AEs after treatment will be likely be reduced as well. Future trials will have to confirm this assumption.

In contrast to the likely benefits for subjects, in-depth parasitological and immunological evaluation of CHMIs could be hampered due to earlier cut-off and reduced parasitaemia. This may, for example, occur in case of partial protection as reflected by a prolonged pre-patent period compared to controls. However, we found that using qPCR-based initiation of treatment did not affect the ability to discriminate partial protected subjects from controls. In addition, new and more sensitive Nucleic acid test (NAT) PCR techniques [12], may be able to detect partial protection between groups with even greater sensitivity.

Using parasite detection by qPCR results in fewer completed asexual multiplication cycles where parasite multiplication rate (PMR) is a proxy for assessing the presence of blood-stage immunity; consequently, calculation of PMR is severely limited or becomes even impossible because of the absence of two consecutive cycles. Depending on the immunization goal, this will remain important in future trials.

Aside from limitations in assessing the PMR, also the elucidation of biomarkers might be hampered using earlier qPCR-based treatment. Biomarkers in vaccine development can be roughly divided into markers that are associated with disease and those that are associated with protection and could be either mechanistically or non-mechanistically correlated [13]. Many different types of biomarkers can act as correlates: changes in cell subset composition in the peripheral blood, (intracellular) cytokine levels, transcriptomic and/or metabolomics markers or antibodies against malaria. All of these individual factors could play a role in the intricate interaction between parasite and host that is triggered by immunizations by malaria parasites. As earlier qPCR-initiation of treatment will only have study-related consequences after challenge, only the discovery of biomarkers that correlate with disease may be affected. Importantly, the detection of biomarkers that correlate with protection should

not be affected as these biomarkers most likely are detectable during or shortly after immunizations.

It has been shown that sterile protective CPS-induced immunity targets pre-erythrocytic parasite stages [10]. Acquisition of protection is reflected by a declining number of adverse events and parasites after each consecutive CPS immunization. Although after the third immunization all of the protected volunteers had a negative qPCR, it appears that also 5 out of 12 eventually unprotected subjects had a negative qPCR after the third immunization. Subsequently, a negative qPCR after the third immunization, does not qualify as marker of protection in this study .

Many cellular changes in peripheral blood take place between the 9th day after challenge and day three after treatment. For example the activation of monocytes and Dendritic Cells (DC) expressing HLA-DR/CD86 was significantly increased on day of treatment till three days after treatment [14]. Also, the contribution of effector memory cells (EM) as a percentage of total Interferon-gamma (IFNγ)-producing cells was increased from C+9 onwards till C+400 after challenge [15]. In this assessment of two previous CHMIs, the presumed median day of qPCR-based treatment would have been 9 days after challenge. Consequently, many of such changes might be less prominent on day of treatment. The balance between reduction in AE's and presumably increased safety versus the potential loss of parasitological or immunological information needs to be carefully balanced.

In a previous study, Kamau et al [16] used qPCR-based initiation of treatment in non-immune malaria naïve volunteers and proposes initiation of treatment if two positive qPCR are found with one qPCR with at least 2000 parasites per mL. Comparing this cut-off with the threshold in our analysis using two consecutive positive qPCR resulting in a cut-off with a relatively low median of 240 parasites per mL (mean 1854; range 38 – 26381 parasites per mL). Using the cut-off used in the study of Kamau allows most probably for more in-depth parasitological and immunological analysis. If similar reduction of AEs/clinical illness with this cut-off could be achieved in this challenge-only study remains unclear.

Due to the lack of global harmonization in CHMI studies worldwide, centres find, partly due to variation in parasite inoculation by mosquitoes bites and fitness of parasites and mosquitoes, different prepatent periods and PMR. These differences make it difficult to compare results between studies and centres. The need for harmonization of CHMIs is important [17, 18] and application of qPCR-based early treatment might be justified for evaluation of pre-erythrocytic vaccine trials.

Conclusion

Here we show in a retrospective analysis of two CPS trials that the qPCR-based initiation of treatment will likely diminish the clinical burden for participants and possibly further increasing the safety and tolerability while retaining the capacity to evaluate of partially protective efficacy. Further harmonization of CHMIs will be a great asset in future malaria vaccine development.

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Chad. March 2005. Savannah landscape of rural Chad.

CHAPTER 6

Diagnosis and treatment based on quantitative PCR after controlled human malaria infection

Authors: Jona Walk*, Remko Schats*, Marijke C.C. Langenberg, Isaie J. Reuling, Karina Teelen, Meta Roestenberg, Cornelus C. Hermsen, Leo G. Visser, Robert W. Sauerwein*

*Contributed equally

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ABSTRACT

Background

Controlled human malaria infection (CHMI) has become well-established in the evaluation of drugs and vaccines. Anti-malarial treatment is usually initiated when thick blood smears are positive by microscopy. This study explores the effects of using the more sensitive qPCR as the primary diagnostic test.

Methods

1,691 diagnostic blood samples were analysed by microscopy and qPCR from 115 volunteers (55 malaria naïve and 60 having received Chemoprophylaxis and Sporozoite immunization) who were challenged by five mosquitoes infected with *Plasmodium falciparum* sporozoites of the NF54 strain.

Results

Retrospective analysis of different qPCR criteria for diagnosis and treatment, showed that once daily qPCR (threshold 100 parasites/ml) had 99% sensitivity and 100% specificity, shortened the median prepatent period from 10.5 to 7.0 days after CHMI when compared to twice daily measurement of thick blood smears (threshold 4,000 parasites/ml). This is expected to result in a 78% decrease of adverse events before initiation of treatment in future studies. Trial outcome related to infection and protective efficacy remained unchanged.

Conclusion

The use of qPCR as primary diagnostic test in CHMI decreases symptoms as well as parasitaemia while obviating the need for twice daily follow-up. The implementation improves safety while reducing the clinical burden and costs without compromising the evaluation of protective efficacy.

BACKGROUND

Controlled human malaria infection (CHMI) has proven to be a valuable tool to evaluate the efficacy of drugs and vaccines and to study the pathogenesis of clinical malaria. These challenge trials have become highly standardized [1] and are considered a critical step in the clinical development of pre-erythrocytic malaria vaccines [2].

Traditionally, volunteers are followed after CHMI by once to three times daily thick blood smears, and anti-malarial treatment is initiated immediately once two or more parasites are detected by microscopy. In 2004, a standardized protocol for CHMI thick blood smears was introduced using a threshold of 4,000 parasites/ml to improve the comparability of study outcomes between centres [3]. Volunteers generally develop submicroscopic parasitaemia for several days before they become thick smear positive. The more sensitive quantitative PCR (qPCR) with a detection limit of 20 parasites/ml was introduced for retrospective analysis feeding a statistical model for more detailed estimation of important parasite parameters including liver load and asexual parasite maturation and multiplication rates [4, 5].

Over the past decade, CHMIs have been performed in over 300 healthy volunteers at Radboud university medical center (Radboudumc), the 'Harbour Hospital' in Rotterdam or the Leiden University Medical Centre (LUMC). Despite an acceptable safety profile, CHMIs inevitably cause mild to moderate malaria symptoms such as headache, myalgia and malaise in almost all volunteers, and severe (grade 3) symptoms in about half of volunteers [3, 6]. Moreover, there have been three serious adverse cardiac events shortly after treatment for parasitaemia that have remained incompletely understood [7, 8]. As clinical malaria symptoms are only associated with asexual blood stages, a shorter duration of parasitaemia may reduce the number and severity of adverse events, thereby further minimizing risks and volunteer burden. In addition, treating volunteers before (severe) symptoms occur, would simplify the conduct and follow-up, thereby lowering costs.

In this retrospective study, different thresholds for qPCR diagnostics were analyzed in relation to prepatent period and occurrence of adverse events as well as effects on assessment of protective efficacy.

METHODS

Study volunteers

Retrospective qPCR data that had previously been generated were collected from nine CHMI trials performed at the Radboud university medical center (Radboudumc), the 'Harbour Hospital' in Rotterdam or the Leiden University Medical Centre (LUMC) between 2007 and 2012 [9-15], **Table 6.1**.

All study subjects were healthy female and male volunteers between the age of 18 and 35 years exposed to bites of five *P. falciparum* NF54 strain infected *Anopheles stephensi* mosquitoes. Prior to challenge infection, 55 volunteers were malaria naïve and 60 had received Chemoprophylaxis and Sporozoite (CPS) immunization. CPS-immunization was administered via infected mosquito bites at different dosages under chloroquine or mefloquine prophylaxis, as described previously [10-14].

Prior to inclusion, study volunteers were medically screened as described previously [13] and provided written informed consent. All clinical trials were approved by the Radboudumc Committee on Research Involving Human Subjects (CMO) or the Central Committee on Research Involving Human Subjects (CCMO) of the Netherlands.

Parasitological data

Treatment was initiated after CHMI when a thick blood smear was found positive for parasites. Thick smears were made twice or three times daily and read according to a standard protocol [11]. In short, a slide was considered positive if after reading the number of fields equivalent to 0.5uL of blood at least two parasites were seen (a threshold of four parasites per µL), and positivity was confirmed by a second independent reader. qPCR assessment was performed according to previously published protocols [16]. qPCR was performed retrospectively from samples taken twice per day from day 5 until day 15 after challenge and once per day from day 16 until day 21.

Recording of adverse events

Subjects were asked to keep a diary recording symptoms while followed up for adverse events (AEs) on an outpatient basis once or twice daily starting on day 5 after challenge infection until day 21. Adverse events were collected until

Table 6.1 Summary of data included in the analysis

Data was included from all malaria naïve or CPS-immunized volunteers undergoing challenge infection with bites from five mosquitoes infected NF54 since 2007.

	Year	Number of volunteers	CPS-immunization	Patent parasitemia	Pre-patent period[1] Median	Range	References
Study 1	2007	10	3 x 12-15 mosquitoes	0/10	-	-	Roestenberg and McCall 2009
		5	-	5/5	9	7 - 10.5	
Study 2[2]	2007	18	-	18/18	10.5	9 - 12.5	
Study 3	2009	6	3 x 12-15 mosquitoes[3]	2/6	16.8	15 - 18.6	Roestenberg 2011
		4	-	4/4	8.5	7.5 - 10.5	
Study 4	2010	5	-	4/5	10.6	10.6 - 11	Teirlinck and Roestenberg 2013
Study 5	2011	5	3 x 15 mosquitoes	1/5	12	-	Bijker and Teirlinck 2014
		9	3 x 10 mosquitoes	1/9	12	-	
		10	3 x 5 mosquitoes	5/10	11	9 - 15	
		5	-	5/5	9.5	9 - 13.5	
Study 6	2011	5	3 x 15 mosquitoes	0/5	-	-	Bijker and Bastiaens 2013
		5	-	5/5	12.5	9.5 - 12.5	
Study 7	2012	15	3 x 8 mosquitoes	5/15	12	11 - 14	Bijker and Schats 2014
		4	-	4/4	8.5	7 - 12	
Study 8	2012	5	-	5/5	10.5	9 - 10.5	
Study 9	2012	5	-	5/5	12	10.5 - 16	Bastiaens 2015

[1] *Only volunteers with patent parasitemia included. In all studies pre-patent period is defined as time to positive thick blood smear.*

[2] *Volunteers received three immunization with a candidate malaria vaccine but were unprotected from challenge infection.*

[3] *Rechallenge of CPS-immunized volunteers from Study 1, 2.5 years after immunization and malaria naive controls.*

end of study visits either on day 28 or day 35 after challenge, depending on the study. An adverse event was defined as any undesirable symptom occurring after challenge infection. AEs were defined as grade 1, no interference with daily activity; grade 2, some interference with daily activity; or grade 3, requiring bed rest. The following symptoms were solicited: fever, headache, malaise, fatigue, myalgia, arthalgia, nausea, vomiting, chills, diarrhoea and abdominal pain.

Statistical analysis

Depending on the study, qPCR data was analysed using Microsoft Excel (version 2007) for Windows or using a specialized electronic Case Report Form program (Hermsen Computer Services) created for Radboudumc CHMI trials. Data was combined using Microsoft Excel 2007 for Windows and statistical analysis was performed using IBM SPSS Statistics 22 for Windows.

RESULTS

Fifty-five malaria naïve volunteers in nine trials received a challenge infection with bites from five NF54 infected mosquitoes. Geometric mean parasitaemia curves generated from retrospective qPCR data were similar between trials, **figure 6.1A**. These volunteers received anti-malarial treatment at positive thick blood smear at a median of 10.5 days post-challenge (range 7.0-16.0). Based on the retrospective qPCR data, initiating treatment based on qPCR can gradually decrease the duration of parasitaemia, depending on the treatment threshold and blood sampling frequency used, **figure 6.1B**. When two consecutive positive qPCR measurements above 500 parasites per millilitre are used as a criterion to initiate treatment, volunteers are treated at a median of nine days post CHMI. When only a single positive qPCR is required to initiate treatment, the mean day of treatment decreases further. Using the threshold of 100 parasites per millilitre blood, the median duration of parasitaemia would decrease by 3.5 days.

All solicited adverse events that were possibly, probably or definitively related to the CHMI occurring between day 5 post-infection and the end of the study were collected. Fifty-five percent of all adverse events and 39% of severe adverse events occurred prior to the initiation of anti-malarial treatment (**Figure 6.1C**). Importantly, only 22% of the total adverse events and 13% of grade 3 adverse events before treatment occurred before parasitaemia reached 100 parasites/ml (**Figure 6.1D**).

Once daily blood sampling for qPCR (threshold of 100 parasites/ml), instead of twice daily sampling, did not influence the median treatment day, **Figure 6.1B**. Five volunteers (9%) would have been treated 24 hours earlier when sampling for qPCR twice daily. However, the mean number of adverse events before treatment increased only minimally when once daily sampling was used, **Figure 6.1C**.

CPS immunization induces dose-dependent protection against CHMI [12]. Partial protection was determined by time to parasitaemia and mean parasite density of the first wave, as estimation of the liver parasite load [4]. Since both parameters depend on the method of parasite detection and treatment threshold used, it was retrospectively assessed whether the proportion of volunteers with partial protection changed with qPCR sampling once daily and initiation of treatment based on a single qPCR above 100 parasites per millilitre. **Table 6.2** shows that differences in pre-patent period and mean parasitaemia of the first wave for 10 partially protected volunteers and controls [11, 12] gave similar outcomes when using microscopy or qPCR.

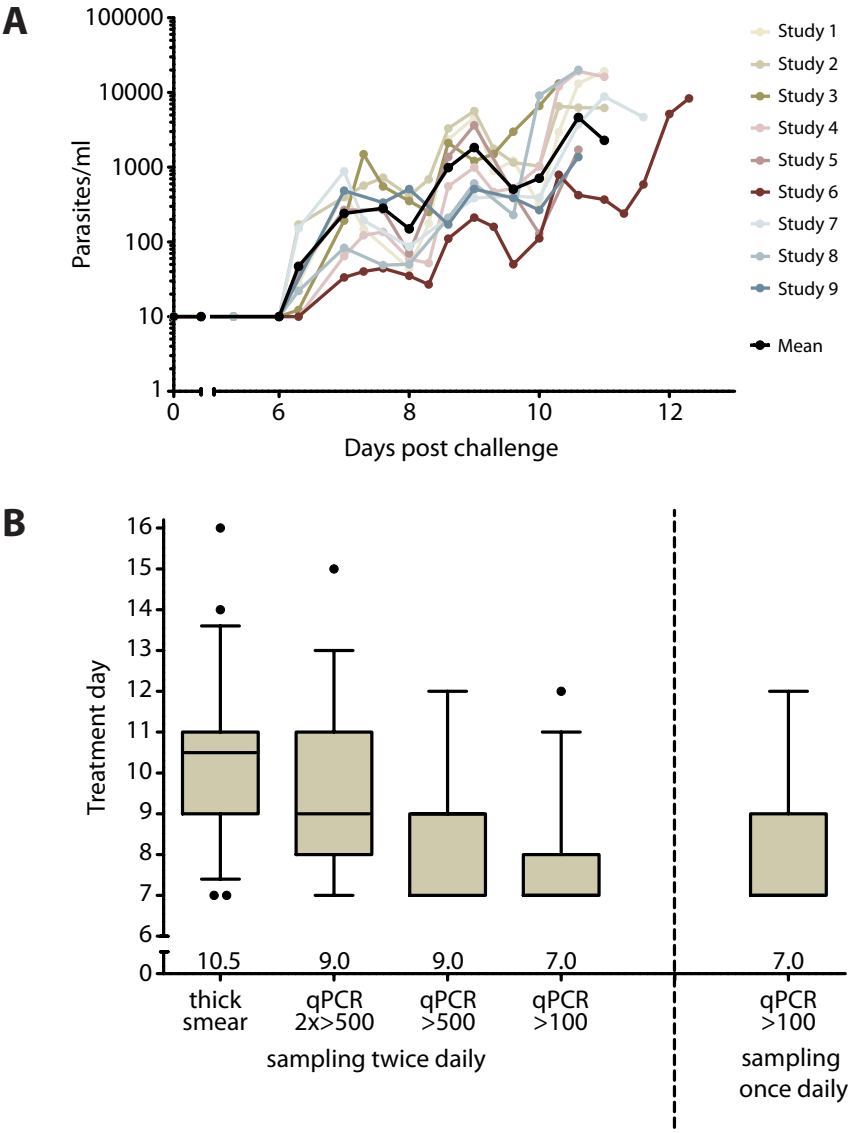

Figure 6.1 Parasitaemia at different thresholds of qPCR and association with adverse events
*(**A**) Mean parasitaemia by qPCR from a total of 55 malaria naïve volunteers undergoing CHMI by five NF54 infected mosquito bites in 9 trials. (**B**) Day of positive thick smear or positive qPCR at different parasite density thresholds as starting day of curative treatment. Box-and-whisker plots show the median, first and third quartiles and 5-95th percentiles. Numbers above the x-axis are median treatment days. (**C**) The mean number of adverse events per volunteer occurring prior to and after treatment. **Brown** = total adverse events; **Red** = grade 3. (**D**) The mean number of adverse events per volunteer occurring prior to thick smear positivity compared to different parasite thresholds for initiation of treatment. Percentages above the bars show the percentage of total AEs that occur relative to thick smear. Red= grade 3; **dark brown** = grade 2; **light brown** = grade 1.*

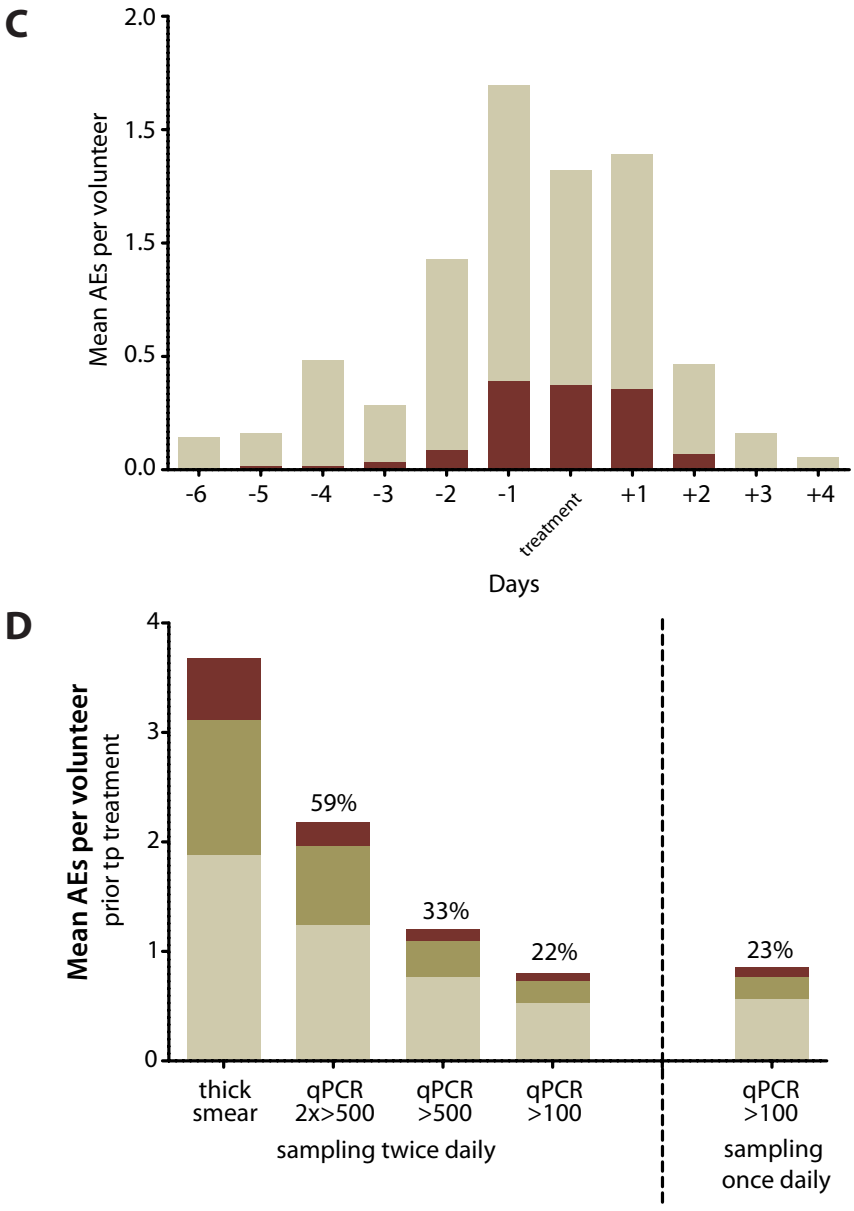

Figure 6.1 *Continued*

Table 6.2 Partial protection after CPS immunization as detected by thick smear or retrospective qPCR

Differences between mean pre-patent periods were determined by Mann-Whitney U test in 10 partially protected and 9 control volunteers after CPS immunization [11, 12]. Parasitaemia of the first parasite wave was estimated by determining the geometric mean parasitaemia from 6.5 until 8.0 days after challenge. Differences in the mean parasitaemia of the first peak was determined by an independent samples t-test.

	Number	Pre-patent period (days)			Parasitemia 1st peak (log)		
		Mean	SD	p value	Mean	SD	p value
POSITIVE THICK SMEAR[1]							
CPS-immunized (partially protected)[2]	10	12.2	1.85	0.006	1.00	0.56	0.02
Controls (unprotected)	9	9.7	2.05		2.07	1.07	
POSITIVE qPCR[3]							
CPS-immunized (partially protected)[2]	10	9.6	2.06	0.035	1.10	0.67	0.04
Controls (unprotected)	9	7.9	1.83		1.99	1.06	

[1] *Threshold of 4000 parasites/ml and twice daily blood sampling*
[2] *Only volunteers with patent parasitemia included in the analyis*
[3] *Threshold of 100 parasites/ml and once daily blood sampling*

A tentative diagnostic replacement of microscopy by qPCR requires a reliable test outcome. A total of 778 retrospective qPCR tests have been performed in 35 fully protected volunteers without a single qPCR above 100 parasites/ml. In the same studies, performed between 2010 and 2012, 107 qPCR standard curves were generated using serial dilutions of blood samples with known parasite densities, diluted from isolated ring stages whose concentration had been determined by microscopy. At densities of 20, 50 and 100 parasites per millilitre, the parasitaemia in these samples was correctly quantified (less than 5% deviation between duplo samples) in 63% (57/107), 87% (93/107) and 96% (103/107) of the samples, respectively. With recent introduction of a new standardized reagents mix for the DNA extraction in 2014 (MagNA Pure LC Total Nucleic Acid Isolation Kit, Roche Diagnostics), 81 of 82 standard curve samples with 100 parasites per millilitre and 79 of 82 samples with 50 parasites per millilitre were correctly measured. The combined data indicate that qPCR with threshold of 100 parasites per millilitre can be reliably used for diagnosis in the CHMI model, with a sensitivity of 99% and a specificity of 100%.

DISCUSSION

This retrospective qPCR analysis shows that the duration of blood stage parasitaemia in CHMI volunteers can be shortened by 3.5 days compared to thick blood smear if a treatment threshold of 100 parasites per millilitre is used. This threshold has a sensitivity of 99% and a specificity of 100%.

Shortening the duration of parasitaemia in volunteers after CHMI has several potential advantages. Most importantly, an increase in safety as malaria symptoms are related to the height and duration of parasitaemia, and the potential to greatly decrease the burden for volunteers. Over half the adverse events after CHMI occur prior to thick smear positivity. This analysis shows that anti-malarial treatment of volunteers when parasitaemia reaches 100 parasites per millilitre will lead to a 78% reduction in the number of adverse events occurring before treatment. Presumably, treatment of volunteers at lower parasitaemia will also lead to a decrease in adverse events occurring after treatment.

If prospective qPCR diagnostics are introduced with a low threshold (100 parasites per millilitre), once daily blood sampling will suffice without the need for a second sample within 24 hours, as there appears to be only a slight effect on the duration of parasitaemia and/or the number of adverse events. Five volunteers (9%) would have been treated 24 hours earlier when sampling for qPCR twice daily. Notwithstanding, we still favour once daily sampling considering the great burden of twice daily blood sampling and the absence of a significant increase in the number of adverse events at that very low parasitaemia. Shortening the duration of parasitaemia and decreasing the frequency of blood sampling will significantly reduce the follow-up of CHMI volunteers. Given the intensive visit schedule for volunteers, requiring multiple personnel and safety laboratory evaluations, the reduced follow-up period will substantially simplify the conduct of these trials, which will also lower CHMI costs.

However, these benefits should not compromise the scientific value of the trial. This study shows that using these diagnostic criteria will not impede the ability to discriminate the delay in parasitaemia and/or reduction in mean first wave parasitaemia as proxy for parasite liver stage development that occurs when a vaccine provides partial pre-erythrocytic protection. Therefore, using once daily qPCR with 100 parasites per millilitre threshold will likely provide a similar primary outcome of protective vaccine efficacy in prospective studies. However, the standard deviations of both mean time to parasitaemia and mean parasitaemia in the vaccination groups increased in this analysis. Consequently, when a relatively smaller difference is anticipated between vaccinees and controls, use of these qPCR criteria may require an increase in sample size to obtain sufficient statistical power.

Evaluation of qPCR data from 35 CPS-immunized and protected volunteers shows that since the introduction of the current qPCR method at Radboudumc, LUMC and the Harbour Hospital in 2010, no immunized and fully protected volunteers developed a positive qPCR after challenge above 100 parasites per millilitre. Using this qPCR method, parasites can be detected at a threshold of 50 parasites/ml with about 96% sensitivity and at 100 parasites/ml with 99% sensitivity. Therefore, the test clearly has sufficient accuracy for diagnostic purposes at these centres. A possible hazard of using a single positive qPCR as a criterion to initiate treatment is the risk of false-positives by cross-contamination or accidental sample switching, especially since treatment will now often be initiated in the absence of clinical symptoms. To minimize this risk, it is important to set up quality control steps not only within the qPCR test but in the conduct and logistics of the qPCR as well. Prior to a CHMI study, qPCR standards are generated and validated, and the same standard is used throughout an entire study. In order to ensure comparability of CHMI data between centres it will be a logical next step to standardize the PCR assay, or make commercially available *P. falciparum* qPCR standards.

Andrews et al. [17] first demonstrated the increased sensitivity of qPCR compared to thick smear, and recognized that qPCR could be used to initiate earlier treatment, at a threshold of 1000 parasites per millilitre [17]. However, recent advances in qPCR methodology, such as the use of an automated system for extraction, has improved sensitivity at low parasite densities. The current analysis shows that this has made it possible to lower the treatment threshold much further. Likewise, other CHMI study centres have also repeatedly shown that qPCR first becomes positive 2-4 days before thick blood smear when both are determined [18-21]. Similarly, studies assessing blood stage drugs or vaccines have already begun to use qPCR as a primary outcome, and have confirmed its sensitivity and specificity [22]. In 2014 Kamau et al. analysed parasitological data from 16 subjects undergoing CHMI in two trials. They also showed that qPCR is positive two to seven days before thick smear [23]. Based on their analysis, the authors recommend treatment after CHMI after two (not necessarily consecutive) positive qPCRs of which one is above 2,000 parasites per millilitre. This threshold was chosen to assess parasite multiplication rates requiring at least two replication cycles. For evaluation of pre-erythrocytic vaccines, however, a treatment threshold of 100 parasites per millilitre will be sufficiently adequate. This analysis shows that different qPCR thresholds can be chosen to assess the duration of parasitaemia. For example, using two consecutive positive qPCRs above 500 parasites per millilitre as a threshold, prolongs the median pre-patent period to 9 days. Different qPCR treatment thresholds

will therefore lead to different durations of parasitaemia. In this way, CHMI can be made a fit-for-purpose model matching the diagnostic qPCR protocol with the considered primary endpoints.

Although retrospective analyses should be interpreted prudently in general, the predictive value of this study can likely be met with confidence since retrospective qPCR data have been remarkably consistent over time between CHMI trials, and CHMI centres [3]. Therefore, PCR may be preferred for diagnosis and treatment when evaluating the protective efficacy of pre-erythrocytic vaccines [19].

CONCLUSIONS

After CHMI, qPCR becomes positive on average 3.5 days before thick blood smear. This analysis shows that depending on the threshold used, treatment based on qPCR diagnostics can greatly reduce the pre-patent period and the number of adverse events occurring before treatment. Furthermore, these data demonstrate for the first time that qPCR has sufficient sensitivity and specificity to use 100 parasite per millilitre as a treatment threshold without affecting trial outcome related to infection and pre-erythrocytic protective efficacy. Therefore, the implementation of these diagnostics would improve safety while reducing the clinical burden and costs without compromising the evaluation of protective efficacy.

Abbreviations

CHMI: Controlled Human Malaria Infection; CPS: Chemoprophylaxis and Sporozoite immunization; qPCR: quantitative Polymerase Chain Reaction; CMO: Radboudumc Committee on Research Involving Human Subjects ; CCMO: Central Committee on Research Involving Human Subjects of the Netherlands; AEs: Adverse events.

Ethics approval and consent to participate

All clinical trials were approved by the Radboudumc Committee on Research Involving Human Subjects (CMO) or the Central Committee on Research Involving Human Subjects (CCMO) of the Netherlands. Prior to inclusion, study volunteers provided written informed consent.

Conflicts of interest

The authors declare no conflicts of interest.

Authors' contributions

JW, RS, MR, CCH, LV and RWS conceived the study. All authors participated in the data analysis and writing of the manuscript.

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Chad. April 2005. Visiting refugees in their tent in Forchana camp.

CHAPTER 7

Discussion

DISCUSSION

The development of a malaria vaccine is far from an easy path and should preferably fulfill several criteria before implementation in the field is acceptable. A malaria vaccine needs, firstly, to be highly efficacious and provide long term protection against a variety of strains. Secondly, this vaccine preferably should also have transmission blocking capacities and provide cross-species protection against *P. vivax*. Thirdly, this vaccine should be easy to administer with a minimum number of immunizations for optimal patient adherence.

Aside from a tremendous number of outstanding questions in current malaria vaccine research, two aspects of vaccine development remain of utter importance. Firstly, to develop a malaria vaccine that protects against a variety of *falciparum* strains. However, none of the current vaccine initiatives have proven to fully protect for years and, up to date, only very few whole parasite vaccine candidates have evaluated heterologous protection to other strains [1-3]. Secondly, progress needs to be made in further identification of immunological mechanisms and markers of sterile protection (and/or disease). Unravelling these mechanisms may enhance vaccine development.

Answers to these questions are still basically absent in current malaria vaccine research and this fundamental lack of knowledge hampers the development of an effective vaccine that could theoretically avoid 438.000 deaths and 214 million clinical cases of malaria annually.

Chloroquine Prophylaxis Sporozoites immunization is a highly efficient strategy to induce sterile protection

In malaria research there are several malaria vaccination models, one of these is the Chloroquine Prophylaxis Sporozoites (CPS) strategy. CPS has proven to effectively induce 100% homologous protection with a minimum of 3x15 mosquito bites [4], is highly reproducible (**chapter 2 and 3**), and has proven to last up to 28 months in two out of three volunteers as was shown by Controlled Human Malaria Infection (CHMI) [5]. Reducing the immunization dose from 3x15 infectious mosquito bites to 3x8 bites (**chapter 3**) or 3x5 bites (**chapter 2**) resulted in a clear dose-dependent profile.

This dose-dependent efficacy we found is remarkable given the breadth of approximately 50 to 200 sporozoites that are inoculated per mosquito bite. It is known that the inoculation dose is dependent on the duration of feeding of the mosquito in one bite session [6], and from animal models it is known that approximately half of the inoculated sporozoites remain in the skin [7]. But

how could this discordant finding between the variation of inoculated sporozoites per bite and the variation in numbers of parasites reaching the liver explain this striking dose-dependent efficacy? The most obvious reason could be that the variability in the number of inoculated sporozoites (within one batch of mosquitoes) may be smaller than postulated [8] and the percentage of parasites reaching the liver in relation to the parasites that remain in the skin is more constant than assumed.

After challenge with five infected mosquito bites and follow-up, volunteers can be grouped to either being sterilely protected to challenge, unprotected to challenge or being partially protected. The latter is characterised by a significantly prolonged pre-patent period compared to the unprotected control volunteers.

The prolonged pre-patent period in immunized subjects is most probably the result of killing of liver stage parasites and not by inhibition of the parasite multiplication during erythrocytic stages [9]. However, it is still unclear if this intrahepatic killing and subsequent reduced release of merozoites is a reflection of either (or a combination of) a reduction or a delayed development of parasites in the liver. This pre-(or intra) hepatic early killing of sporozoites by adaptive immune responses is characterized by inter-individual variation and may bias trial outcome especially when using small number of volunteers per study arm in trials [10].

Chloroquine and mefloquine equal as prophylaxis in the CPS model

Partly by unknown mechanisms [11], chloroquine (CQ) is able to kill the intra-erythrocytic parasite by blocking the transformation of haem into non-toxic haematoin crystals, resulting in the accumulation of a highly toxic haem. CQ also possesses immune-modulatory properties and is used in auto-immune diseases like rheumatoid arthritis or SLE diseases [12]. It is hypothesised that the efficient induction of sterile protection in CPS, found in **chapter 2 and 3**, might have been partially explained by these immune-modulating properties of CQ. CQ is known to enhance $CD8^+$ T cell responses by induction of cross-presentation in which malaria antigens are presented on MHC class I molecules to cytotoxic CD8 T-cells without the usual proteosomal processing and presentation in dendritic cells [13].

Due to widespread resistance of malaria parasites to CQ, other chemoprophylactic drugs need to be assessed for use in the CPS strategy. Mefloquine (MQ) is a registered chemoprophylactic drug that also acts on blood stages of *falciparum* and could in theory be used in the CPS model. In **chapter 3** we compared CQ to MQ which is not known for such immune-modulatory properties. Protective efficacy was expected to be reduced using MQ as prophylaxis. However, we were unable to demonstrate a difference in protective efficacy

between the use of CQ or MQ using an immunization dose of 3x8 mosquito bites (**chapter 3**). However, concerns about neuro-psychiatric side-effects of MQ, further fuelled by a FDA black-box warning, may limit its clinical use [14].

Several factors could have hypothetically affected the protective efficacy we found in the CPS model. Remaining drug concentrations of CQ or MQ could have aided parasite clearance in conjunction with suboptimal (protective) immune responses and may have led to a longer pre-patent period, even up to day of treatment and in this way could have resulted in misclassification of partially protected individuals into protected individuals. However, in **chapter 3**, all volunteers had remaining plasma levels of CQ (and desethyl-chloroquine) between 7–10 µg/L or MQ (no active metabolites) between 5–116 µg/L on the day before challenge (C-1). These concentrations are well below therapeutic or prophylactic plasma levels of CQ (30 µg/L) [15] or MQ (406-603 µg/L) [16, 17] and therefore could not have biased the protective efficacy we found. In addition, the highest MQ levels at C-1 were present in two control subjects, and their pre-patent periods (by thick smear) were 9,5 and 12 days, similar to pre-patent periods of historical controls (ranging between 7 and 12,3 days). In addition, none of the protected subjects had a positive qPCR during the entire challenge period of 21 days. Also parasite multiplication rates after challenge in both CQ and MQ groups were similar to earlier infection studies without malaria prophylaxis, suggesting that blood-stage parasite multiplication was not significantly inhibited [4] and therefore excludes anti-parasitic effects of remaining anti-malaria drug concentrations.

Another factor that could have biased the striking efficacy of CPS is that in current trials, volunteers are only monitored up to 21 days after challenge after which all subjects are curatively treated with antimalarials. A combination of remaining drug levels and an extremely low inoculation dose and/or liver load could theoretically have delayed the thick smear pre-patent period beyond 21 days. In one CPS trial [9] one volunteer did become qPCR positive, retrospectively analyzed on day 21 after challenge. It should therefore be taken in consideration to further extend the observation period after challenge in future trials to detect volunteers who may have an extreme late pre-patent period.

Adverse events, parasitaemia and safety of volunteers in CHMIs

During and after CPS and CHMI safety and adverse events are constantly monitored. Part of these adverse events can be clinical manifestations of (immune-) reactions to parasites. We observed a declining or even absence of parasites and adverse events after each subsequent immunization, suggesting early acquisition of sterile protection in subjects (**chapter 2 and 3**). This could be explained by increasing pre-erythrocytic killing of parasites by the immune sys-

tem leading to absence of circulating blood stage parasites. Unfortunately, the absence of parasites during immunizations was not a very sensitive or specific marker of protection. Nine out of 25 protected subjects (**chapter 2 and 3**) and one unprotected subject (**chapter 3**) did not show any positive qPCR signal during the entire immunization period, not even after the first immunization. Two factors may have contributed to the absence of parasitaemia during immunizations in the unprotected subject. Firstly, it is possible that the early primary innate immune response (Interleukin 1 and 6), responsible for killing of pre-erythrocytic stages, may lead to a liver load that is insufficient to induce an adaptive immune responses and establish a sterile immune response [18]. Secondly, aside a reduced liver load, the chemoprophylaxis also might have reduced parasitaemia during immunizations, keeping the parasitaemia below the qPCR detection limit of approximately 20-50 parasites per millilitre.

The predictive values calculated from both the studies combined, taking either a positive or a negative qPCR after the first immunization as a predictor, the positive predictive value for protection (PPV) was 39% and the negative predictive value (NPV) is 79%. Conclusively, a negative qPCR in the days after the first immunization is a poor predictor of protection after CHMI. When taking respectively the second and the third immunization as a predictor the NPV (91%, 83%) and in particular the PPV (67%, 100%) becomes more reliable.

Using qPCR instead of thick smear leads to lower parasitaemia levels at day of treatment in the CHMI model

Volunteers in CHMIs are closely monitored for safety reasons, and adverse events are recorded during the entire study. Serious adverse are rare in CHMIs but cardiac complications have occurred. Up to date, several instances of cardiac complications in CHMIs have occurred: one case of suspected acute coronary syndrome after immunization with a recombinant vaccine (PfLSA3) and subsequent treatment with arthemeter/lumefantrine [19], one case of myocardial infarction [20] and one case of myocarditis [21] . Despite intensified cardiac screening and selection of volunteers in the trials conducted afterwards, another case of myocarditis occurred recently. This myocarditis occurred in a CPS study 12 days after CHMI infection on the second day treatment [21].

Although parasite densities are already very low at initiation of treatment in CHMIs when compared to natural infections [22], parasite numbers on day of treatment can be considerably reduced further if treatment would be initiated based on qPCR detection of parasitaemia instead of thick smears. Although no causal relationship has been proven between cardiac complications and parasitaemia, a qPCR-based initiation of treatment, to further reduce parasitaemia in volunteers, was proposed to increase trial safety.

In **Chapter 5 and 6** we evaluated the advantages and disadvantages of using qPCR to initiate treatment. In **chapter 6** we calculated, in a retrospective analysis of nine trials, the reduction of the prepatent period and adverse events using different qPCR cut-offs.

Taking a positive qPCR as point in time to initiate treatment reduces the number of parasites a volunteer is exposed to by 90%, by shortening the pre-patent period and reducing the number of erythrocytic parasite multiplication cycles. Shortening of the prepatent period reduces the peak and the cumulative number of parasites during an infection and correspondingly reduces the number of adverse events in volunteers by approximately 70% because of earlier initiation of treatment compared to treatment after a positive thick smear. Using the studies in **chapter 2 and 3** as fictive test trials in **chapter 5**, the impact of qPCR-based initiation of treatment on adverse events and parasitaemia could only be evaluated up to day of treatment.

Unfortunately, it is unclear if this reduction in parasitaemia also will reduce the risk of cardiac events in CHMIs. For example, in the case of the myocardial infarction, no detectable parasitaemia was present and it raises the question whether reducing parasitaemia by using a low qPCR cut-off for initiation of treatment could effectively prevent cardiac complications in future trials. In **chapter 6** we evaluated what the most optimum cut-off for parasitaemia by qPCR should be and how frequent blood samples should be tested for optimal trial results. Using a qPCR threshold of 100 parasites per millilitre the prepatent period can theoretically be reduced by 3,5 days and, together with twice daily sampling, is the most optimal strategy to reduce costs and clinical burden for volunteers [23, 24].

Besides a clear benefit for subjects, the detection of immunological markers of disease, like for example cytokines or blood cells, may become more difficult to detect or can even missed because of limited immunological stimulation due to earlier treatment. Depending on the study objective, studies can be designed to either use thick smears or a positive qPCR for initiation of treatment.

qPCR based initiation of treatment most probably won't affect assessment of biomarkers for protection as they are per definition found in protected individuals without parasitaemia and before the pre-patent period of controls or unprotected individuals. Future trials have to confirm whether (early) qPCR-based treatment truly will enhance safety for volunteers and simultaneously does not (profoundly) hinder immunological assays by using different cut-offs for parasitaemia depending on the goal of the (immunological) study.

Heterologous protection in the CPS model

In malaria affected areas many different strains of *P. falciparum* exist. These strains are genetically diverse both in and between regions and are under constant selective pressure by the human immune system and anti-malaria drugs [25]. It is unclear to what extent this diversity in strains is immunologically relevant in malaria vaccine development as it is unknown which antigens of these strains are exactly involved for induction of full sterile (long lasting) heterologous protection [25]. The NF54 *P. falciparum* clone used in **chapter 2 and 3**, together with clones 3D7 and 7G8, have been extensively used in CHMI trials worldwide and were the only strains available for CHMI for a long time [10]. The NF135.C10 clone used in **chapter 4**, from a patient from Cambodia, is available for CHMI studies since a few years [7] and made testing of heterologous protection in our model feasible. New strains like NF166, originating from Ethiopia, were very scarcely used in CHMIs [26] before but were recently reintroduced in trials to study infectivity (Clinicaltrials.gov NCT01627951; McCall et al unpublished). Unfortunately, up to date, it is unknown which (combination of) strains should be used in malaria vaccine models to induce sufficient heterologous protection for future field application.

The reason why acquisition of natural immunity in malaria endemic areas (probably) takes years and years of repeated exposure is partly due the large variety of genetically different strains that hosts are exposed to in the field. It is hypothesised that each infection with a different strain creates its own unique immune response [27]. Repetitive small inoculation doses with (highly) antigenic different strains over time are insufficient to accomplish sufficiently high liver loads required for an adequate immune response and to generate subsequently sterile protection.

It is even hypothesised that each immunological different (sub)strain might need its own sufficient liver load to reach the threshold for sterile protection against that specific strain [27, 28].

Obviously, for a malaria vaccine to be efficacious, it is essential to cover this variety of strains present in an endemic area. Up to now, studies assessing heterologous protection after whole radiation- or chemically attenuated sporozoite vaccination are scarce [1-3]. In **chapter 4** we re-challenged volunteers previously immunized with the West-African NF54 strain, using the Cambodian NF135.C10 strain, and found a rather low heterologous protective efficacy of 15%. Several factors could have contributed to this relative low efficacy. Waning immunity over time could have resulted in lower efficacy as subjects were challenged 14 months after the last immunization. In addition, subjects previously received different and maybe suboptimal immunization doses which could have added to the relative low efficacy. Moreover, it is hypothesised that

NF135 has a higher infectivity and leads to a subsequent higher liver loads compared to NF54. This is supported by the finding that the NF135.C10 controls had an extremely short pre-patent period suggesting a higher liver load compared to NF54 controls. This is further underpinned by a higher first peak of NF135.C10 (2871 *Pf*/ml) parasitaemia compared to NF54 (456 *Pf*/ml) in previous controls [29]. This could implicate that the dose of five infectious bites with the NF135.C10 strain might equal ten or even fifteen infectious bites with NF54. Because of the small number of subjects, we could not include a NF54 control group in our re-challenge study by which we could have compared the heterologous efficacy we found with NF135.C10. Therefore, to assess the optimal dose for challenge, dose-escalating infection studies with NF135.C10 have been performed (Clinicaltrials.gov NCT02149550; Wammes et al unpublished).

It is hypothesised that for the induction of full homologous or even heterologous protective immunity a certain antigen magnitude is needed to overcome an immunological threshold. The required immunization dose could be further increased to amplify the (pluriform) antigen exposure and increase the immune response to the parasite. It is known that the malaria parasite exhibits profound immuno-evasive techniques preventing maximum exposure to the immune system. Genetic variability between individual parasites and cross-stage variability of antigen exposure during the parasite's lifecycle are important factors in the immuno-evasive techniques of parasites [28]. By increasing both the quality and quantity of antigens exposed to the immune-system (effectively the liver load), a more effective immune response could be mounted and overcome the induced immune-evasive capacity by the parasite. This in turn could lead to an increased (long lasting) heterologous efficacy or even cross-species protection against for example *P. vivax*. Currently, studies are ongoing assessing heterologous protection against three different strains after full effective CPS NF54 immunizations (Clinicaltrials.gov NCT02098590). In addition, recent work showed that 33 weeks after immunizations with 3 times $9x10^5$ irradiated P*f*SPZ (3D7 clone) i.v. injections, 5 out of 6 of these previously fully homologous protected individuals were also heterologously protected with strain 7G8, a clone of Brazilian origin [3].

And albeit with very limited evidence, it is hypothesised that vaccination efficacy found in malaria naïve volunteers could predict similar results in field settings [10] as the homologous NF54 challenge, used commonly in CHMIs, might be even too stringent compared to a 'natural challenge' in endemic areas with far less sporozoites inoculated by mosquito bites compared to the number of sporozoites in trials [30].

Field application of the whole-sporozoites model

The current most important and relevant phase 3 vaccine initiatives are whole sporozoite vaccines and the RTS,S subunit vaccine. The RTS,S vaccine has already been scheduled for field implementation although it has a known low efficacy. Despite the fact that the whole parasite model (e.g. CPS) is safe and is able to efficiently induce long-lasting homologous protection, several hurdles need to be taken before field application becomes suitable. Obviously, immunizing communities through mosquito bites lacks full applicability. Nonetheless, whole sporozoites vaccine candidates could be further optimized in several ways for field use. These whole sporozoite vaccine candidates could be further altered using irradiated (*Pf*SPZ), chemically attenuated (*Pf*SPZ-CVac) or genetically attenuated (GAP) parasites and could be either injected intravenously, intramuscularly of subcutaneously.

Recently progress has been made with intravenous injection of irradiated sporozoites (*Pf*SPZ), and is currently tested in several African countries [31, 32]. In this model infected mosquitoes are irradiated, dissected and sporozoites are harvested. These extracted sporozoites are purified and cryopreserved, and injected in humans after reconstitution. The irradiation dose needs to be carefully chosen to limit the development of these parasites during the liver stage, and to subsequently prevent breakthrough to blood stages, and simultaneously allow these live parasites to develop as long as possible in the liver stage to mount an adequate immune response in the human host.

The *Pf*SPZ-CVac method uses aseptic, purified, cryopreserved, non-irradiated P*f*SPZ injected intravenously whilst taking (for example) chloroquine as a chemo-prophylactic to prevent full erythrocytic multiplication and subsequent progress to disease of malaria. Also other chemo-prophylactic anti-malaria drugs can be used like mefloquine or for example ferroquine; a new drug still under phase IIb research. A single 800mg dose of ferroquine is able to provide for more than 8 days of erythrocytic parasite killing [33] and could be the ideal partner-drug for *Pf*SPZ-CVac to secure adequate serum drug concentrations while mass-vaccinating communities.

Although previous studies showed a relative low protective efficacy [34], recently, a study using three intravenous doses of 5.12×10^4 *Pf*SPZ, with an interval of 28 days, conferred short-term sterile homologous protection in 100% of subjects ten weeks after immunizations [35].

The reason previous studies using *Pf*SPZ-CVac were less effective is most probably due to both the quality and quantity of injected sporozoites, as well as the inoculation route that were suboptimal for sufficient numbers of viable parasites to reach the liver and mount an adequate immune stimulation needed for sterile protection. It is known that the route of inoculation is crucial for

the number of sporozoites that are able to reach the liver and the resulting parasite liver load is known to correlate with protective efficacy [36]. In the murine model it has been shown that injection of whole sporozoites by intravenous (i.v.) injection results in 2 to 50 fold higher liver loads compared to intramuscular (i.m.), subcutaneous (s.c.) or intradermal (i.d.) inoculation [36, 37]. The use of smaller volumes by multiple intradermal injections can increase the liver load further and might mimic probing and injection of saliva and sporozoites in the skin by *Anopheles* mosquitoes.

However, aside from the laborious process of extracting of these parasites from mosquitoes both in the *Pf*SPZ-CVac and in the *Pf*SPZ model, vialling and delivering cryo-preserved parasites to field settings remain a challenge. In addition, intravenous injection is far more time-consuming and risky than intramuscular or subcutaneous injection. And last but not least, only short-term homologous protection has been evaluated and no heterologous protection.

An alternative to the whole sporozoite *Pf*SPZ-CVac or *Pf*SPZ model is the use of genetically attenuated sporozoites (GAP) and this might be the most attractive option for future field application [38]. With GAP as a vaccine, a new field of research is entered, and it could be an alternative to the other whole parasite vaccines being either impractical because of the use of chemoprophylaxis during immunizations or because of using injection of large numbers of (irradiated) sporozoites [39]. Recently, intensive research in the production of genetically attenuated sporozoites has been performed [40-42] and has already proven a superior efficacy compared to irradiated *Pf*SPZ immunizations in humans [43]. However, the genetic alterations in the parasite, resulting in arrest of parasites in the liver, must be carefully chosen [44]. Essential genes for parasite survival in the liver are altered to arrest development and proliferation while still allowing exposure of antigens to the host during the liver stage. These genetic alterations leading to arrest of parasites could be either early or late during the development in the liver. Early liver arrest of parasites could be safer because of lower risk of breakthrough to blood-stages but might be inadequate to mount an immune response needed for sterile protection. Alternatively, late liver stage arrest of parasites might induce sufficient sterile protection but could implicate breakthrough to blood-stages and therefore being unsafe for vaccinees. This delicate balance between full arrest of parasites in the liver, allowing maximum antigen exposure, and acquisition of protective immunity but without breakthroughs to blood-stages, are critical for safety of vaccinees and vaccine efficacy. To improve efficacy and to enhance immune stimulation, adjuvants can be added, either separately or embedded in het parasites genome. Despite all current research, the production and implementation of a GAP vaccine, as any other candidate whole sporozoite vaccine, is still

a major challenge. A first-in-human trial of *Pf*Δp52Δp36GAP failed because of lack of safety due to break through to blood-stages [45]. Recently, trials in the mouse model show promising results with a double knockout of genes p52 and p36 (*Py*Δp52Δp36GAP) [46] or the genes Slarp and B9 (PbΔb9ΔslarpGAP) [47] and showed protective immunity without break through to blood-stages. In addition, in vitro studies using *Pf*Δb9ΔslarpGAP were able to infect humanized mice hepatocytes. Currently, *Pf*Δb9ΔslarpGAP is evaluated in humans for safety, immunogenicity and efficacy of protection (Clinical trial NCT03163121). Other revolutionary novel techniques using alterations in the genome of the parasite are attenuations in the CRISPR-CAS9 gene [48] and form a complete new field for both drug targets and well as for vaccine purposes [49] but have not been evaluated in humans yet.

Immunology in Malaria

Despite decades of research, up to date, it is still unknown what exactly contributes to natural or (artificial) sterile protection against *P. falciparum* in the human host. In the CPS model, the host immune system is exposed to all stages of the parasite, including early blood stages after which parasites are killed by the prophylactic drug. In whole sporozoite CPS vaccination, it is shown both in mice and humans, that sterile protection against *P. falciparum* is induced in the liver and is T-cell mediated [9, 50-52]. In line, challenge with i.v. asexual blood stages after CPS immunizations did not lead to protection. Instead, immunized subjects showed earlier fever and higher inflammation markers like IFNγ compared to controls and indicates a response sufficient for immune recognition but insufficient for killing of parasites [9]. Together these data suggest that protection is mediated by pre-erythrocytic immunity and next, raises the question how immunity is acquired during this clinically silent liver phase. *Plasmodium* can infect and replicate undetected in hepatocytes. In absence of clinical symptoms, presentation of parasite RNA in liver cells by the cytosolic pattern recognition receptor Melanoma differentiation-associated (Mda) protein, which acts as a Pathogen-associated Molecular Pattern (PAMP), induces Interferon (IFN) cytokines and triggers the recruitment of cytotoxic $CD8^+$ T cells for later killing [53].

CD8 T-cells play an important role in sterile protection

In the CPS model we were only able to assess the peripheral blood compartment for immunology taken as a reflection for the liver compartment. Taking the peripheral blood compartment as a proxy we found sterile pre-erythrocytic

protection to be likely mediated by cytotoxic $CD8^+$ T-cells, in conjunction with Th1 lineage effector mediators like IFNγ, IL2, TNF and other cytotoxic mediators (like Granzyme B and perforin) produced by innate and adaptive immune cells like NK-cells, $CD8^+$ and $CD4^+$ T-cells and γδ CD3+T-cells (**chapter 2 and 3**). These Th1 effector mediators have been assessed in several platforms: the murine model [54], non-human primates [55] and humans [56, 57].

Two mechanisms of $CD8^+$ T-cell mediated killing of infected liver cells are currently proposed, but mouse models show contradictory results. One method of killing is mediated by $CD8^+$ T-cells releasing perforin and granzyme B. The second method is mediated by the Fas receptor and its ligand on the activated effector T-cells (T_{eff}). However, granzyme$^{-/-}$, perforin pore protein (ppo)$^{-/-}$ and apoptosis ligand FasL/CD95L$^{-/-}$ deficient mice were fully protected after immunizations with irradiated sporozoites (either *P.yoelii* or *P.berghei*) [58]. This suggests that, in the mouse model, induction of sterile protection is indirectly mediated by a $CD8^+$ T-cell associated cytokine cascade and suggests to act independent of granzyme B. However, the mechanism of sterile protection might be dependent on the strain used, and additionally, it remains unclear if these findings can be extrapolated to humans.

The role of $CD4^+$ T cells in the CPS model remains unclear

In addition to $CD8^+$ T-cells, also $CD3^+$ γδ T-cells [59], NK-cells [58] and cytotoxic $CD4^+$ T-cells (**chapter 2 and 3**) [50] may play a role in pre-erythrocytic immunity but their exact contribution remains unclear. It is known that $CD4^+$ T-cells are needed to control blood-stage (natural) infections by IFN-γ production in assisting B-cells for antibody production [50]. In **chapter 2** we found cytotoxic CD4+ T-cells to be correlated in the induction of sterile protection and indirect killing of hepatocytes might take place via effector mediators despite hepatocytes lack MHCII receptors for antigen presentation.

B-cells in the CPS model correlate poorly with sterile protection

Aside from T-cell involvement, also B-cells and several antigens like AMA, MSP1-3, GLURP and CSP may play a role in the induction of sterile immunity in malaria. Unfortunately, levels of these antibodies appear to correlate poorly with sterile protection both in malaria naïve subjects [60] and in field trials [61], and show large intra-individual variation making clear that avidity of antibodies in general appear to be more important than the quantity of antibodies in sterile protection from malaria [62]. Recent immuno-epidemiological work showed antibodies (like AMA, MSP and GLURP) are associated with protection against clinical malaria in Malian [63, 64] and in Gabonese children [65]. Blood-stage parasites are able to alter both the number and function of B-cells in clinically

immune adults and further inhibit mounting of sterile protection [66]. This lack of number and function of B cells might be the reason of the insufficient association with sterile protection.

The role of regulatory T-cells in sterile protection

It is known that during natural infection T-cell responses are reduced [67, 68]. Studies in mice have demonstrated that after natural infection, $CD8^+$ T-cell responses against liver stage antigens were lower compared to immunization with irradiated sporozoites, even after repeated infections [69]. Also liver-stage specific T-cells in mice were reduced after infection [70]. Clearly, $CD8^+$ T-cell responses are down-regulated by blood-stages of malaria.

In natural infections, clinical immunity is slowly acquired but without effectively killing of all parasites leading to sterile immunity. Nonetheless, death by malaria can prevented by just one or two clinical infections [71]. In high endemic areas, when humans are repeatedly exposed to parasites, a delicate balance exists between: *i* controlling the infection and simultaneously acquisition of clinical protection and *ii* limiting collateral immunological damage whilst combatting parasites. This balance is partly effectuated by regulatory T cells (T_{reg}) which control the damage by down-regulating the force of the inflammatory response caused by T_{eff}.

During combat against malaria parasites, pro-inflammatory cytokines and chemokines, to a large part produced by Th1 $CD4^+$ T-cells, recruit inflammatory cells to the site of malaria infection. After recruitment, cytotoxic T-cells (CTL or Effector T cells (T_{eff})) and Natural Killer (NK) cells kill intracellular malaria parasites in the liver. However, the timing and degree of the response and the ratio between T_{reg} and T_{eff} [72] and subsequent inflammatory response, is crucial in successfully combatting malaria infections. Both pro-inflammatory (IFN-γ, TNF-α, IL-12) and anti-inflammatory/regulating (IL-10 and TGF-β) responses need to be carefully orchestrated and timed, and, unless tightly controlled, unlimited pro-inflammatory cytokine responses can lead to severe immune-pathology and eventually to death [73, 74]. Alternatively, too early activation of T_{reg} responses can induce immune-suppression by inhibiting Th1 responses and subsequently increase of parasitaemia [74]. It is known from malaria infections studies in humans that a high parasitaemia correlates with induction of T_{reg} and lower inflammatory responses [75] resulting in a persisting blood stage infection. On the contrary, data in mice regarding the role of T_{regs} in malaria infection are contradictory, depending on the mouse–parasite strain combination used, and large differences in T_{reg} immunological responses exist between murine and human model [76].

The necessity of (early) T_{eff} induction and IFN-γ production by immune cells has been repeatedly proven to be related with sterile immunity in malaria, both in the murine model [50] as in humans [56] [77], but could not be proven to correlate with protection and subsequently be used as a biomarker in our CPS model (**chapter 2**; [52]). It is hypothesised that *because of* the very low parasitaemia in CPS during immunizations, the high T_{eff} / T_{reg} ratio is able to induce sterile protection [67] and shape memory responses [78]. Alternatively, prolonged parasitaemia during blood-stages can suppress T cell responses and IFN-γ production both by vaccination and by natural exposure [79] and can inhibit acquisition of protection through the activation of T_{reg} [57, 80-82]. However, it remains unclear how regulatory T-cells exactly control pro-inflammatory and anti-inflammatory responses in vaccine-induced responses [82].

The importance of biomarkers in the malaria vaccine model

Molecular techniques like transcriptomics, metabolomics and proteomics can be of assistance in finding biomarkers in malaria by elucidating the immunological processes that form the basis of protection against malaria [83]. Simultaneously, new software and internet-based integrated analysis (e.g. Ingenuity or Cytobank) provide researchers in systems biology and systems immunology [84] powerful information to solve complex multi-dimensional cellular and molecular interactions that underlie malaria pathogenesis and protection. Already key gene-expression signatures have been found for licensed vaccines against for example yellow fever [85] and for other infectious diseases like tuberculosis [86]. Similar approaches for malaria could be of benefit. Up to date, no markers are known that unequivocally correlate with sterile protection in any malaria vaccine model.

Nonetheless, we found in **chapter 3** markers that are associated with parasite exposure. Merozoite Surface Protein 1 (MSP-1) antibodies, a marker of parasite exposure, were elevated in all unprotected volunteers but not in protected individuals and therefore can't be used a predictor of sterile protection [62]. A recent proof-of-principle study, comparing RNA-seq profiles before and after malaria infection between malaria-experienced (Malian) individuals and malaria-naïve (CHMI) individuals showed that activation of pro-inflammatory, interferon-mediated, immune responses were highest in the malaria naïve individuals and lowest in malaria-experienced individuals from Mali [87] showing a reduced inflammatory response which suggests both reduced manifestations of clinical malaria and simultaneously increased B-cell receptor signaling demonstrating build-up of adaptive immunity. Differences in acquisition of clinical protection are considered caused by an inflammatory ('pyrogenic') threshold. The absence of fever and concomitantly low activation of pro-in-

flammatory responses in the malaria-experienced individuals, and lack of sterile protection, might be caused by the co-infection of helminths down-regulating these CD8+ T-cell inflammatory responses.

The role of parasitic infections in malaria vaccination

A limitation of the CPS model or CHMIs in humans is that these trials can only perform immunological assays in the blood-compartment and this may not reflect processes in important sites like the liver or the spleen. Additionally, assessing T-cell responses and efficacy of vaccination in malaria endemic areas might even be more difficult than in malaria naïve subjects in hyper-controlled trial settings in the western hemisphere. Repeated exposure of individuals to malaria parasites leads generally to naturally acquired immunity (NAI), and the level of acquired immunity depends on the combination of the individuals' specific immune-system and the previous level of exposure to parasites [88]. A complicating factor of assessing efficacy after vaccination in a field setting might be the lack of tools to assess the degree of (immunological) magnitude of pre-existing NAI in subjects [89] and assessment of for example the influence on the level of T_{reg} induction and IFNγ production [90]. Also co-infections with other parasites like for example helminths could dampen the vaccine efficacy in vaccinees. A quarter of the world population is infected with helminths, of which most infections are in highly endemic low income countries [91]. Helminth infections are known to induce strong regulatory mechanisms for survival in its host and have proven to inhibit Th1 responses to infections [92, 93] and can reduce protection after vaccinations [94, 95].

Future

Given the tremendous suffering of communities of malaria it might seem that quick deployment of any malaria vaccine is necessary, nonetheless, several aspects might need to be taken into consideration. RTS,S is the first licensed, and soon mass scale distributed, vaccine against malaria. However, it is questionable if the deployment of this vaccine is justifiable at this moment. And although the vaccine averted clinical episodes of malaria shortly after vaccination in children, the short and long term clinical protection has been proven to be poor, and this vaccine does not significantly protect against severe malaria or malaria hospitalization as being demonstrated after a trial conducted in eleven African sites [96]. Most positively seen this vaccine will avert clinical malaria cases, and will be a try-out and form a base for future vaccines to be rolled out in endemic areas. Additionally, this vaccine could assist in reducing (clinical) malaria to-

gether with other existing tools like the use of bednets, insecticide spraying, adequate diagnosis and treatment of malaria. Given the poor immunogenicity and protection of RTS,S, implementation of this vaccine in endemic areas could have other effects. If vaccines do not fully protect, resistance can develop as a result of selection of the remaining resistant parasite population. Additionally, (partial) clinical vaccine-induced protection might reduce the existing natural clinical immunity of the population over time. This waning natural clinical immunity might lead to more severe malaria infections in former vaccinees and this could theoretically intensify the already existing malaria burden even more. In contrast, once-yearly administration of a malaria vaccine that is only effective short-term to communities living in areas with seasonal short malaria episodes might be considered being useful [97].

What needs to be done while in the meantime while Africa waits for an effective vaccine? One option is to further improve the effectiveness of the two most promising vaccine model: the whole parasite vaccines like *Pf*SPZ, *Pf*-SPZ-CVac or GAP, and the subunit vaccine RTS,S. The whole parasite vaccines could be further optimised by improving longevity, heterologous protection and applicability: the use of cryopreserved parasites in combination with an adjuvans or the further development of a genetically attenuated plasmodium (GAP) vaccine. The laborious manual harvesting of (genetically, (non) irradiated parasites used in whole sporozoite vaccines is in the process of being automated and new parasite culturing techniques may improve mass-scale applicability in the future. Additionally, new injection techniques like the use of multiple small volume intradermal inoculations [98] might overcome the impracticability of repeated intravenous injections.

The subunit vaccine RTS,S could be made more effective by adding different (cocktail) multi-stage immuno-potent antigens, development of new adjuvants or combination with other vaccines like ChAd63/MVA ME-TRAP [99, 100]. These multi-antigen, multi-stage or even cross-species subunit vaccines (NCT01883609, NCT02252640) using a combination of carefully selected (but yet unknown) antigens. These highly immunogenic antigens at different - including sexual - stages, might be the key solution in malaria vaccine development [101]. It is even possible that it may be necessary to produce a vaccine for different regions or continents each containing a different cocktail (of antigens) of strains. The current deployment of the RTS,S vaccine in Africa could be taken as a platform for further deployment of this vaccine or others.

Malaria vaccine community needs to combine knowledge and strengths

It is under debate whether the international malaria vaccine community should proceed on the current research route [102]. Even when new antigens are found, new delivery systems or cocktails of vaccines are used, without basic knowledge of the immune biology of malaria and without correlates of protection this may not work in the case of finding the first in-human anti-parasite vaccine. Maybe other directions need to be explored, given the current status of vaccine research and the availability of a field vaccine of only limited efficacy, even after decades of research. Malaria vaccine development could benefit maybe more from the use of genome-based research to find important immunogenic antigens and further explore immunological pathways that are responsible for protective immunity [103]. In addition, with the current highly heterogeneous landscape of vaccine research, studies need to be further harmonized worldwide to combine strengths and to further facilitate comparability between studies [104]. The success of malaria research lies in multi-disciplinary approach where disciplines like malariology, epidemiology, bio-informatics, immunology and clinicians bundle expertise and enable efficient research in conjunction with next-generation molecular and cellular techniques.

Despite all existing shortcomings in current malaria vaccine research, eradication of malaria using all available anti-malaria tools, including a highly efficacious vaccine [102], might be feasible in the coming decades as declared by Bill Gates in 2007 [104]. However, the availability of sufficient funds for now and for the future, both for vaccine-research as well as for further implementation of current malaria tools, remains a tremendous additional challenge.

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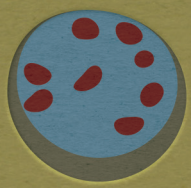
CHAPTER 8

Summary
Samenvatting



SUMMARY

Using a variety of anti-malaria tools, numerous efforts have been made to stop malaria over the past decades. These tools include DDT fumigation, improved diagnosis (rapid tests) and treatment (ACT), distribution of bed nets and spraying of houses and water places near houses. The combined strengths of these tools resulted in a steady decline of malaria in several endemic countries worldwide but unfortunately so far without elimination of malaria. Unfortunately, it has been shown that if one or a combination of these tools are no longer stringently applied, malaria incidence can rapidly increase. A more permanent solution is urgently needed and availability of an effective vaccine, together with all other existing tools will be critical to halt malaria or even succeed to final eradication.

In that perspective, we studied the potential of whole sporozoite immunization. Dutch healthy volunteers were immunized according to the CPS model, which encompasses immunizations under chemoprophylaxis via bites of *Anopheles* mosquitoes, infected with P. *falciparum* sporozoites. It has been previously shown that three inoculations by 15 *P. falciparum* infected *Anopheles* mosquitoes results in 100% sterile homologous protection. Protection is evaluated in the Controlled Human Malaria Infection (CHMI) model.

In this thesis we further explored the CPS model and assessed different immunizing doses, type of chemoprophylaxis and immunological determinants of disease and protection.

In **chapter 2** we reduced the immunization dose from 3x15 bites to 3x10 bites and to 3x5 bites and found a clear dose dependent efficacy in the CPS model; when reducing the immunization dose to 3x5 bites the efficacy was reduced to 50% protection. After challenge and follow-up, volunteers can be grouped to either being sterilely protected, completely unprotected or partially protected as defined by a significantly prolonged pre-patent period compared to control volunteers.

In addition we evaluated immune responses related to protective immunity. In vitro re-stimulation tests showed CD4 T cells expressing CD107a and CD8 T cells producing granzyme B are associated with protection in het CPS model.

Chloroquine (CQ), has known immuno-modulating properties with potential effects on the high protective efficacy. In **chapter 3** we, therefore, evaluated whether Mefloquine (MQ), is also effective in inducing protective immunity. Although the number of volunteers in each arm in this study was relatively low, we found no differences in efficacy when comparing these two drugs using three times eight infective bites. Also, no differences in immune responses were found.

In most CPS trials, the P. *falciparum* strain NF54 is used both for immunizations and challenge to assess homologous protection. In the field many genetically different strains circulate and a future vaccine should be able to cover multiple strains. To be able to use the CPS model in field settings in the future protection to a wide range of other strains are needed.

In **chapter 4** we re-challenged a subset of the volunteers who participated in the study in **chapter 2** with a different strain to assess heterologous protection. Re-challenging with a strain NF135 from Cambodia, showed that two out of thirteen volunteers were also heterologously protected at 14 months after immunization.

Despite the use of malaria chemoprophylaxis during the immunization period, participants in the CPS model usually experience symptoms and signs of malaria. Volunteers can experience usually mild symptoms of fever, muscle pain, headache and general malaise. Unprotected volunteers may develop signs and symptoms of clinical malaria after challenge before treatment is initiated.

To reduce the severity of such symptoms after challenge as well as the associated delay of treatment, more sensitive molecular parasite detection methods such as qPCR can be used to potentially reduce the clinical symptoms of malaria for volunteers. In **chapter 5** we retrospectively assessed the parasitological dynamics and adverse events in case of a positive qPCR rather than thick smear. Analysing parasite data of the clinical trials described in **chapters 2 and 3**, we found that parasite density and adverse events on day of treatment considerably reduce if treatment would be initiated based on positive qPCR . However, different end-points in studies will change parasitological and immunological assessment when treating volunteers shorter after challenge.

In **chapter 6** we retrospectively assess which qPCR cut-off parasite densities should be used to optimise both the reduction in adverse events for volunteers and simultaneously be able to acquire sufficient relevant immunological and parasitological data. A qPCR threshold of 100 parasites/ml was accompanied with a 99 % sensitivity and 100 % specificity and resulted in a shortening of the prepatent period with 3,5 days when compared to thick smear.

The world is waiting for an highly effective malaria vaccine. CPS is currently not suitable for large scale field applicability yet. In the meanwhile an injectable variant of radiation attenuated sporozoites (*Pf*SPZ-CVac) is being further optimized and might be effective and implementable on a larger scale. Additionally, other vaccine initiatives such genetically attenuate parasites (GAP) are being tested. Finally, improving existing vaccines like RTS,S with multistage antigens and new adjuvants might be an option to improve its efficacy.

The implementation of malaria vaccines in endemic areas will most probably face several new tremendous challenges. For instance, many people in malaria endemic areas are infected with helminth infections which may impact on vaccine efficacy. Introducing a malaria vaccine that does not cover all immunological relevant strains might select immuno-resistant strains and thereby affecting vaccine efficacy and could theoretically make the vaccine useless.

Successful malaria eradication will be more likely to be achieved with a multi-disciplinary approach including all relevant disciplines like clinicians, parasitologists, epidemiologists and bio-statisticians. But most important the affected populations and their governments need to accept vaccination. Additionally, sufficient and continuous funds will proof to be of tremendous necessity.

NEDERLANDSE SAMENVATTING

De laatste twintig jaar heeft men met een grote verscheidenheid aan anti-malaria maatregelen getracht malaria een halt toe te roepen. Deze maatregelen bestonden onder andere uit het vernevelen van langwerkende insecticiden (zoals DDT) in huizen, het verbeteren van de malaria-diagnostiek, het gebruik van krachtig werkzame anti-malaria behandeling (artemisinin combination therapy; ACT) en het bevorderen van het slapen onder geïmpregneerde klamboes. Samen resulteerden de gecombineerde maatregelen in een gestage afname van malaria in vele landen in de wereld waar deze ziekte voorkomt. Helaas heeft het echter niet geleid tot de totale uitroeiing van deze dodelijke bloedparasiet.

Bovendien kan malaria weer zeer snel toenemen als deze maatregelen niet langer strikt worden nageleefd. Een meer definitieve oplossing is daarom dringend nodig. Zo zal de beschikbaarheid van een effectief vaccin, samen met alle andere bestaande anti-malaria maatregelen, uitermate belangrijk zijn om malaria te stoppen of zelfs volledig uit te roeien.

In dit proefschrift bestudeerden wij de veiligheid en effectiviteit van de herhaalde toediening van muggenbeten met het infectieuze stadium (sporozoïeten) van de malariaparasiet *Plasmodium falciparum* onder bescherming van een anti-malariamiddel (ook wel 'whole sporozoite immunisation under chemoprophylaxis' of CPS genoemd). Eerder werd aangetoond dat Nederlandse vrijwilligers na CPS volledig beschermd waren tegen een infectie met dezelfde stam (homologe bescherming). Deze bescherming werd getest door vrijwilligers bloot te stellen aan een 'gecontroleerde malaria infectie' ('Controlled Human Malaria Infection' (CHMI) model) via geïnfecteerde muggenbeten, maar dit keer zonder chemoprofylaxe. Het bloed van de vrijwilligers werd daarna op vaste tijdstippen nauwgezet microscopisch onderzocht op de aanwezigheid van malariaparasieten. Zodra malariaparasieten werden gezien, werd gestart met de behandeling.

In dit proefschrift werd CPS verder geëxploreerd waarbij het effect van lagere immunisatie doses, andere malariaprofylaxe en gevoeligere malariadiagnostiek werden getoetst. Hierbij werd gekeken naar mate van ziekzijn en bescherming na CHMI en naar de afweermechanismen die hierbij een rol spelen.

In **hoofdstuk 2** werd de immunisatiedosis verlaagd van 3 x 15 naar 3 x 10 en 3 x 5 geïnfecteerde muggenbeten. We vonden een duidelijke relatie tussen dosis en effectiviteit van CPS: wanneer de dosis werd verlaagd naar 3 x 5 beten, nam de bescherming met de helft af.

Niet alle vrijwilligers waren echter volledig beschermd na een gecontroleerde infectie. Bij sommige vrijwilligers trad malaria wel op, maar dat gebeurde significant later dan bij controle vrijwilligers die geen enkele vorm van bescherming hadden. Door deze verschillen in bescherming (volledig of gedeeltelijk beschermd en onbeschermd) was het mogelijk om de afweerreactie te onderzoeken die samenhangt met bescherming. In het laboratorium werd aangetoond dat bepaalde typen afweercellen, namelijk CD4 T-cellen (CD4 en CD8 T cellen) met speciale afweercelkenmerken (CD107a) en CD8 T-cellen die bepaalde afweerstoffen (granzyme B) produceerden, die vaker voorkomen bij personen die beschermd zijn tegen malaria. Deze stoffen en celkenmerken en stoffen worden geproduceerd door het immuunsysteem om de met malaria geïnfecteerde lichaamscellen te kunnen doden.

Chloroquine (CQ) is een antimalariamiddel dat al sinds de veertiger jaren bestaat. CQ heeft ook afweer-veranderende eigenschappen waarvan bij auto-immuunziekten, zoals reuma, gebruik wordt gemaakt bij auto-immuunziekten, zoals reuma. Deze eigenschappen van CQ zouden mogelijk een belangrijk deel van de beschermende effectiviteit van CPS kunnen verklaren. In **hoofdstuk 3** werd daarom onderzocht of mefloquine, een verwant malariamedicijn, ook effectief is in het opwekken van beschermende immuniteit na CPS. Alhoewel het aantal vrijwilligers in elke studie-arm relatief klein was, vonden we geen verschil in effectiviteit van CPS met 3 x 8 muggenbeten. Ook vonden we geen verschillen in afweerrespons.

In de meeste onderzoeken wordt zowel voor de immunisatie (CPS) als voor de beoordeling van bescherming (CHMI) de Afrikaanse *P. falciparum* stam NF54 gebruikt.

In gebieden waar malaria veel voorkomt, circuleren echter verschillende stammen van *P. falciparum*. Het vaccin van de toekomst zou dan ook tegen al deze stammen bescherming moeten bieden. Om CPS te kunnen toepassen in verschillende landen waar malaria voorkomt moet het dus bescherming bieden aan een breed scala aan stammen.

In **hoofdstuk 4** werd een deel van de vrijwilligers, die 14 maanden eerder deelnamen aan de studie van **hoofdstuk 2**, opnieuw blootgesteld aan een gecontroleerde infectie. Echter, ditmaal met een *P. falciparum* stam uit Cambodja (NF135). Bij (slechts) twee van de 13 vrijwilligers werd volledige bescherming tegen deze vreemde stam aangetoond (heterologe bescherming).

Er zijn veel meer parasieten nodig in het bloed (parasietendichtheid) om malaria te kunnen aantonen met microscopisch onderzoek van een dikke druppel bloed, dan met een kwantitatieve polymerasekettingreactie (qPCR) waarbij een stukje van het kernmateriaal (DNA) van de malariaparasiet meer dan een miljoen keer wordt vermenigvuldigd. Het gebruik van deze veel

gevoeligere malariadetectiemethode zou dus kunnen leiden tot het eerder vaststellen van malaria, het sneller starten van de anti-malaria behandeling en het verminderen van de duur en de ernst van het ziekzijn door malaria bij vrijwilligers na een gecontroleerde infectie.

In **hoofdstuk 5** werd daarom teruggekeken naar de gegevens van de studies uit **hoofdstuk 2 en 3**. Als de veel gevoeligere qPCR zou zijn gebruikt in plaats van het dikkedruppelonderzoek, dan zou er anderhalve dag eerder met de malariabehandeling gestart zijn, was de parasietendichtheid met 90% afgenomen en waren de klachten op de dag van behandeling 70% minder uitgesproken.

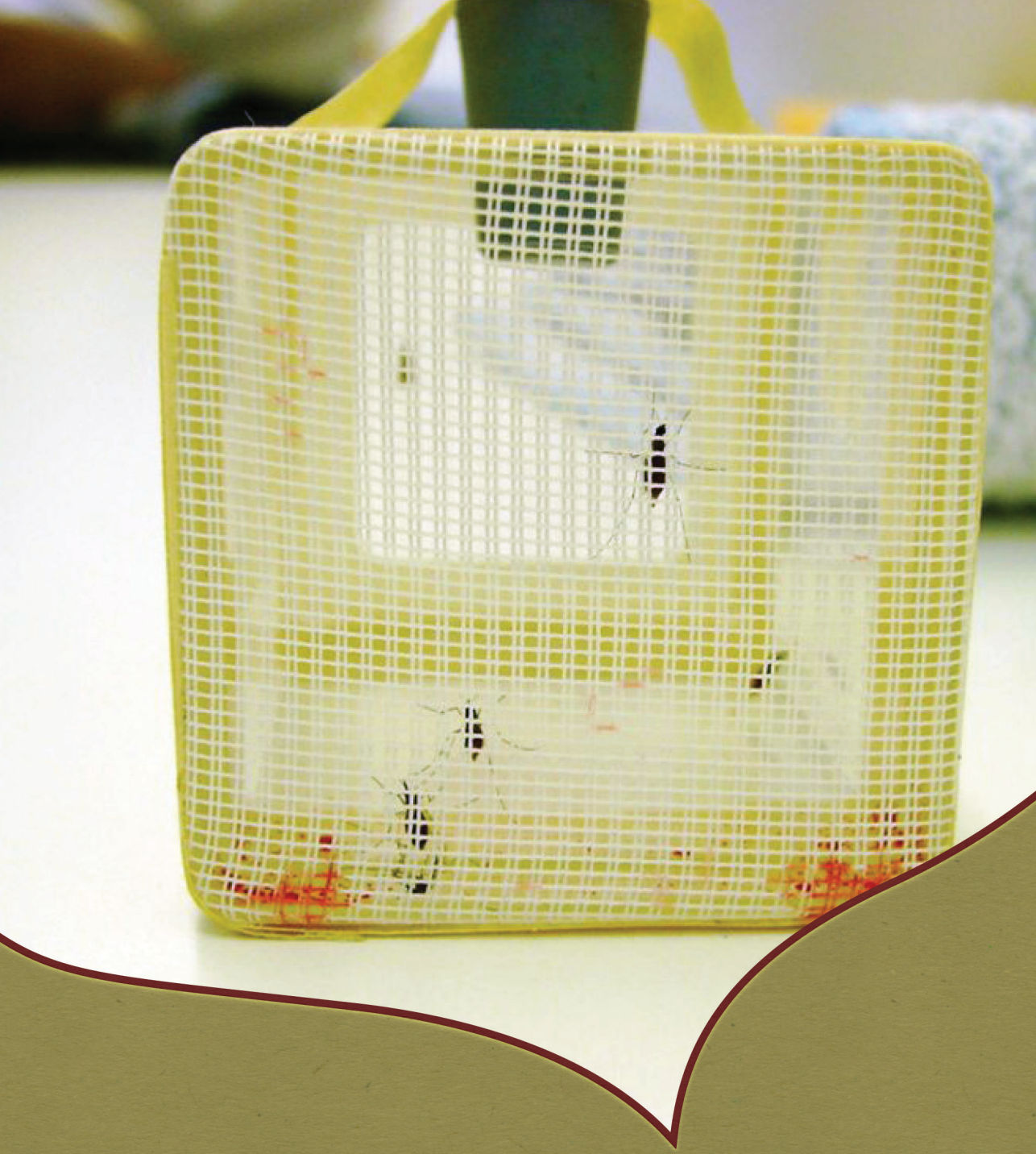
In **hoofdstuk 6** werd aan de hand van negen eerder uitgevoerde CHMI studies uitgezocht wat het optimale qPCR afkappunt is om enerzijds de klachten bij vrijwilligers zo veel mogelijk te beperken en anderzijds voldoende betrouwbare gegevens over parasietendichtheid en afweerreactie te verkrijgen. Een drempelwaarde van 100 parasieten per milliliter had een sensitiviteit van 99% en een specificiteit van 100% en resulteerde in een verkorting van tijd tot het stellen van de diagnose met 3,5 dagen gesteld in vergelijking met het dikkedruppelonderzoek.

De wereld wacht al lange tijd op een effectief malariavaccin. CPS is op dit moment echter nog niet geschikt voor grote vaccinatiecampagnes. Ondertussen wordt hard gewerkt aan een injecteerbare variant van door bestraling geïnactiveerde sporozoïeten (Sanaria *Pf*SPZ vaccine). Met dit vaccin worden fase-2 studies in verschillende Afrikaanse landen uitgevoerd. Na verdere optimalisatie zou dit vaccin mogelijk op een grotere schaal ingezet kunnen worden en effectief zijn. Daarnaast worden andere methoden voor het verzwakken van sporozoïeten uitgetest zoals door wijziging van genetische code van de malariaparasiet (genetically attenuated malaria parasite of GAP).

Tenslotte wordt het reeds bestaande vaccin RTS,S, waar in Afrika al mee gevaccineerd wordt, verder worden geoptimaliseerd met antigenen uit andere stadia van de parasiet of met andere hulpstoffen (adjuvans) om de effectiviteit daarvan te vergroten.

De implementatie van malariavaccins in malaria gebieden zal ongetwijfeld weer nieuwe uitdagingen met zich meebrengen. In veel malariagebieden zijn mensen vaak geïnfecteerd met wormen die mogelijk de effectiviteit van vaccinatie negatief zouden kunnen beïnvloeden. Daarnaast kan de introductie van een malariavaccin, dat als het niet tegen alle stammen voldoende afweer biedt, er voor zorgen dat er een selectie plaatsvindt van stammen waartegen het vaccin minder werkzaam is en mogelijk uiteindelijk hierdoor onwerkzaam wordt.

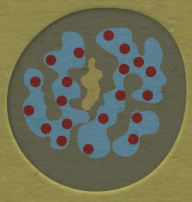
Succesvolle uitroeiing van malaria zal waarschijnlijk alleen bereikt kunnen worden door betrokkenheid en intensieve samenwerking van vele disciplines zoals artsen, parasitologen, epidemiologen en bio-statistici. De allerbelangrijkste factor is wellicht dat de lokale bevolking de vaccinatie accepteert en er een continue voldoende geldstroom zal zijn om malaria tot de laatste parasiet uit te roeien.



Netherlands. 2011. Anopheles mosquitoes infected with malaria tropica after taking a blood meal on the arms of a volunteer in the Controlled Human Malaria Infections.

CHAPTER 9

Curriculum Vitae
Publications
Dankwoord



CURRICULUM VITAE

Remko Schats was born in Eindhoven on the 25th of March 1975. He graduated from Atheneum at the Bisschop Bekkers College in Eindhoven in 1994 and started medical school at the University of Utrecht in the same year.

Already during his medical school Remko showed his immense drive for Tropical Medicine and International Health. He followed lectures in tropical medicine in his second year and this inspired him to go to Ghana for a clinical internship Surgery at the Korle Bu Hospital the following year. Afterwards, he went to Calcutta for an internship in primary health care in 1999 followed by a gynaecology internship in Suriname in 2000.

Straight after medical school he specialized into Tropical Medicine & International Health and did two years of training into surgery, gynaecology/ obstetrics and traumatology in a Dutch hospital. He finished his training at the Royal Institute for Tropical Medicine (KIT) in Amsterdam involving a three months training in tropical diseases and public health. During his entire Tropical Medicine training he was chairman of the Dutch 'tropical medicine doctors in training' (Troie) and fulfilled also several other positions within the board of The Netherlands Society for Tropical Medicine and International Health (NvTG).

He started his career as a global health doctor in 2003 and he has been on three international missions since. The first mission was located in Ghana in a rural 50-bed hospital part of a large timber company, serving both 2000 employees and their families as well as expats and people living in villages in the surrounding areas. In collaboration with GSMF Int, Pharmaccess Int and the Royal Dutch Embassy he initiated an HIV prevention and treatment programme. In 2005 he worked in Chad for Doctors Without Borders/ Médecins Sans Frontières (MSF) as the only doctor in a camp serving 17.000 refugees from Darfur bearing both the physical and mental scars of a prolonged ethnic conflict. In one episode of R_x for Survival, a PBS documentary, footage of his mission in Chad was broadcasted to gain awareness for this humanitarian crisis in Darfur. In the aftermath of the 2004 tsunami in Asia, he worked as a clinical trainer of malaria and other vector-born tropical diseases in Aceh. Indonesia was severely hit and nearly 130.000 people died in Aceh during and after the tsunami. Remko and the team of The Mentor Initiative made efforts to prevent a malaria epidemic by controlling malaria in these flooded areas.

His fieldwork, among others, was recently recognized by the NvTG and was highlighted in the media campaign 'Into the World'. A campaign to engage other physicians to work in International Health.

After returning to the Netherlands in 2007, he started the specialization to become a general practitioner (GP). Besides his work as a GP, he has contin-

ued his efforts in global health as board member for the WHIG, a platform for family medicine and international health.

During his GP specialization, he made efforts to establish a PhD research project in Urban Malaria in Ghana by himself. In joint venture with several national and international universities, he applied for funding but turned out to be difficult to achieve. Shortly afterwards, Remko started malaria vaccine research, as part of this PhD, at the Leids Universitair Medisch Centrum (LUMC) department of Infectious Diseases, in collaboration with Radboud University Nijmegen (Radboudumc). He performed clinical trials immunizing and challenging healthy volunteers with live, deadly malaria parasites by bites of malaria mosquitoes. At the annual conference of 'The American Society of Tropical Medicine and Hygiëne (ASTMH)' in 2013 his work was recognized and rewarded with the first prize of the 'Elsevier Clinical Research Award'.

Remko Schats is currently a GP and works mainly with patients having (relatively) poor access to health care: seamen, prisoners, arrested people, refugees and illegal patients. Furthermore, he continues to be involved in International Health by teaching malaria master classes and other travel medicine related topics for health care professionals throughout the Netherlands. He recently became a board member of SANO; a Dutch foundation facilitating doctors to aid in developing countries.

His work was several times covered in national and international media.

The Netherlands Society for Tropical Medicine and International Health Campaign 'Into the World':
https://www.artsinternationalegezondheidszorg.nl/throwback-thursday-21-remko-schats/
https://www.nvtg.org/uploads/MTb-PDF/2018_MT_1_IntoTheWorld.pdf

The mission in Chad:
https://www.pbs.org/wgbh/rxforsurvival/series/about/episodes.html
https://www.pbs.org/wgbh/rxforsurvival/series/champions/remko_schats_lina_gustin.html
https://www.pbs.org/wgbh/rxforsurvival/series/dispatches/pebble-dropped-pond.html

The mission in Aceh Indonesia:
https://www.rd.nl/vandaag/buitenland/i-atjehs-grote-meevaller-epidemie%C3%ABn-bleven-uit-i-1.7606

Malaria research in the LUMC:
http://archief.mareonline.nl/artikel/1011/24/0101/

During the Ebola crisis in 2014, he was interviewed by a Dutch newspaper in order to gain awareness for the urgent need of doctors in the Ebola crisis. This resulted in a front-page media coverage in 'Trouw':
https://www.trouw.nl/nieuws/net-als-anderen-heb-ik-verplichtingen-op-het-werk~bf6b07c7/
http://whig.nl/schrijnend-tekort-aan-ebola-artsen/

Radio interview:
https://www.nporadio1.nl/de-ochtend/onderwerpen/228335-stand-nl-europa-moet-meer-doen-in-de-strijd-tegen-ebola

PUBLICATIONS

Whole-blood transcriptomic signatures induced during immunization by chloroquine prophylaxis and Plasmodium falciparum sporozoites. Tran TM, Bijker EM, Haks MC, Ottenhoff THM, Visser L, **Schats R**, Venepally P, Lorenzi H, Crompton PD, Sauerwein RW.

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Ghana. July 2004.
Hospital Ward.

Ghana. June 2004.
Peer-educators trained by NGO's GSMF Int. and Pharmaccess Int. to inform other company workers and their peers about HIV and STD prevention.

Ghana. September 2004.
Generous gift from the company workers for having protected their Human Rights and initiation of an HIV-prevention programme.

Chad. April 2005.
Medical examination of a refugee at Forchana camp.

Chad. April 2005.
Health centre camp Gaga performing a vaccination campaign against measles and meningitis.

Chad. March 2005.
Footage taken for the PBS documentary
Rx for Survival: Delivering the Goods.

Indonesia, Aceh.
December 2005.
Clinical Malaria
training to local
doctors and nurses.

Indonesia. April 2006.
Malaria survey team in Aceh province.

Indonesia, Aceh. January 2006.
Taking a helicopter flight to the tsunami destroyed
and flooded region of Calang was the only means
of travel.