CLINICAL TRIAL PROTOCOL

Safety and protective efficacy of chemoprophylaxis and sporozoite immunization with Plasmodium falciparum NF135 against homologous and heterologous challenge infection in healthy volunteers in the Netherlands

CPS135

Version 7.0
Date: 3 November 2020

CONFIDENTIAL

PROTOCOL TITLE

'Safety and protective efficacy of chemoprophylaxis and sporozoite immunization with Plasmodium falciparum NF135 against homologous and heterologous challenge infection in healthy volunteers in the Netherlands' (CPS135)

Protocol ID	CPS135
Short title	Safety and efficacy of NF135 CPS immunization
ToetsingOnline nummer	NL63594.091.17
Version	7.0
Date	3 November 2020
Protocol author	Radboudumc Department of Medical Microbiology Jona Walk, MD Manon Alkema, MD Saskia van der Boor, MD
Sponsor	**Stichting Katholieke Universiteit Nijmegen**
Principal investigator	Radboudumc Department of Medical Microbiology **Matthew McCall, MD, PhD** E-mail: Matthew.mccall@Radboudumc.nl Tel : +31 (0)24 3615363
Senior Co-Investigator	Radboudumc Department of Medical Microbiology **Robert Sauerwein MD PhD** Tel : +31 (0)24 3610577 Fax :+31 (0)24 3614666

Clinical investigators	Radboudumc Department of Medical Microbiology Tel: +31 (0)24 3619515 Fax: +31 (0)24 3614666 **Saskia van der Boor, MD** E-mail: Saskia.vanderBoor@radboudumc.nl **Manon Alkema, MD** E-mail: Manon.Alkema@radboudumc.nl
Subsidising party	**PATH MVI** 2201 Westlake Avenue, Suite 200 Seattle, Washington 98121, USA
Independent expert	Radboudumc Department of Internal Medicine **Dr. Arjan van Laarhoven** Tel : +31 24 361 69 80 Arjan.vanLaarhoven@radboudumc.nl
Laboratory sites	**Radboudumc Clinical Chemical Laboratory** **Eric Aaldring** Tel: +31 (0)24 3616997 Fax: +31 (0)24 3568408 e-mail: Eric.Aaldring@radboudumc.nl **Clinical Microbiology Laboratory** **Dr. Heiman Wertheim** Tel: +31 (0)24 36 14281 e-mail: Heiman.Wertheim@radboudumc.nl
Pharmacy	**Radboudumc Clinical Pharmacy** Clinical Trial Unit **Dr. Rob ter Heine** Tel: +31 (0)24 3616405 Fax: +31 (0)24 3668755 e-mail: R.terHeine@radboudumc.nl

CRO	**CROMSOURCE** Lange Dreef 11H 4131 NF Vianen The Netherlands

PROTOCOL SIGNATURE SHEET

Name	Signature	Date
Principal Investigator ***Matthew McCall, MD, PhD*** *Department of Medical Microbiology* *Radboud university medical center*		
Investigator ***Saskia van der Boor, MD*** *Department of Medical Microbiology* *Radboud university medical center*		
Investigator ***Manon Alkema, MD*** *Department of Medical Microbiology* *Radboud university medical center*		

1. TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	**ABR form, General Assessment and Registration form, is the application form that is required for submission to the accredited Ethics Committee (In Dutch, ABR = Algemene Beoordeling en Registratie)**
AE	**Adverse Event**
A/L	**Artemether/lumefantrine (Riamet®)**
A/P	**Atovaquone/proguanil (Malarone®)**
AR	**Adverse Reaction**
CA	**Competent Authority**
CCMO	**Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek**
CV	**Curriculum Vitae**
DASS	**Depression Anxiety Stress Scales**
DSMB	**Data Safety Monitoring Board**
eCRF	**electronic Case Report Forms**
EU	**European Union**
EudraCT	**European drug regulatory affairs Clinical Trials**
GCP	**Good Clinical Practice**
IB	**Investigator's Brochure**
IC	**Informed Consent**
IMP	**Investigational Medicinal Product**
IMPD	**Investigational Medicinal Product Dossier**
METC	**Medical research ethics committee (MREC); in Dutch: medisch ethische toetsing commissie (METC)**
MINI	**Mini-International Neuropsychiatric Interview**
MVI	**Malaria Vaccine Initiative**
PATH-REC	**PATH Research Ethics Committee; the funding partner's ethical committee.**
PI	**Priniciple Investigator**
(S)AE	**(Serious) Adverse Event**
SPC	**Summary of Product Characteristics (in Dutch: officiële productinfomatie IB1-tekst)**

Sponsor	**The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.**

SUSAR	**Suspected Unexpected Serious Adverse Reaction**
Wbp	**Personal Data Protection Act (in Dutch: Wet Bescherming Persoonsgevens)**
WMO	**Medical Research Involving Human Subjects Act (in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen**
WIRB	**Western Institutional Review Board**

SUMMARY

Rationale: Malaria, a disease caused by the parasite *Plasmodium*, is one of the world's major infectious diseases. Ultimately, the key to malaria control and hopefully eradication would be an effective vaccine. Chemo-Prophylaxis and Sporozoite immunization (CPS) has repeatedly shown to be an extremely efficient regimen for induction of long lasting sterile homologous protection. However, it provided only 20% and 10% sterile protection against heterologous NF135.C10 and NF166.C8 clones, respectively. We propose to make use of the increased liver infectivity of NF135.C10, to increase the late liver stage load without the need for increasing the number of sporozoites administered. The presumably generated higher titers and broader repertoire of specific antibodies can increase heterologous protection.

Objectives

Primary Objective: To determine the safety and tolerability of NF135.C10 sporozoite immunization under chemoprophylaxis

Secondary Objectives:

- To determine the dose-dependent protective efficacy of NF135.C10 CPS-immunization against homologous controlled human malaria infection
- To determine the protective efficacy of NF135.C10 CPS-immunization against heterologous NF54 controlled human malaria infection
- To assess the longevity of protective immunity after NF135.C10 CPS-immunization against homologous challenge in cohort A

Study design and intervention: In an open label, randomized, controlled clinical trial, a maximum of 52 volunteers in two cohorts will be allocated to receive immunizations with NF135.C10 infected *Anopheles* mosquitoes. In cohort A, volunteers will receive 3 immunizations with 15 (n=10) or 5 (n=10) NF135.C10 infected mosquitoes or no immunizations (controls, n=3). Immunizations will be performed under mefloquine prophylaxis, spaced 4 weeks apart. A thick smear will be performed on days 7-9 after each immunization and symptomatic volunteers with a positive thick smear will be treated with a curative regimen of atovaquone/proguanil (A/P).

In cohort B, 20 volunteers will receive a first immunization with 15 NF135.C10 infected mosquitoes and a second immunization with 5 NF135.C10 infected mosquitoes. Volunteers will not take mefloquine prophylaxis. Instead all volunteers will be treated presumptively on day 7 after the first immunization with a curative regimen of A/L, and on day 5 after the second immunization with a curative regimen of A/P, regardless of parasitaemia or symptoms. Seventeen - nineteen weeks after the last immunization, all volunteers plus 6 naïve controls will be challenged either by the bites of 5 NF135.C10 (n=36) or 5 NF54 (n=13) infected mosquitoes. After challenge infection, volunteers will be followed up on an out-patient basis once daily for qPCR and safety lab measurements from day 6 until day 21 post challenge.

All volunteers will be treated with a curative regimen of A/P, either at the time of detection of blood stage parasitemia, or 28 days after challenge infection. If ≥50% of subjects in the immunization groups in cohort A are protected against primary challenge, protected subjects, along with 3 newly recruited controls will undergo a (second) CHMI with NF135.C10 approximately one year after the last immunization.

Study population: Healthy, malaria-naive male and female volunteers aged 18-35.

Main study endpoints

Primary study endpoint: Frequency and magnitude of adverse events after NF135.C10 CPS immunization

Secondary study endpoints:

- Time to blood stage parasitemia detectable by qPCR after malaria challenge infection
- Sterile protection after controlled human malaria infection

Exploratory study endpoints:

- The phenotype and cytokine profile of *P. falciparum* specific T cell responses induced by NF135.C10 CPS immunization
- The antigen specificity of T cell responses induced by NF135.C10 CPS immunization
- The antigen specificity and/or functionality of *P. falciparum* specific antibodies induced by NF135.C10 CPS immunization
- The phenotype and/or function of innate and semi-innate immune responses to NF135.C10 CPS immunization and/or CHMI, including γδT cells, invariant T cells, antigen presenting cells, NK cells and granulocytes
- Epigenetic profiles of innate immune cell subsets, with emphasis on both activation (H3K4me3, H3K4me1, H3K27Ac) and repression (H3K9me3, H3K27me) markers
- RNA transcriptome profiling through whole mRNA-sequencing, PCR and/or microarray

Nature and extent of the burden and risks associated with participation, benefit and group relatedness: The study is associated with several short periods of intense clinical monitoring with frequent clinic visits and blood examinations. As it is unpredictable when subjects will develop a positive qPCR after challenge, it is impossible to state the exact number of clinical visits and blood examinations. However, in groups 1 and 2 (cohort A) the number of scheduled visits will be minimally 33 and maximally 71. In groups 3 and 4 (cohort B), the number of scheduled visits will be minimally 34 and maximally 50. For naïve controls (groups 5-8) the number of visits will be minimally 9 and maximally 24. The maximum amount of blood collected will be 500mL for the immunization period and 500mL per malaria challenge infection period. Additionally, periodical physical examinations will be performed and subjects are asked to complete a diary during the periods listed in the protocol.

1. INTRODUCTION AND RATIONALE

1.1 Introduction

Malaria, a disease caused by the parasite *Plasmodium*, is one of the world's major infectious diseases. With approximately 429,000 deaths in 2015, it is both a chief cause of morbidity and mortality as well as a significant contribution to ongoing poverty in endemic countries. Most malaria deaths occur in Africa and the victims are predominately young children who have yet to develop protective immunity to the disease [1].

Human malaria is caused by five species of *Plasmodium* protozoa: *P. falciparum*, *P. vivax*, P. *ovale*, *P. malariae*, and *P. knowlesi*. Parasites (sporozoite stage) are injected into the skin by an infected female *Anopheles* mosquito. From the skin capillaries they travel via the bloodstream to the liver, where they develop and multiply in liver cells before entering the bloodstream again (merozoite stage) and invading red blood cells for further multiplication. Clinical malaria is caused only by the cyclical proliferation of asexual stages in red blood cells, the sporozoite and liver stages are asymptomatic.

The international community spends approximately $2.9 billion a year to combat malaria using insecticide-impregnated bednets (ITN), insecticides and antimalarial drugs [1]. Unfortunately widespread implementation of these interventions are hampered by poor health care infrastructure in many endemic countries. Additionally the *Plasmodium* parasites are rapidly developing resistance to antimalarial medications, including the recent development of resistance to artemisinin-based derivatives [2, 3], the current frontline treatment.

Ultimately, the key to malaria control and hopefully eradication would be an effective vaccine. Though a number of vaccine-candidates have entered the pipeline of pre-clinical and clinical development, they have yet to achieve the level of efficacy necessary for effective malaria prevention [4]. RTS,S (Mosquirix) is the first and only malaria vaccine that recently received a positive opinion from the European Medicines Agency. This sub-unit vaccine, however, confers only modest and rather short-lasting protection [5].

1.2 Rationale

Chemo-Prophylaxis and Sporozoite immunization (CPS), exposure to non-attenuated P. falciparum sporozoite- infected mosquitoes under blood stage prophylaxis, has repeatedly shown to induce long lasting sterile homologous protection in malaria- naive Dutch volunteers in the CHMI model. In three studies CPS immunization by the bites of a total of only 30-45 infected mosquitoes under chloroquine prophylaxis (3 immunizations with 15 mosquitoes per immunization, spaced 4 weeks apart) protects 19/20 volunteers (95%) [6-8].

A re-challenge of 6 CPS immunized volunteers 2,5 years after the last immunization showed that 4 out of 6 were still protected, demonstrating the lasting protective efficacy of this immunization regimen [9]. In a dose-finding study, immunization with a total of 30 and 15 infected mosquitoes protected 9 of 10 and 5 of 10 volunteers, respectively [7]. In contrast, bites from 1000 mosquitoes infected with radiation-attenuated sporozoites are required for protection [10, 11]. Sanaria, Inc, a United States biotech company, has incorporated the CPS approach in their product development portfolio as PfSPZ-CVac. GMP-compliant, cryopreserved NF54 sporozoites have been manufactured, and the first trial has been conducted in Germany, showing 100% protective efficacy against homologous challenge confirming our previous data using infected mosquito bites [12]. PfSPZ-CVac, administered by direct intravenous inoculation, is currently tested in malaria endemic areas.

Induction of protection against genetically distinct (heterologous) *P. falciparum* strains is a major stumble block and critical for understanding protective efficacy as well as for clinical vaccine development. Recently only 20% and 10% sterile protection was obtained after CPS- immunization with NF54 against heterologous NF135.C10 and NF166.C8 clones [13]. This reduced protection was reflected in the in vitro finding that plasma from CPS-NF54 volunteers less efficiently inhibited heterologous sporozoite invasion/maturation in hepatocyte cultures.

NF135.C10 and NF166.C8 sporozoites are more infectious for liver cells than NF54, indicated by a 10-100 fold higher 'first peak', parasitemia post-infection, as proxy for liver parasite load. In vitro comparison shows a 2-3 times higher invasion rate of these clones compared to NF54 and about twice the number of merozoites per schizont [14]. Therefore the observed reduced heterologous protection could at least partly be due to increased sporozoite infectivity and thus greater stringency of the challenge. However, this seems unlikely as liver cell invasion of these three clones in vitro is equipotently inhibited using serial dilutions of anti-CSP monoclonal antibodies [13].

For the future clinical development, it will be essential to improve the CPS-immunization regimen to obtain improved heterologous protective efficacy. Though the low number of heterologous protected volunteers did not allow for statistical analysis to find a correlate of protection, volunteers that were protected against a heterologous parasite challenge had relatively high antibody and/or cellular responses to *P. falciparum*, figure 1. This suggests that the decreased efficacy of CPS-induced immunity against heterologous parasites can be overcome by increasing the intensity of these immune responses[13].

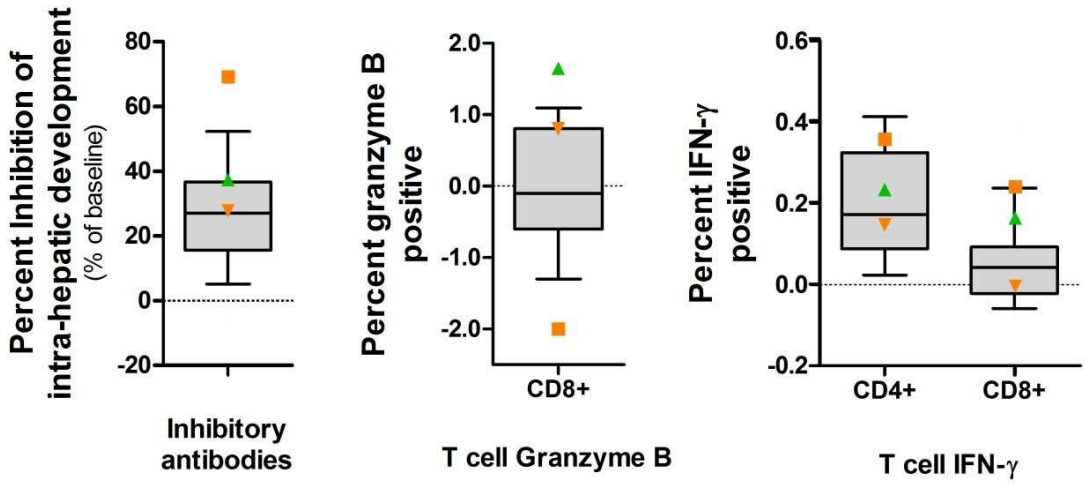

Figure 1: Analysis of *in vitro* intra-hepatic sporozoite development inhibition by CPS-induced antibodies, cellular responses and protection status *in vivo*.

CPS-induced antibody-mediated inhibition of challenge strain sporozoites (NF135.C10 or NF166.C8) *in vitro* and cytotoxic and cytokine-producing T cell responses to NF54 infected RBCs are shown. The 10th and 90th percentile of each response in all (n=19) CPS-immunized volunteers are shown as grey box-and-whisker plots. The green triangle represents the one CPS-immunized volunteer sterilely protected against NF166.C8 challenge infection, while the orange square and upside down triangle represent the two CPS-immunized volunteers sterilely protected against NF135.C10 challenge infection (Source: [13]).

This could be achieved by increasing NF54 sporozoite dose, as shown by studies using *Pf*SPZ immunization of radiation-attenuated sporozoites delivered by direct venous inoculation [15]. However, instead we propose to make use of the increased liver infectivity of NF135.C10. We postulate that using these more potent parasites will generate a far greater late liver stage load without the need for increasing the number of sporozoites administered. Presumably this will generate higher antibody titers, a broader repertoire of specific antibodies and stronger T cell responses that may be sufficient for the induction of heterologous protection.

In this study we will use the same numbers of infectious mosquito bites 5 and 15, that were used in previously published NF54 CPS immunization studies [7, 8]. In support of possible clinical development of CPS-NF135, it will be important to generate clinical data on CPS- NF135 potency as compared to CPS-NF54. Based on previous trial results with CPS-NF54, CPS-NF135 will be compared to both 3 x 15 mosquitoes (>95% homologous protection [8]), and a dose of 3 x 5 mosquitoes inducing about 50% homologous protection in the CPS-NF54 model [7]. In addition we will investigate whether CPS-NF135 can provide superior heterologous protection compared to CPS-NF54. As higher sporozoite dosages are required

for heterologous protection [16], we initially intend to use the high dose immunization regimen, 3 x 15 NF135 infected mosquitoes, in these groups.

As the NF135 strain has reduced sensitivity to chloroquine, but similar sensitivity to mefloquine as NF54 *in vitro* [17], immunization in cohort A will be performed under mefloquine prophylaxis. Mefloquine has similar activity to chloroquine and we have previously demonstrated that safety and efficacy of NF54 CPS immunization under mefloquine is similar to immunization under chloroquine [18].

1.3 Controlled Human Malaria Infection

Controlled human malaria infection (CHMI) has proven to be a valuable tool to study the pathogenesis of clinical malaria and to evaluate the efficacy of drugs and vaccines. These challenge trials have become highly standardized [19] and are an important step in the clinical development of malaria vaccines [20].

The first human malaria challenge study was performed in 1917, and since 1986, when the modern protocol using laboratory adapted *P. falciparum* strains was first performed by the US army, >3,500 subjects have been challenged by the bites of mosquitoes fed on cultures of *P. falciparum* gametocytes to produce sporozoites [21]. Infecting humans by the bite of *P. falciparum* sporozoite-infected mosquitoes is an established clinical trial methodology and is considered a reproducible, predictable and safe method of inducing *P. falciparum* malaria. The results of such studies were summarized in 1997 [22], in 2007 [23] and in 2012 [24].

The Radboud university medical center (Radboudumc) has the experience and infrastructure to conduct CHMI trials. Over 350 subjects have been enrolled in CHMIs by the Radboudumc since 2001, and the Central Committee for Research on Human Subjects (CCMO) has reviewed these protocols since 2007. Standard operating procedures according to international standards are in place for both clinical and laboratory activities, and the Radboudumc mosquito and parasite culture was positively audited in 2014. We have also developed a very sensitive method of parasite detection by real-time quantitative PCR (qPCR) in whole blood that allows us to detect malaria infection in an early stage providing early treatment and which is able to detect small differences in parasite density [25].

In two previous CHMI trials (NL48301.091.14; NL48732.091.14) qPCR was performed daily during follow-up and volunteers were treated with anti-malarial drugs after a single positive qPCR. During these two studies, 39 volunteers developed patent parasitemia. Of these volunteers 26% developed no malaria symptoms, 38% had only grade 1 symptoms (no influence on daily activities), 28% had grade 2 symptoms (some influence on daily activities) and only 13% had grade 3 symptoms (requiring bedrest). All grade 3 symptoms lasted for less than one day.

1.4 Safety Aspects

Adverse events in CHMIs:

After CHMI, most malaria-naive volunteers experience symptoms such as headache, chills or fatigue during 1-3 days. During the extensive experience with CHMI, severe or life- threatening malaria has never been reported. Real-Time Quantitative PCR (qPCR) allows for sensitive detection of low parasite densities, abrogating malaria infection in an early stage [26].

In the twenty-three CHMI studies conducted at Radboudumc prior to CPS135* (involving 376 healthy volunteers), three cardiac events have occurred after infection with the NF54 strain with the confirmed or differential diagnosis of myocarditis [27, 28]. The occurrence of cardiac events in both natural uncomplicated malaria and CHMI has not been described elsewhere. However, the three cardiac events share no specific factor other than malaria infection.
With regards to the malaria infection, these myocarditis cases do share a number of characteristics: i) they took place 1-5 days after start of antimalarial treatment but different anti-malarial drugs were used in all cases ii) there was no parasitemia detectable at the time of the event, and iii) next to the *P. falciparum* infection with NF54, other and known possibly triggering factors (e.g. preceding vaccinations, concomitant infections, cannabis use) were present during or preceding the event. More information on these SAEs is discussed in detail in section 13.

As a result of these cardiac SAEs our safety procedures for CHMI have been strongly intensified. In the current trial, we will adhere to those stringent procedures that are relevant, including:

- Individuals are excluded from participation if they have first or second degree relatives who had cardiovascular events (including ischemic events and myocarditis) under the age of 50
- A positive urine toxicology test for amphetamines, cocaine and cannabis is an exclusion criterion
- Volunteers who took standard vaccinations within 12 weeks before the start of the trial or are planning to take standard vaccinations during the trial period up to 8 weeks after CHMI are excluded from participation
- Increased control of hs troponin T as a marker of cardiac damage; treatment is initiated in consultation with the cardiologist
- Daily measurements of platelets; volunteers will be treated when platelet levels are <120x 10^9/L

Curative Malarone treatment after a single positive qPCR after CHMI to minimize period of parasitemia

* Additionally, during the first immunization of cohort B of CPS135, a fourth cardiac event occurred after immunizing with the NF135 strain. Similar to the previous three SAEs, the event took place within 1-5 days after start of presumptive antimalarial treatment and there was no parasitemia detectable at time of the event. No other triggering factors were present during or preceding the event. This case is discussed in more detail in section 13.

LFT elevations:

Transient, asymptomatic liver function derangements have been reported in volunteers in previous CHMI studies, and are likely to be related to the challenge infection [29]. A retrospective analysis of 13 CHMI studies conducted in the Radboudumc showed that 72/120 (60%) of the volunteers that were treated at thick smear parasitemia levels have mild (38%), moderate (10%) or severe (12%) asymptomatic increases of liver transaminases (ALT/AST). However, volunteers treated based on qPCR threshold of 100 *Pf*/mL showed a lower percentage and severity of LFT abnormalities. 13/58 volunteers (22%) showed LFT derangements, with 11/58 (19%) mild, 1/58 (2%) moderate, and 1/58 (2%) severe abnormalities. Importantly, all volunteers showed normalized LFT values at study end.

A clear explanation for the transient elevated transaminases in CHMI studies is not obvious. A combination of the malaria infection, antimalarial use, paracetamol use, and individual susceptibility may have triggered the observed abnormalities. Given the rapid and spontaneous resolution of the observations and the absence of significantly elevated bilirubin these events were judged to not cause permanent subclinical liver damage, and to not preclude further studies utilizing *Plasmodium falciparum* challenge. After review, the CCMO in the Netherlands has approved of further CHMI studies.

As a result of the LFT elevations our safety procedures for CHMI have been intensified. In the current trial, we will adhere to those stringent procedures that are relevant, including:

- Regular safety monitoring to assess asymptomatic liver function test abnormalities: prior to CHMI, on day 6 post-CHMI, at antimalarial treatment, two days post antimalarial treatment and 35 days post CHMI
- Avoiding additional triggers that may cause elevations in liver enzymes including alcohol from baseline up to 1 week post treatment
- A maximum dose of 3000 mg per day of paracetamol/acetaminophen
- Initiation of curative anti-malarial treatment if ALT/AST levels >5x ULN

Rationale for modifications to the first immunization phase of cohort B

During the immunization phase of cohort A, it was observed that NF135.C10 was unexpectedly insufficiently susceptible to MQ prophylaxis (see also section 7.3 Summary of findings from clinical studies) and that all subjects required rescue treatment with A/P, resulting in heterogeneous exposure to liver- and blood-stage parasites which may impact on the evaluation of induced immunity. Since no suitable alternative registered *chemoprophylactic* drug exists in the Netherlands (NF135.C10 is resistant to CQ and A/P has a pre-erythrocytic effect), an alternative is to treat all subjects presumptively with a *therapeutic* drug on day 7 after each immunization. Such a presumptive therapeutic regime results in parasite killing at almost exactly the same point in its life cycle (i.e., as soon as parasites first emerge from the liver into the blood) and thus exposure of the immune system to the same range of parasite stages, as would be expected to occur under an *effective* blood-stage prophylactic (=classic CPS) regime.

A/P itself nevertheless is unsuitable for presumptive treatment in this context, as residual atovaquone levels in cohort A subjects who received rescue treatment with A/P were so high that they plausibly interfered with liver-stage development during subsequent immunizations (see also section 7.3) and consequently with the development of protective immunity. A similar effect would be expected following presumptive treatment, even with a longer (6-week) interval between immunizations. A preferential drug for presumptive treatment is therefore A/L, which has no pre-erythrocytic effect. Considerations relating to the (cardiac) safety profile of A/L are discussed in section 13.2 (Cardiac events following Controlled Human Malaria Infections).

Since simultaneous intake of MQ and A/L is contra-indicated due to potential QT-prolongation as a result of drug-drug interactions between MQ and lumefantrine and since presumptive A/L treatment inhibits blood-stage multiplication, MQ prophylaxis should and can be omitted from the immunization regime altogether.

Rationale for modifications to the second immunization of cohort B

After the first immunization of cohort B, one volunteer developed a cardiac SAE. Although the underlying pathological mechanism remains unknown, it cannot be excluded that this may be directly or indirectly related to either the height of (the first wave of) parasitemia, or the level of systemic inflammation. Moreover, two subjects experienced recrudescent parasitemia after presumptive A/L treatment. Potential explanations could include *de novo* development of resistance *in vivo*, in particular against the lumefantrine component, and unfavorable A/L pharmacokinetics in these volunteers. The second immunized of cohort B will therefore take place with 5 instead of 15 NF135 infected mosquitoes to reduce inflammation by lowering the parasite load, whilst still boosting immunity compared to the 1st immunization. Priming with a high dose (15 mosquitoes) and boosting with a lower dose (5 mosquitoes) is a common approach in vaccinology in order to improve the quality of memory responses by mobilizing the highest quality memory cells induced by priming.

This is supported by the NF54 CQ CPS model: after a second immunization a greater proportion of subjects developed sterile immunity compared to the first immunization (59/67 vs 42/67, p<0.001). The relatively lower dose given during the second immunization is offset to an extent by the higher intrinsic infectivity of NF135.

Secondly, volunteers are treated presumptively with atovaquone/proguanil (A/P) starting on day 5 after immunization 2. A/P treatment is preferred over A/L because of i) risk of treatment failure after A/L treatment, and ii) the higher inflammatory responses after A/L treatment (potentially due to more rapid-acting mechanism) as is also illustrated by the higher number and severity of adverse events following the first immunization of cohort B compared to the 15 mosquito-bite dose arm in cohort A, despite similar parasite densities. Treatment will be initiated on day 5 to potentially block or reduce release of merozoites from the liver, thus further reducing inflammation, whilst still allowing late liver-stage development and the consequent development of pre-erythrocytic immunity. Even if merozoite release is not fully blocked, the presence of circulating drug concentrations upon merozoite release will help to ensure that intra-erythrocytic development is suppressed from the start.

Furthermore, addition follow up visits including qPCR on days 13, 16, 20 and 23 (all +/- 1 day) will be added after immunization 2, to actively screen for recrudescence (as an additional precaution, since although recrudescence of NF135 has never been observed following A/P treatment, the effect of starting treatment at day 5, instead of day 7, has never previously been formally documented).

Finally, the second immunization will be followed by a challenge at ~17-19 weeks after the second (last) immunization (allowing sufficient interval to clear residual circulating atovaquone), with 5 NF54 or NF135 infected mosquitoes. A third immunization improves protection relatively little in the NF54 CPS model and would be logistically infeasible due to the long half-life of atovaquone/proguanil following presumptive treatment.

2. OBJECTIVES

Primary Objective: To determine the safety and tolerability of NF135.C10 sporozoite immunization under chemoprophylaxis

Secondary Objectives:

- To determine the dose-dependent protective efficacy of NF135.C10 CPS-immunization against homologous controlled human malaria infection
- To determine the protective efficacy of NF135.C10 CPS-immunization against heterologous NF54 controlled human malaria infection

- To assess the longevity of homologous protective immunity after NF135.C10 CPS-immunization, if primary homologous protection against challenge in cohort A is >50%

Exploratory Objectives:

- To analyse *P. falciparum* specific T cell responses in NF135.C10 CPS-immunized volunteers
- To delineate the antibody repertoire directed against *P. falciparum* in NF135.C10 CPS-immunized volunteers
- To evaluate changes in phenotype and function of innate and semi-innate immune cells following NF135.C10 immunization
- To explore the (innate) immunology of early malaria infection, with specific attention to γδ-T cells, monocytes, antigen presenting cells and natural killer cells
- To analyse changes in epigenetic and transcriptome profiles of (innate) immune cells after NF135.C10 CPS immunization and/or after malaria infection

3. STUDY DESIGN

In an open label, randomized, controlled clinical trial, a maximum of 52 volunteers in two cohorts will be allocated to receive immunizations with NF135.C10 infected *Anopheles* mosquitoes. In cohort A, volunteers will receive 3 immunizations with 15 (n=10) or 5 (n=10) NF135.C10 infected mosquitoes under mefloquine prophylaxis, spaced 4 weeks apart, or no immunizations (controls, n=3). A thick smear will be performed on days 7-9 after each immunization and symptomatic volunteers with a positive thick smear will be treated with a curative regimen of atovaquone/proguanil (A/P). Nineteen weeks after the last immunization, all volunteers plus 3 naïve controls will be challenged by the bites of 5 NF135.C10 infected mosquitoes.

In cohort B, 20 volunteers will receive 1 immunization with 15 NF135.C10 infected mosquitoes and a second immunization with 5 NF135.C10 infected mosquitoes. Volunteers will not take mefloquine prophylaxis. Instead all volunteers will be treated presumptively on day 7 after the first immunization with a curative regimen of A/L, and on day 5 after the second immunization with a curative regimen of A/P, regardless of parasitaemia or symptoms (see considerations in protocol section 1.4). Six controls will receive no immunization. Seventeen - nineteen weeks after the second immunization, volunteers plus naïve controls will be challenged either by the bites of 5 NF135.C10 or 5 NF54 infected mosquitoes (table 1A and 1B). After challenge infection, volunteers will be followed up on an out-patient basis once daily for qPCR and safety lab measurements from day 6 until day 21 post challenge. All volunteers will be treated with a curative regimen of A/P, either at the time of detection of blood stage parasitemia (for treatment criteria see paragraph 8.3.3), or 28 days after challenge infection. All volunteers will be checked for parasites by qPCR after treatment.

If ≥50% of subjects in the groups that received NF135.C10 CPS- immunization in cohort A (groups 1 and 2) are protected against primary challenge, protected subjects along with 3 newly recruited controls (group 8) will undergo a (second) CHMI with NF135.C10 approximately one year after the last immunization. The criteria for proceeding to this second challenge are described in more detail in section 8.3.5. For cohort B (groups 3 and 4) there will not be a second CHMI. During the immunization and challenge phases, blood will also be drawn for exploratory immunology and parasitology objectives. These samples will be analyzed by Radboudumc and its collaborators. The total study period will last approximately fourteen months for cohort A (including the second challenge) and nine months for cohort B. An overview of the schedule is given in the flowchart in section 8.3.14. If one of the volunteers is not fit to participate in the study at inclusion or withdraws at any time before the first immunization, another volunteer who passed screening will be included as replacement. To allow for subjects intolerant of mefloquine to leave the study prior to the first CPS immunization without impacting the study sample size, in cohort A, three reserve volunteers will also begin mefloquine prophylaxis. If they are not used to replace a withdrawn volunteer, these reserve volunteers will stop mefloquine prophylaxis after the fourth dose.

				CHALLENGE 1					**CHALLENGE 2**
	Cohort	**Group**	**Number of volunteers**	**Immunization**		**Challenge**	**Continuation criterium**	**Volunteers**	**Challenge**
				Strain	**Mosquito #**	**Strain**			**Strain**
Immunization groups	**A**	**1**	10	NF135.C10	3 x 15	NF135.C10	if ≥50% protected	protected	NF135.C10
	A	**2**	10	NF135.C10	3 x 5	NF135.C10	if ≥50% protected	protected	NF135.C10
	B	**3**	10	NF135.C10	1 x 15 1 x 5	NF54	N/A	N/A	N/A
	B	**4**	10	NF135.C10	1 x 15 1 x 5	NF135.C10	N/A	N/A	N/A
Control Groups	**A**	**5**	3	-	-	NF135.C10			-
	B	**6**	3	-	-	NF54			-
	B	**7**	3	-	-	NF135.C10			-
	A	**8**	3	-	-	-			NF135.C10

Table 1: Study design.

4. STUDY POPULATION

4.1 Population (base)

The study population will be comprised of healthy, malaria naive male and female subjects between age 18 and 35 years old. A total of maximally 52 subjects will be enrolled to participate in the study. The investigator will ensure that all subjects being considered for the study meet the following eligibility criteria. Subject selection is to be established by checking through all inclusion/exclusion criteria at screening and baseline (inclusion visit). A relevant record of the eligibility criteria will be stored with the source documentation at the study site.

4.2 Inclusion criteria

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

1. Subject is aged ≥ 18 and ≤ 35 years and in good health.
2. Subject has adequate understanding of the procedures of the study and agrees to abide strictly thereby.
3. Subject is able to communicate well with the investigator and is available to attend all study visits.
4. The subject will remain within the Netherlands during the challenge period, not travel to a malaria-endemic area during the study period, and is reachable (24/7) by mobile telephone throughout the entire study period.
5. Subject agrees to inform his/her general practitioner about participation in the study and to sign a request to release by the General Practitioner (GP), and medical specialist when necessary, any relevant medical information concerning possible contra- indications for participation in the study.
6. The subject agrees to refrain from blood donation throughout the study period and for a defined period thereafter according to current guidelines.
7. For female subjects: subject agrees to use adequate contraception and not to breastfeed for the duration of study. Acceptable forms of contraception include: established use of oral, injected or implanted hormonal contraceptives; intrauterine device or intrauterine system; barrier methods (condoms or diaphragm with additional spermicide); male partner's sterilisation (with appropriate post-vasectomy documentation of absence of sperm in the ejaculate); true abstinence when this is in line with the preferred and usual lifestyle of the subject. Periodic abstinence (e.g., calendar, ovulation, symptothermal, post-ovulation methods) and withdrawal are not acceptable methods of contraception.

8. Subject agrees to refrain from intensive physical exercise (disproportionate to the subjects usual daily activity or exercise routine) during the malaria challenge period.
9. Subject agrees to avoid additional triggers that may cause elevations in liver enzymes including alcohol from baseline up to 1 week post treatment.
10. Subject has signed informed consent.

4.3 Exclusion criteria

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

1. Any history, or evidence at screening, of clinically significant symptoms, physical signs or abnormal laboratory values suggestive of systemic conditions, such as cardiovascular, pulmonary, renal, hepatic, neurological, dermatological, endocrine, malignant, haematological, infectious, immunodeficient, psychiatric and other disorders, which could compromise the health of the volunteer during the study or interfere with the interpretation of the study results. These include, but are not limited to, any of the following.
    1.1 Body weight <50 kg or Body Mass Index (BMI) <18 or >30 kg/m^2 at screening.
    1.2 A heightened risk of cardiovascular disease, as determined by: an estimated ten year risk of fatal cardiovascular disease of ≥5% at screening, as determined by the Systematic Coronary Risk Evaluation (SCORE); history, or evidence at screening, of clinically significant arrhythmia's, prolonged QT-interval or other clinically relevant ECG abnormalities; or a positive family history of cardiovascular events (including ischemia and myocarditis) in 1st or 2nd degree relatives <50 years old.
    1.3 A medical history of functional asplenia, sickle cell trait/disease, thalassaemia trait/disease or G6PD deficiency.
    1.4 History of epilepsy in the period of five years prior to study onset, even if no longer on medication.
    1.5 Screening tests positive for Human Immunodeficiency Virus (HIV), or active Hepatitis B Virus (HBV) or Hepatitis C Virus (HCV).
    1.6 Chronic use of i) immunosuppressive drugs, ii) antibiotics or antimalarials, iii) or other immune modifying drugs within three months prior to study onset (inhaled and topical corticosteroids and oral anti-histamines exempted) or expected use of such during the study period.
    1.7 History of malignancy of any organ system (other than localized basal cell carcinoma of the skin), treated or untreated, within the past 5 years.
    1.8 Any history severe psychiatric disease diagnosed by a psychiatrist.
    1.9 History of drug or alcohol abuse interfering with normal social function in the period

of one year prior to study onset, positive urine toxicology test for cocaine or amphetamines at screening or inclusion, or positive urine toxicology test for cannabis at inclusion.

2. For female subjects: positive urine pregnancy test at screening or at inclusion.
3. Any history of malaria, positive serology for *P. falciparum,* or previous participation in any malaria (vaccine) study.
4. Known hypersensitivity to or contra-indications (including co-medication) for use of Mefloquine, Malarone or artemether-lumefantrine, or history of severe (allergic) reactions to mosquito bites.
5. Receipt of any vaccinations in the 3 months prior to the start of the study or plans to receive any other vaccinations during the study period or up to 90 days thereafter.
6. Participation in any other clinical study in the 30 days prior to the start of the study or during the study period.
7. Being an employee or student of the department of Medical Microbiology of the Radboudumc or the department of Internal Medicine.
8. Any other condition or situation that would, in the opinion of the investigator, place the subject at an unacceptable risk of injury or render the subject unable to meet the requirements of the protocol.

4.4 Sample size calculation

This is a pilot study, with the primary objective to assess the safety and tolerability of CPS immunization with NF135.C10 in healthy volunteers. Based on previous experience with CPS immunization and convention, the study will recruit 10 volunteers per intervention group.

Cohort A – homologous protection

The (short term) homologous protective efficacy, protection after controlled human malaria infection with NF135.C10 will be compared between study groups 1 and 2, and three unimmunized controls (Cohort A). In five previous challenge studies using the NF135.C10 parasite, unimmunized volunteers became parasite positive by qPCR (if once daily sampling was performed) at a mean of 7.1 days with a standard deviation of 0.21 days. Therefore, a delay in time to parasitemia (prepatent period) of 0.5 days can be detected (α=0.05, 1-ß=0.80) in the proposed study.

Cohort B – homologous and heterologous protection

To assess (short term) homologous protective efficacy, protection after controlled human malaria infection with NF135.C10 will be compared between group 4 and three unimmunized controls (Cohort B). As described for homologous protection in Cohort A above, assuming a mean of 7.1 days with a standard deviation of 0.21 days, a delay in time to parasitemia (prepatent period)

of 0.5 days can be detected (α=0.05, 1-ß=0.80) in a group of 10 volunteers. In case of withdrawal of subjects during the study prior to challenge, a delay of 0.5 days (rounded) can still be detected in a group of at least 7 volunteers.

To asses (short term) heterologous protective efficacy, protection after controlled human malaria infection with NF54 will be compared between study group 3 and three unimmunized controls. In nineteen previous challenge studies using the NF54 parasite, unimmunized volunteers became parasite positive by PCR at a mean of 7.8 days with a standard deviation of 1.7 days. Therefore, a delay in time to parasitemia (prepatent period) of 3.5 days can be detected in a group of 10 volunteers, and of 3.8 days in a group of at least 7 volunteers (α=0.05, 1-ß=0.80).

These group sizes will provide sufficient power to detect differences in prepatency of 3.8 days (or greater). A difference in prepatent period less than ~3.8 days would mean that the heterologous protective efficacy of CPS-NF135 is lower than that of CPS-NF54 (NL48732.091.14; [1 3]).

Cohort B – total protection

The total protective efficacy of immunization against controlled human malaria infection with either NF135.C10 (homologous) and NF54 (heterologous) may be assessed and compared to 6 unimmunized volunteers. Assuming on average 50% protection in immunized volunteers and theoretical 1% protection in control volunteers, a significant difference in proportion of protected volunteers between immunized and controls can be shown with a minimum of 14 volunteers assuming α=0.05, 1-ß=0.80.

5. TREATMENT OF SUBJECTS

5.1 Investigational product/treatment

There is no investigational product in this study.

5.2 Mefloquine prophylaxis

5.2.1 Dosage

Volunteers in cohort A will receive weekly mefloquine according to standard prophylactic regimen (Source: LCR Richtlijn, March 2018). Volunteers will receive 250mg weekly starting three weeks prior to immunization, where the fourth tablet is taken the day before the first immunization, until four weeks after the last immunization. (Note: in cohort B, no mefloquine prophylaxis will be given.)

5.2.2 Contraindications

- Severely decreased liver function
- Severe haemolysis
- Hypersensitivity for quinine or related substances
- A history of depression, suicidal tendencies, anxiety disorders, psychoses, schizophrenia or other psychiatric disorders
- A history of convulsions

5.2.3 Possible side effects

The most commonly reported frequencies of side-effects of mefloquine are registered for curative and prophylactic dosages combined, however the frequencies of adverse events are lower for prophylactic dosages than curative regimens. (Source: farmacotherapeutischkompas.nl; SmPC Mefloquine)

Very frequent side effects (>10%):

- Sleeplessness
- Abnormal dreams

Frequent side effects (1-10%):

- Anxiety
- Depression
- Dizziness and vertigo
- Headache
- Vision abnormalities
- Nausea, vomiting, abdominal pain and diarrhea

- Pruritus

Recently, a Cochrane Database Systematic Review analysed the frequency of side-effects compared with other malaria prophylactic drugs, including atovaquone-proguanil and chloroquine, drugs more commonly used in CHMI studies [30]. This meta-analysis concluded that there was a very low incidence of severe adverse events with mefloquine, and no difference in severe adverse events when comparing mefloquine and chloroquine as prophylaxis. When comparing mefloquine with atovaquone-proguanil, the best estimates of absolute effect sizes for mefloquine versus atovaquone-proguanil were 6% versus 2% for discontinuation of the drug, 13% versus 3% for insomnia, 14% versus 7% for abnormal dreams, 6% versus 1% for anxiety, and 6% versus 1% for depressed mood.

5.3 Atovaquone/proguanil treatment

5.3.1 Dosage

The treatment will consist of the drug A/P. Dosing will be as follows: once daily 4 tablets of 250 atovaquone/100mg proguanil for three consecutive days according to Dutch SWAB guidelines.

5.3.2 Contraindications

- Severely reduced kidney function (creatinine clearance <30 ml/min)
- Concomittent use of metoclopramide, tetracycline, rifampicine, rifabutine, efavirenz, nevirapine or protease inhibitors

5.3.3 Possible side effects

Very frequent side effects (> 10%):

- Headache
- Nausea, vomiting, diarrhoea and abdominal pain

Frequent (1-10%):

- Allergic reactions, rash, pruritus
- Fever, anorexia
- Coughing
- Abnormal dreams, sleeplessness
- Depression
- Dizziness
- Hyponatremia, elevated liver enzymes

5.4 Artemether/lumefantrine treatment

5.4.1 Dosage

The treatment will consist of the drug A/L and will be taken on day 7 after the first immunization in cohort B only. Dosing will be as follows: 4 tablets of 20mg artemether and 120mg lumefantrine taken on T= 0, 8, 24, 36, 48 and 60 hours, according to Dutch SWAB guidelines.

5.4.2 Contraindications

- (History of) Congestive heart failure with reduced left ventricular ejection fraction.
- Symptomatic arythmia's, clinically relevant bradycardia
- Disturbances in electrolyte balance
- Prolonged QT-interval
- Concomitant use of other drugs that induce QT-prolongation (including potentially mefloquine), CYP2D6 metabolized drugs with small therapeutic window or CYP3A4 inducers.

5.4.3 Possible side effects

Very frequent side effects (> 10%):

- Headache, dizziness
- Palpitations
- Nausea, vomiting, abdominal pain
- Fatigue, asthenia, loss of appetite
- Myalgia, arthralgia

Frequent (1-10%):

- Paresthesia, clonus, walking disorder
- Insomnia
- Cough
- Diarrhea
- Skin rash, itching
- Prolonged QT interval (ECG)

5.5 Symptomatic treatment

Volunteers will be provided tripelennamine crème for the local treatment of mosquito bites.Volunteers in cohort A and during the 1st immunization of cohort B will be provided paracetamol for complaints secondary to CHMI and onset of parasitemia (fever, muscle aches,

headache, etc.). The maximum dose of paracetamol is 3 grams a day. Given their anti-inflammatory effect, volunteers in cohort B from immunization 2 onwards will be provided with NSAIDs. Should volunteers experience severe nausea, domperidon tablets or suppositories can be prescribed by the study physician. The maximum dose of domperidon is 10mg 3 times daily orally or 30mg 2 times daily rectally. Tripelennamine crème, paracetamol or any other symptomatic treatment will be supplied to the volunteers by the study physician.

6. INVESTIGATIONAL PRODUCT

Not applicable, there is no investigational product used in this study.

7. NON-INVESTIGATIONAL PRODUCT

7.1 Name and description of non-investigational product(s)

P. falciparum isolate NF135.C10 infected mosquitoes for the purpose of CPS-immunization and controlled human malaria infection.

P. falciparum isolate NF54 infected mosquitoes for the purpose of controlled human malaria infection.

7.2 Summary of findings from non-clinical studies

The *P. falciparum* NF135 strain (clone C10) was isolated from a patient visiting Cambodia [17]. In *in vitro* studies, this parasite has been shown susceptible to multiple antimalarials *in vitro*, including A/P (IC50 atovaquone 1.277nM, IC50 proguanil $2.8*10^6$nM), arthemeter/lumefantrine (IC50 artemisinin 24.36nM, IC50 lumefantrine 188.8nM) and mefloquine (IC50 115.4nM), but it has reduced sensitivity to chloroquine. The *P. falciparum* NF54 strain was isolated from a patient living in the Schiphol-area in the Netherlands. This parasite is susceptible to A/P, arthemeter/lumefantrine, mefloquine and chloroquine *in vitro*.

7.3 Summary of findings from clinical studies

Safety findings

CHMIs are well accepted as a powerful tool for the evaluation of parasite development in humans. Radboudumc has the experience and infrastructure to conduct CHMIs. Radboudumc also developed a very sensitive method of parasite detection by real-time qPCR that will allow us to detect malaria infection in an early stage providing early treatment and to detect small differences in parasite density [26]. There is a large clinical experience with infecting humans by the bite of *P. falciparum*- infected mosquitoes. Since 1986 more than 3500 volunteers have had CHMI by the bites of mosquitoes fed on cultures of *P. falciparum* gametocytes to produce *P.*

falciparum sporozoites [21]. This has proved to be a reproducible, predictable and safe method of inducing *P. falciparum* malaria. The results of such studies were summarized in 1997 [22], in 2007 [23] and in 2012 [24].

When treatment after CHMI was initiated based on thick blood smear, mild-moderate solicited adverse events were generally experienced by all study subjects post-infection, included particularly headache (95%), general malaise/fatigue (65%) and fever (90%). Gastro-intestinal complaints, including nausea, diarrhea and abdominal pain were experienced by several subjects, mainly following intake of atovaquone-proguanil. Symptoms that were severe enough to prohibit daily activities occurred in 49% of participants. When treatment was initiated based a single positive qPCR, 75% of participants experienced any solicited adverse event, but severe adverse events occurred in only 10% of participants.

Since 2007 the Radboudumc has performed CPS immunization with the NF54 parasite on healthy volunteers. A total of 62 subjects have received CPS immunization by bites from 3x 12-15 NF54-infected mosquitoes (EHMI8, NL14967.091.06; EHMI9, NL33904.091.10; ZonMw1, NL34273.091.10; BMGF1, NL48301.091.14; BMGF2b, NL48732.091.14). Combined data of 30 subjects from three studies showed that all volunteers experienced mild (possibly or probably) related adverse events (AEs) during CPS immunization, 57% (17/30) experienced moderate, and 23% (7/30) experienced severe AEs (table 2). Severe AEs were malaise, headache, fatigue, fever and myalgia. The majority of AEs occurred after the first immunization (97% of subjects), while 33% (10/30) of subjects experienced AEs after the second and third immunization (see table 2). A single volunteer in the BMGF1 study (NL4830.091.14) experienced a serious adverse event after CPS immunization, diagnosed as an asymptomatic myocarditis. This event is discussed in detail in section 1.5 and section 13.

	Immunization							
	1st		**2nd**		**3rd**		**Any**	
Grade	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
1	27	90	10	33	9	30	30	100
2	16	53	3	10	2	7	17	57
3	6	20	0	0	1	3	7	23
Any	29	97	10	33	10	33	30	100

Table 2: Subjects with AEs during CPS immunization.

This is the first study of CPS immunization with the NF135.C10 strain. However, the NF135.C10 strain has been used extensively in CHMI, with 29 volunteers receiving a challenge with 5 infected mosquitoes (TIP1, NL303050.058.09; TIP3, NL41004.078.12; BMGF2a, NL48704.000.14;

BMGF2b, NL48732.091.14). In 26 volunteers that developed parasitemia after challenge with 5 NF135.C10 (n=13) or NF54 (n=13) infected mosquitoes, there was no difference in reported adverse events or elevation of liver enzymes, table 3 and figure 2.

	Challenge			
	NF135.C10		**NF54**	
Grade	**No.**	**%**	**No.**	**%**
1	11	85	11	85
2	5	38	4	31
3	1	8	0	0
Any	11	85	11	85

Table 3: Subjects with AEs after challenge

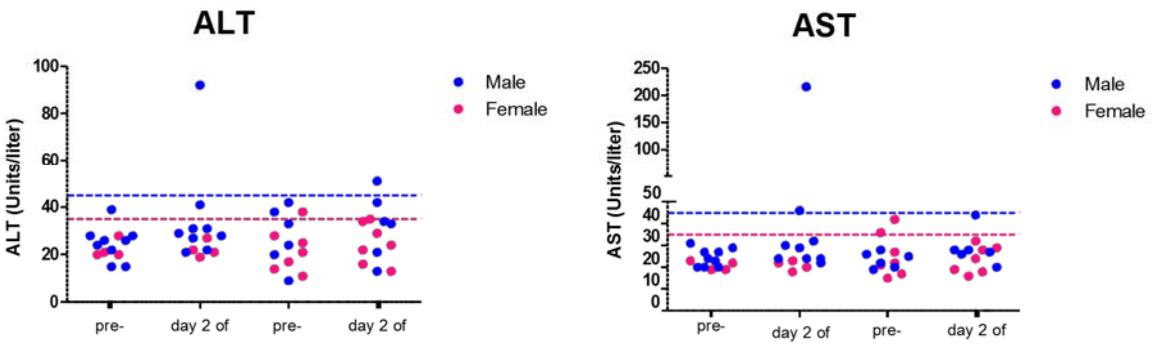

Figure 2: Graphs show the number of ALT or AST elevations in volunteers undergoing a malaria challenge infection with NF54 (n=13) or NF135.C10 (n=13), and treated at first positive qPCR (above 100 parasites per milliliter). Data from the BMGF1 (NL48301.091.14) and BMGF2b (NL48732.091.14). Dotted lines show the upper limit normal for male (blue) and female (pink) volunteers.

Efficacy findings

The *P. falciparum* isolate NF54 has been tested in 18 studies involving 251 volunteers. In these studies generally all unimmunized volunteers developed patent parasitemia with NF54. The *P. falciparum* isolate NF135.C10 has been tested in 5 studies involving 58 volunteers. In a single CHMI study (TIP1, NL30350.058.09, [17]), only 3 out of 5 volunteers developed patent parasitemia with NF135.C10. However, it is unlikely that this is an inherent characteristic of this strain. In the same study, 1 out of 5 volunteers in the NF54 group also failed to develop parasitemia, in contrast to 17 other studies when NF54 generated 100% parasitemia. Instead, technical problems with the parasite culture because of interrupted gas supply likely affected the culture medium quality. These problems have since been solved and have not recurred in the 4 subsequent studies using NF135.C10, where all volunteers challenged with 5 mosquitoes developed patent parasitemia.

In 2012 CHMI with NF135.C10, NF166.C8 and NF54 was compared in malaria naive volunteers (TIP3, NL41004.078.12). Five out of five subjects in each group developed patent parasitemia (assessed by microscopy, not by qPCR) following bites of 5 NF54, NF135.C10 or NF166.C8 infected mosquitoes, respectively; median [range] pre-patent periods for these strains were 10.5 [9.0-10.5], 7 [6.5-8.5] and 7 days [6.5-8.5] [14]. There was no statistically significant difference in frequency, duration or gradation of adverse events between the three groups.

Summary of findings from CPS135 – Cohort A

During immunization in cohort A of the CPS trial, all n=20 subjects taking mefloquine prophylaxis required rescue treatment with A/P due to positive thick smear in combination with symptoms following their first immunization and n=6 and n=9 required A/P rescue treatment following their 2nd and 3rd immunizations, respectively. MQ levels at time point of parasite release from the liver were found to be in the expected range for subjects taking MQ prophylaxis. Circulating breakthrough parasites that were re-cultured from subjects showed similar *in vitro* MQ IC50s (med 56 [range 11 – 194nM]) as compared to the parent strain used for inoculation. These results are indicative of *in vivo* resistance of NF135.C10 to MQ and of as yet unexplained discrepancy between *in vitro* IC50s and *in vivo* resistance. Moreover, residual atovaquone levels following rescue treatment and measured immediately prior to 2nd and 3rd immunization, were also considerably higher (med 54.5 ng/mL [range <20-225ng/mL]) than IC50s atovaquone for NF135.C10 liver- and blood-stage parasites *in vitro*.

Summary of finding CPS135 – Cohort B immunization 1

After the first immunization in Cohort B, two volunteers developed recrudescent parasitemia on day 19 and day 21 after treatment on day 7 with A/L. Both subjects were successfully treated with rescue

treatment (standard curative 3-day regimen of A/P per protocol), with good clinical and parasitological response (qPCR negative by 23 days post-inoculation onwards). A/L treatment failure has been reported more frequently in large European Males [31]. Drug susceptibility of the recrudescent isolate in comparison to the parent strain, as well as circulating A/L levels in these two plus the remaining Cohort B volunteers is pending.

7.4 Summary of known and potential risks and benefits

Cohort A

The risks associated with both mosquito feeding and exposure to P. falciparum are discussed below. In this part of the trial, the risk of developing clinical malaria is low, as volunteers will be taking standard mefloquine prophylaxis and the P. falciparum strain used, NF135.C10, is sensitive to mefloquine. In cohort A, thick smears (TS) will be made on day 7, 8 and 9 after each immunization (the days with highest parasitemia after immunization) in order to monitor that volunteers do not develop parasitemia. Thick smears made on day 7 and 8 will be read only of subjects are symptomatic, all thick smears on day 9 will be read regardless of symptoms. A thick smear will always be read in any volunteer with a temperature above 38.0° Celsius after any immunization. Symptomatic volunteers with a positive thick smear during the immunization phase of cohort A will be treated with A/P. In asymptomatic parasitaemic volunteers A/P treatment will be initiated in case of parasite increment, or if judged clinically prudent by the investigator.

Mefloquine is a marketed medication registered for use as a malaria prophylactic agent for *Plasmodium* strains sensitive to it. Common side effects include sleeplessness and vivid dreaming (>10%), psychiatric symptoms such as fear and depression (1-10%), headache (1- 10%) and gastro-intestinal symptoms (1-10%). In line with clinical practice, all volunteers will be screened for contra-indications in their medical and family history to mefloquine (section 5.2.2). Volunteers with contra-indications will not be given mefloquine. Furthermore, ten days after start of mefloquine, the presence of any side-effects possible related to mefloquine (section 5.2.3) will be discussed in a follow-up visit. Volunteers will be asked to complete the Depression Anxiety Stress Scales (DASS) questionnaire and 20 items on positive symptoms from the Community Assessment of Psychic Experience (CAPE) questionnaire to detect changes in emotional states of depression, anxiety, stress and psychotic symptoms. These questionnaires will be taken during subsequent follow-up visits only on indication.

Cohort B

In cohort B, all volunteers will in presumptively receive a curative treatment of A/L on day 7 after the first immunization, and A/P on day 5 after the second immunization. Daily qPCR will be

performed from day 6-10 following the first immunization, and from day 6-10, day 13, 16, 20 and 23 (+/- 1 day) after the second immunization In addition, a thick smear will be performed in any volunteer with a temperature above 38.0° Celsius or if judged clinically prudent by the investigator. Volunteers with recrudescent parasitaemia during the immunization phase of cohort B will be treated with A/P, or an appropriate alternative.

In general:

There is no benefit expected for subjects participating in this study. The risk to subjects after exposure to *P. falciparum* infected mosquitoes in this trial will be minimized by adherence to the inclusion/exclusion criteria and close clinical monitoring, which ensures that subjects with malaria are detected and treated early. The risks associated with CPS immunizations are those related to exposure to *P. falciparum* infected mosquitoes and those associated with mefloquine prophylaxis and/or treatment with A/P and A/L.

The risks of a CHMI for malaria-naïve subjects include the discomfort sustained by mosquito bites, the discomfort associated with periodic blood draws and the risk of acquiring clinical *P. falciparum* malaria.

The exposure to infected P. falciparum infected mosquito bites will occur at Radboudumc insectary which has a double-door barrier system along with a double blower (negative pressure wind blockade) to prevent flight across entryways. Mosquito bites are known to cause mild discomfort associated with mosquito feeding. A small amount of inflammation and pruritus typically accompanies the bite of the insect. Anaphylaxis to the bite of a mosquito is extremely rare and has never been reported after CHMI. While significant allergic reactions are extremely rare, in the event of an allergic reaction, epinephrine, anti-histamines, on-call physician and resuscitation equipment are available on site. The Radboudumc, an established site for CHMIs, is fully equipped to manage anaphylaxis and any other medical emergency. Frequent blood draws will be necessary to closely monitor the subjects and to perform qPCR for early detection of P. falciparum parasitemia after challenge infection. Universal precautions will be maintained for the protection of the volunteer and the study personnel during venapuncture. Throughout this study, the amount of blood collected will be maximally 500 mL during the immunization period and 500 mL during each challenge period. This amount is similar to widely accepted guidelines used by the Sanquin blood bank.

Intensive follow-up with qPCR performed on samples taken once daily will allow for detection of parasites at a very early stage. As therapy will be initiated at this early stage, dangerously high levels or prolonged duration of parasitemia that would put the subject at undue risk, will not occur. Severe malaria has never been described in a CHMI. Mild malaria symptoms include headache,

myalgia, fever, chills, sweats, nausea, vomiting, and diarrhea. Researchers at the Radboudumc have extensive experience with the care of clinical malaria. Although subjects often become symptomatic with mild malaria after CHMI, rapid diagnosis by qPCR and treatment quickly attenuates the illness so that the infection does not place the subject at undue risk. A/P, which is a marketed first-line treatment for malaria will be used to treat subjects after the second immunization in cohort B, after the challenge and in case of treatment failure of mefloquine or A/L, and is generally well tolerated. Common adverse reactions (≥5%) in adults associated with A/P treatment include abdominal pain, nausea, vomiting, headache, diarrhoea, asthenia, anorexia, and dizziness. Those volunteers intolerant of A/P will be given alternative treatment according to the national malaria treatment guidelines.

Additional information about the potential risks associated with CHMI and a summary of relevant reported Serious Adverse Events is given in section 1.5 and section 13.

7.5 Description and justification of route of administration and dosage

Study subjects will be exposed to malaria through (cutaneous) bites of P. falciparum-infected female Anopheles stephensi mosquitoes. This is the natural route of infection and the one with which most experience has been accumulated in CHMI trials. The dosages selected for this study will allow us to directly compare the relative homologous and heterologous protective efficacy of NF135.C10 CPS immunization within the context of this trial, as well as to compare this ratio to that induced by NF54 CPS immunization in previous studies.

Cohort A

Study subjects will be immunized 3 times with the bites of 5 or 15 NF135.C10 *P. falciparum* infected mosquitoes at intervals of four weeks. Experience from CHMI studies by our group has shown immunization with three monthly bites from 15 NF54 *P. falciparum* infected mosquitoes to be a very effective dosage to ensure the volunteers are fully protected against homologous challenge [6 - 8]. While immunization with three monthly bites from 5 NF135.C10-infected mosquitoes leads to 50% homologous protection [7]. In contrast, immunization with three monthly bites from 15 NF54 infected mosquitoes leads to only 10-20% protection against NF135.C10 and NF166.C8 challenge [13].

Cohort B

Subjects in cohort B will be exposed to 15 NF135.C10 infected mosquitoes after the first immunization and 5 NF135.C10 infected mosquitoes after the second immunization, an approach commonly adopted in vaccinology (protocol section 1.5).

Challenge

The gold-standard dosage for CHMI studies at the Radboudumc is five infective bites per subject, based on extensive experience with the NF54, NF135.C10 and NF166.C8 strains.

7.6 Dosages, dosage modifications and method of administration

. No dose modification will take place.

7.7 Preparation and labelling of Non Investigational Medicinal Product

The culture of *P. falciparum* parasites and infection of mosquitoes has been a routine procedure for over 20 years in the Malaria Unit of the Central Animal Facility of the Radboudumc, Nijmegen. The *P. falciparum* isolates used in this study were originally derived from patient material (see also section 6.3) and were cultured in vitro in RPMI-1640 medium with 10% serum and 5% haematocrit red blood cells. Both the serum and the red blood cells are obtained from the Nijmegen department of the Sanquin Bloedbank region Zuid-Oost. Both are negative for malaria and Hepatitis B surface Antigen (HBsAg), and seronegative for HIV, HCV, HEV, HumanT-lymphotropic Virus (HTLV) 1+2 and syphilis. The cultures are checked for bacterial, fungal and *Mycoplasma* contamination.

To produce infectious gametocytes for this study, the asexual parasites will be cultured *in vitro*. After 14 days of culture, the sexual stage parasites will be harvested for feeding to 1-5 days old laboratory cultured *A. stephensi* mosquitoes via a 'membrane feeder'. The percentage *P. falciparum*-infected mosquitoes will be assessed 6-9 days after feeding and one day prior to the CHMI.

Mosquitoes are kept in the same midi-cage from Membrane Feed for Sporozoite production (MFS) until the day before CHMI. Mosquitoes infected with different strains are kept in different midi-cages, labeled with distinguishable coloured labels identifying the study, the strain, the date of MFS, and the feeder-number. On the day before CHMI, a sample of 10 mosquitoes from each batch is checked for the presence of sporozoites and a sample of 10 mosquitoes is assessed for the average number of sporozoites per mosquito. Based on these results, the best batch is chosen for CHMI. Criteria that must be met for use of mosquitoes for CHMI are provided in the Product Information. Mosquitoes are then transferred from midi- cages to small CHMI-

cages. Each step in this process is performed by an experienced technician, and checked and recorded on standardized forms by another technician.

7.8 Drug accountability

The date, time of collection and person collecting the mosquitoes is filled in on a standard table. This form will be signed by the responsible employee.

8. METHODS

8.1 Study parameters/endpoints

8.1.1 Main study endpoint

- Frequency and magnitude of adverse events after NF135.C10 CPS immunization

8.1.2 Secondary study endpoints

- Time to blood stage parasitemia detectable by qPCR after malaria challenge infection
- Sterile protection after controlled human malaria infection

8.1.3 Exploratory study endpoints

- The phenotype and cytokine profile of *P. falciparum* specific T cell responses induced by NF135.C10 CPS immunization
- The antigen specificity of T cell responses induced by NF135.C10 CPS immunization
- The immunoglobulin repertoire and functional activity of *P. falciparum* specific sera and monoclonal antibodies induced by NF135.C10 CPS immunization and derived from plasmablasts
- The phenotype and/or function of innate and semi-innate immune responses to NF135.C10 CPS immunization and/or CHMI, including γδT cells, invariant T cells, antigen presenting cells, NK cells and granulocytes
- Epigenetic profiles of innate immune cell subsets, with emphasis on both activation (H3K4me3, H3K4me1, H3K27Ac) and repression (H3K9me3, H3K27me) markers
- RNA transcriptome profiling through whole mRNA-sequencing, PCR and/or microarray

8.2 Randomisation and treatment allocation

This is an open-label study. For logistical reasons the trial will need to be performed in two time-

separated cohorts (cohort A: group 1, 2, 5 and 8; cohort B: group 3, 4, 6 and 7).

Volunteers will be recruited separately for groups 1 and 2, groups 3 and 4, group 5, groups 6, 7, and group 8. Between these groups (i.e. group 1 and group 2), included volunteers will be randomly assigned. Volunteers will be allocated to a group according to a randomization list derived from the Microsoft Excel command ASELECTUSSEN (0,1000). Every volunteer number will be linked to a random number between 0 and 1000. For example, the ten lowest numbers will be assigned group 1, and the ten highest numbers will be assigned to group 2. If two identical numbers are produced, the whole procedure is repeated. One of the investigators at the Radboudumc will be responsible for performing the randomization and for assigning volunteers according to the randomization list. A second employee, not involved in the assignment of volunteers, will check to see if randomization is done correctly. The master randomization list is kept in a fireproof clinical trial cabinet at the Radboudumc Malaria Unit.

8.2.1 Baseline screening

Subjects who sign informed consent will undergo complete screening including a patient history, physical examination, vital signs and laboratory evaluations (see sections 8.3.6- 8.3.9). If physical examination, vital signs or laboratory values are out of the normal range a repeat measurement may be obtained.

Following a screening period of up to 120 days, subjects who meet the eligibility criteria (Section 4.2 and 4.3) will present to the study site for baseline assessments. Patient history will be taken and all adverse events that have occurred since screening will be noted. Only subjects who still meet the inclusion criteria will be included into the study. Subjects that wish to participate in cohort A will be screened for contra-indications to mefloquine. Volunteers with contra-indications to mefloquine (section 5.2.2) will not be allowed to take mefloquine and will therefore not be included in an immunization group in cohort A. For each subject in cohort A, study start will be defined as the day of the first dose of mefloquine prophylaxis. In cohort B, mefloquine prophylaxis will not be a part of the immunization procedure, therefore study start (inclusion) will be defined as the day before the first immunization.

Mefloquine prophylaxis has been associated with mild side effects including vivid dreams, anxiety, headache and gastro-intestinal symptoms in 1-10% of individuals (see also section 5). In most cases (around 80%) these side effects present during the first two to three weeks of use. For this reason, ten days after start of mefloquine, the presence of any side- effects possible related to mefloquine (section 5.2.3) will be discussed in a follow- up visit and volunteers will be asked to complete the DASS questionnaire and 20 items on positive symptoms from the CAPE questionnaire to detect changes in emotional states of depression, anxiety, stress and psychotic symptoms. To allow for subjects intolerant of

mefloquine to leave the study prior to the first CPS immunization without impacting the study sample size, three reserve volunteers will also begin mefloquine prophylaxis. If they are not used to replace a withdrawn volunteer, these volunteers will stop mefloquine prophylaxis after the fourth dose. They will be compensated with 100 euros and offered a chance to participate in the challenge infection in study groups.

8.2.2 CPS immunization with mosquito bite

On the first day of the study, subjects in cohort A will be seen by the investigators to initiate mefloquine prophylaxis. Volunteers that will undergo CPS immunization in cohort A (groups 1 and 2) will receive 250mg mefloquine once a week according to a standard prophylactic regime. They will receive four doses prior to the first CPS immunization (on day 22). Volunteers will continue mefloquine prophylaxis throughout CPS immunization and for four weeks after the last immunization, for a total duration of 16 weeks. In cohort B mefloquine prophylaxis will not be a part of immunization procedures, for subjects in this cohort the day before the first immunization will mark the first day of the study.

For subjects in cohort A, sufficient prophylaxis is a requirement for receipt of the next immunization and will be evaluated prior to each immunization. Compliance with the prophylaxis is monitored at all study visits. Additionally, study subjects will receive a weekly reminder via either e-mail or text message. Twenty-two days after initiation of mefloquine prophylaxis, subjects of cohort A will receive CPS immunization with bites from 5 or 15 NF135.C10 *P. falciparum* infected *A. stephensi* mosquitoes, depending on allocation (see section 3). This procedure will be repeated three times at four week intervals. CPS immunizations of cohort B will consist of bites from 15 (immunisation 1) and 5 (immunisation 2) NF135.C10 *P. falciparum* infected *A. stephensi* mosquitoes.

On each of the days of immunization, the volunteers will be exposed to the bites of 5 or 15 NF135.C10 *P. falciparum* infected *A. stephensi* mosquitoes. Mosquitoes will be prepared by technicians of the Radboudumc malaria unit and placed in identical boxes, numbered to correspond with the participant's study code. Treatment allocation will not be blinded. The infections will be performed by placing a box containing mosquitoes on the forearm of the volunteer. (For more information concerning the production of mosquitoes, please refer to section 'Mosquito preparation' under 6.3.5). Directly after the feed, the mosquitoes will be dissected by a technician of the mosquito unit. This will be done to assure that the mosquito has fed and the presence of sporozoites in the salivary glands of the mosquitoes. Exposure will be repeated until the exact number of infected mosquito bites has been reached. In a previous study, the prophylactic dose of mefloquine was sufficient to overcome bites of 15 NF54 infected mosquitoes [18], and the *in vitro* sensitivity of NF135.C10 to mefloquine is similar to NF54 (see

Product Information).

As long as there are volunteers present in the mosquito unit, there will be supervision by one of the clinical investigators. Another clinical investigator will be on call, in case of emergency. Emergency aid kits will be present and readily available at any location, whenever there are volunteers present.

All volunteers in cohort A and B will be seen by the trial clinicians on days 6 – 10 after each immunization for safety assessments (see flow chart, section 8.3.14). Additionally, volunteers of cohort B will also be seen on 13, 16, 20 and 23 (all +/- 1 day) for follow-up visits including qPCR after the second immunization.

For cohort A, to ensure no break-through infections occur under mefloquine prophylaxis, on day 7 and 8 after each immunization blood will be taken for thick smears and only immediately read in subjects with symptoms that could be related to parasitemia. Additionally, blood will be taken for thick smears and read on day 9 after each immunization. A thick smear will also be performed in any volunteer with a temperature above 38.0° Celsius after immunization. If a symptomatic subject develops a positive thick blood smear at any time point after CPS immunization, the subject is immediately treated with A/P, during three days (see also section 8.3.3). Should an asymptomatic volunteer be blood smear positive on day 9, the day 7 blood smear will be read to detect if parasitemia is increasing. In asymptomatic parasitaemic volunteers A/P treatment will be initiated in case of parasite increment, which is defined as two consecutive positive thick smears of which the second thick smear has a higher parasitemia than the first, or if judged clinically prudent by the investigator. If there is no increase, the volunteer will continue with regular follow-up. Further blood smears may be performed in volunteers with a positive thick smear without treatment. If a treatment with A/P is required during the immunization phase, the subject will continue with the next immunization(s) and a blood sample will be taken to retrospectively determine atovaquone levels at the time of the subsequent immunization.

Subjects that will receive immunizations in cohort B (groups 3 and 4) will not receive mefloquine prophylaxis. Instead, subjects in cohort B will be treated presumptively with a curative regimen of A/L on day 7 after the first immunization, and A/P on day 5 after the second immunization. To ensure treatment is adequately initiated, the first dose of A/L or A/P will be taken under direct observation by one of the study clinicians during the study visit on day 7 or day 5, respectively. Treatment effectiveness will be monitored by performing daily qPCR on day 6 through day 10 after immunization 1 and additionally day 13, 16, 20 and 23 after the second immunization. Should volunteers develop a temperature above 38.0° Celsius before day 7, or when judged clinically prudent by the investigator, a thick smear will be performed. If the thick smear is positive, the volunteer will start with a curative treatment of A/P, or an appropriate alternative.

During the entire study period all subjects of cohort A and B will be instructed to call the trial physicians at any time if they experience symptoms. The trial physician can at all times decide

to initiate any additional diagnostics (including laboratory safety evaluations and/or diagnostics for malaria parasites) considered clinically indicated. Blood will be drawn for retrospective qPCR on day 6 through 10 after each immunization in each cohort. Subjects in cohort A will continue mefloquine prophylaxis until four weeks after the last CPS- immunization.

8.2.3 Controlled Human Malaria Infection after drug washout (challenge)

Seventeen - nineteen weeks after the last CPS immunization, all subjects from group 1, 2, 3 and 4, plus nine newly recruited subjects in group 5, 6 and 7, will undergo malaria challenge infection.

On the challenge day, all subjects will be exposed to the bites of five NF135.C10 or NF54 strain *P. falciparum* infected mosquitoes. Mosquito feeding will be allowed for 10 minutes. Volunteers may receive a local treatment (tripelennamine crème) for mosquito bites and will be observed for 15 minutes after the feed. Directly after the feed, the mosquitoes will be dissected by a technician of the mosquito unit as described above in section 7. Exposure will be repeated until five infected mosquitoes have fed on each volunteer.

As long as there are volunteers present in the mosquito unit, there will be supervision by one of the clinical investigators. Another clinical investigator will be on call, in case of emergency. Emergency aid kits will be present and readily available at any location, whenever there are volunteers present.

After malaria challenge infection subjects will be observed closely according to an intensive out-patient follow-up schedule including frequent safety analyses (see section 8.3.9 for details). From the sixth day until the twenty-first day post-CHMI, assessments of parasite densities using qPCR will be performed once daily. Subjects will also visit the clinical site for a follow-up visit on day 1, 2 and 3 after treatment (TD+1, 2 & 3). Between day 21 and day 28 post challenge, volunteers will be seen only if they have symptoms. All subjects will be seen for a final control visit on day 35 after CHMI. The study design is illustrated in more detail in the flowchart in section 8.3.14.

qPCR assessment of parasite densities will be performed directly in volunteer samples. As soon as a qPCR is deemed positive for malaria parasites, the technician will inform the trial clinician. Treatment will be initiated after a single positive qPCR. If treatment has to be initiated, the trial clinician will contact the volunteer who will return to the clinic to receive A/P treatment.

For unexpected laboratory abnormalities during the study, the laboratory test will be repeated. Any elevation of troponin-T outside normal limits will be discussed with the clinical supervisor. If the elevation is judged to be significant, a cardiologist is consulted and a full cardiac work up is done according to standard medical practice. If there is any ambiguity regarding the decision to include or exclude a volunteer, the study physician or the clinical supervisor will discuss the case with the local safety monitor and make the final decision after that, if necessary with consultation of a specialist. If volunteers prove to be eligible, they will be

invited to the next visit.

All subjects who are exposed to *P. falciparum* will be treated with A/P either upon positive qPCR for malaria or on day 28 after the malaria challenge infection. Additionally, treatment can be initiated in any of the following situations:

1. By decision of study doctor or the safety monitor
2. On request of the volunteer
3. In consultation with the cardiologist
4. When thrombocytes <120 x 10^9/L
5. When ALT/AST levels >5x ULN

8.2.4 Treatment with atovaquone/proguanil after challenge

After the challenge, all volunteers from cohort A and B will be treated with A/P based on the predetermined criteria mentioned above.

The treatment will consist of the drug A/P. Dosing will be as follows: once daily 4 tablets of 250 atovaquone/100mg proguanil for three consecutive days according to Dutch SWAB guidelines. This drug has been chosen because of its fast clinical response and the few side-effects. Furthermore, it has not been reported to have any cardiac side-effects. During treatment, complaints of malaria infection will be treated symptomatically. In addition to specific treatment with A/P, symptomatic treatment will be administered at the discretion of the study physician.

After the challenge, during and one day after A/P treatment, qPCR is performed directly in collected blood samples. If qPCR remains positive on day three after A/P treatment (usually the result of parasite debris remaining in the bloodstream) a thick blood smear may be performed to confirm the absence of intact malaria parasites and the volunteer will receive additional follow-up to confirm decrease in parasitemia Volunteers will not be admitted to the hospital during this study, unless the study doctors or the safety monitor deems it necessary, or on request of the volunteer.

8.2.5 Re-challenge infection

One year after the last CPS immunization, immunized volunteers of cohort A may undergo a second challenge infection to assess the duration of homologous protection. In each of the immunized groups of cohort A (1 and 2), a re-challenge will only be performed if at least 50% of the volunteers of that group were protected against the primary challenge. To assess the duration of homologous protection, volunteers in group 1 and 2 that were protected against primary challenge will receive a second challenge with NF135.C10. Previous studies using radiation-attenuated sporozoites have not shown a boosting effect of a homologous challenge infection [15, 16]. Three new malaria naïve infection controls will be recruited for group 8 (n=3), and will be challenged with NF135.C10. There will not be a second challenge infection for cohort B (groups 3 and 4).

8.2.6 Physical examination

A complete physical examination will include the examination of general appearance, skin, neck, eyes, throat, lungs, heart, abdomen, back and extremities, and a routine vascular and neurological examination.

Height (cm) and body weight (to the nearest 0.1 kilogram [kg] in indoor clothing, but without shoes) will be measured. Body mass index (BMI) will be calculated using the following formula: BMI = Body weight (kg) / [Height (m)]^2 and converted to an integer.

8.2.7 Vital signs

Vital signs including body temperature, blood pressure (BP) and pulse measurements will be determined and recorded at set time points during the study. Systolic and diastolic BP will be measured while the subject is sitting, with back supported and both feet placed on the floor, using an automated validated device, with an appropriately sized cuff. In case the cuff sizes available are not large enough for the subject's arm circumference, a sphygmomanometer with an appropriately sized cuff may be used.

If vital signs are out-of-range at baseline, the investigator may obtain two additional readings, so that a total of up to three consecutive assessments are made, with the subject seated quietly for approximately five minutes preceding each repeat assessment. At least the last reading must be within the normal range in order for the subject to qualify.

Temperature will be measured according to local practice, consistently throughout the study. The thermometer used should have a precision of 0.1ºC. The same route should be used throughout the study.

8.2.8 Electrocardiogram

A standard 12 lead ECG will be performed at screening. Additional ECG assessments may be performed at any time throughout the study at the discretion of the investigator.

All assessments will occur after the subject has rested for approximately 10 min in the supine position. Calibration should be performed per the local site/requirements. Each ECG tracing should be labeled with the subject number and date, and kept in the source documents at the study site. Interpretation of the tracing must be made by a qualified physician and documented in the Case Report Form (CRF). Minimally, the CRF will contain date and time of ECG, heart rate, PR interval, QRS duration and QT interval (corrected). Clinically significant abnormalities should also be recorded in the CRF.

8.2.9 Blood sampling and safety laboratory evaluations

During the study, blood samples will be drawn for screening, safety and research purposes. The

blood sampling schedule in the flowchart (section 8.3.14) indicates the timing and volume of blood drawn.

Regular hematology safety laboratory evaluations will include: hemoglobin, hematocrit, red blood cell count, white blood cell count with differential (e.g. neutrophils, basophils, eosinophils, monocytes, lymphocytes) and platelet count.

Biochemistry safety laboratory evaluations includes, alkaline phosphatase, total bilirubin, creatinine, γGT, LDH, potassium, AST, ALT, sodium, highly sensitive troponin T and urea. Glucose, triglycerides and cholesterol will be measured only at screening.

At the screening visit plasma samples will be tested for HIV, hepatitis B surface antigen and hepatitis C. Additionally plasma will be tested for reactivity to malaria parasites by ELISA.

A midstream urine sample (approx. 30 ml) will be obtained at screening and the day before the study start. In this sample the presence of cannabis, amphetamines and cocaine will be assessed. Additionally, for female participants a commercially available hCG urine test will be used to test for pregnancy. HCG urine tests will also be obtained prior to each immunization and prior to CHMI 1 and 2.

If a laboratory assessment is outside the reference range for the laboratory, a decision regarding whether the result is of clinical significance shall be made by the investigator and shall be based, in part, upon the nature and degree of the observed abnormality.

In all cases, the investigator must document in the source documents, the clinical considerations (i.e., result was/was not clinically significant and/or medically relevant) in allowing or disallowing the subject to continue in the study.

8.2.10 Analysis of parasite densities after challenge infection

qPCR for assessment of parasite densities will be performed directly in volunteer samples, as discussed previously (see section 8.3.3).

qPCR is performed according to a standard procedure described previously [26, 32]. In short, qPCR will be performed on the multicopy 18S ribosomal RNA gene. All samples are spiked with the extraction control Phocine Herpes Virus (PhHV) to determine efficacy of DNA isolation.

Thick smears will be performed during immunization, and at any time it is deemed necessary by the clinical investigator (see section 8.3.3), according to a standard operating procedure which is based on an internationally harmonized protocol for thick smears in CHMIs (Moorthy et al., 1998) and (WHO, 2010). Per slide, the number of fields correlating to 0.5 µl of blood will be read. Slides are considered positive if they contain 2 or more parasites in these fields.

8.2.11 Case report forms and data collection

All data collected by the investigators is registered in electronic case report forms. The investigator's notes are collected in subject study files and are considered source data. Since all

subjects will be healthy, there is no medical file for the study subjects, with exception of the medical file in case of adverse events/reactions resulting in a medical consultation or hospitalization. In this case the medical file will also be considered as the source data. The diaries, produced by the study volunteers are also considered source data. They will be kept as source documents together with the investigator's notes.

8.2.12 Patient-reported outcomes (study diary)

At the inclusion visit, subjects will be issued symptom diaries and thermometers. They will be asked to record all symptoms and medication use daily from the day of inclusion until day 35 after the malaria challenge infection. The subject diary will be reviewed at each study visit and used as a basis for discussion of possible adverse events or medication use. If the occurrence of an adverse event or use of medication is confirmed by the study physician, it is recorded in the subject's study file (see also: section 9.4.1).

Subjects will also be asked to measure their temperature orally every morning and record this temperature in their study diaries from: day 6 until day 10 after each immunization (cohort A), from day 6 until day 23 after each immunization (cohort B), and from the day of the malaria challenge infection until the third day of A/P treatment, (challenge cohort A and cohort B).

At the end of the study, the diary will be collected and kept as source data with the subject's study file.

8.2.13 Exploratory immunological assessments

Blood will be drawn for exploratory immunological assessments at specific time points (see section 8.3.14). From these samples, assays can be performed in whole blood and/or peripheral blood mononuclear cell (PBMC) populations and plasma can be isolated. Samples can be analysed for phenotype or functionality directly, or PBMCs and plasma can be frozen for possible later analyses by the Radboudumc or its collaborating partners as described in the exploratory objectives (section 2). If necessary to assess (antigen specific) T cell responses, the HLA-type of volunteers may be determined from PBMC samples.

Phenotype, cytokine profile and function of immune cells can be measured by intracellular flow cytometry or cytokine specific ELISA or multiplex assays. T cell, B cell antibody antigen specificity can be determined by protein or peptide library screening or by B- or T cell receptor sequencing. RNA transcription levels can be analyzed by specific gene RNA-PCR or total mRNA sequencing. Epigenetic profiles can be investigated using Chromatin Immunoprecipitation (ChIP) followed by sequencing or specific gene PCR.

8.2.14 Flow chart study design

COHORT A

	Screening visit	Inclusion/ controle mefloquine	Mefloquine Check	First immunization			Second immunization			Third immunization			
Visit Number	V1	V2	V3	V4	V5	V6-10	V11	V12	V13-17	V18	V19	V20-24	V25
Reference timepoints				I-1	I1	I1+6 – I1+10	I2-1	I2	I2+6-I2+10	I3-1	I3	I3+6- I3+10	
Number of visits	1x	1x	1x	1x	1x	1x / day	1x	1x	1x / day	1x	1x	1x / day	1x
Eligibility criteria + Demographic data[1]	X												
Physical examination	X	X		X[7]		X[7]	X[7]		X[7]	X[7]		X[7]	
ECG	X												
Temperature	X	X		X		X	X		X	X		X	
Immunization with NF135.C10-infected mosquitoes					X			X			X		
Mefloquine prophylaxis		X	X	X	X	X	X	X	X	X	X	X	
A/L treatment						X[14]			X[14]			X[14]	
Haematology tests[2]	X	X		X		X	X		X	X		X	X
Biochemistry tests[4]	X (5 ml)	X (3 ml)											
HsTropT + LDH only				X		X	X		X			X	
Serology[5]	X (5 ml)	X (3 ml)											
Parasitology[6] (2.0ml)				X		X	X		X	X		X	
Exploratory immunology													
Citrate plasma[10]				I1-1		I1+6; I1+7; I1+10	X		I2+6; I2+10	X		I3+7	X
PBMC for cellular studies				I1-1: 80mL		I1+6: 24 ml; I1+7: 24mL;	I2-1: 40mL		I2+6: 24 ml; I2+10: 24mL	I3-1: 32ml		I3+7: 24mL	I3+28: 40ml
PBMC for plasmablasts				I1-1		I1+6 I1+10	I2-1		I2+6; I2+10	I3-1			
Whole-blood transcriptomics (2.5 ml)				X		D28, D32	X		D56, D60	X			
Pregnancy and Urine Toxicology	X	X		X			X			X			

Supply diary		X											
Reviewing diaries				X		X	X		X	X		X	X
A/P or A/L blood levels[12]							X[12]			X[12]			
Mefloquine blood levels[13]						X[13]			X[13]			X[13]	
Blood Draw Estimate	12 ml	8 ml		88.5 ml		112.5mL	54mL		83mL	46 ml		94mL	
Cumulative Blood Drawn	12 ml	20 ml		108.5mL		221mL	275mL		358 ml	404ml		498 ml	498ml

[1] Visit may be performed 1-3 days earlier
[2] Hemoglobin, hematocrit, platelets, white blood cell count + differentiation
[4] Creatinin, urea, sodium, potassium, bilirubin, AF, γGT, ASAT, ALAT, additional at screening: cholesterol + glucose
[5] Screening: HIV, HBV, HCV, *P. falciparum* (5.0ml). Inclusion: HIV, HBV, HCV (3.0ml)
[6] Immunization: retrospective qPCR day 6-10; Cohort A: thick smear day 7-9
[7] On indication.
[8] After one positive qPCR
[10] Taken from the same tubes as PBMCs
[11] Only if treated after positive qPCR
[12] 1 EDTA tube (2ml) and 1 serum tube (3,5mL)
[13] Mefloquine samples may be collected from volunteers requiring rescue treatment after immunization (cohort A): 1 EDTA tube (2ml) and 1 serum tube (3,5mL)

COHORT B

	Screening visit	Inclusion	First immunization			Second immunization		
Visit Number	V1	V2	V4	V5	V6-10	V11	V12	V13-17
Reference timepoints			I-1	I1	I1+6 – I1+10	I2-1	I2	I2+ (6-10); I2+13, I2+16, I2+20; I2+23[13]
Number of visits	1x	1x	1x	1x	1x / day	1x	1x	1x / day
Eligibility criteria + Demographic data1	X							
Physical examination	X	X	X[7]		X[7]	X[7]		X[7]
ECG	X							
Temperature	X	X	X		X	X		X
Immunization with NF135.C10-infected mosquitoes				X			X	
Presumptive treatment (A/L or A/P)					X[14]			X[14]
Haematology tests3	X	X	X		X	X		X
Biochemistry tests4	X (5 ml)	X (3 ml)						
HsTropT + LDH only			X		X	X		X
Serology5	X (5 ml)	X (3 ml)						
Parasitology6 (2.0ml)			X		X	X		X
Exploratory immunology								
Citrate plasma10			I1-1		I1+6; I1+7; I1+10	X		I2+6; I2+10
PBMC for cellular studies			I1-1: 80mL		I1+6: 24 ml; I1+7: 24mL; I1+10: 24 ml	I2-1: 40mL		I2+6: 24 ml; I2+10: 24mL
PBMC for plasmablasts			I1-1		I1+6 I1+10	I2-1		I2+6; I2+10
Whole-blood transcriptomics (2.5 ml)			X		D28, D32	X		D56, D60
Pregnancy and	X	X	X			X		
Supply diary		X						
Reviewing diaries			X		X	X		X
A/P or A/L blood levels12						X[12]		
Blood Draw Estimate	12 ml	8 ml	88.5 ml		74,5mL	48,5mL		91mL

Cumulative Blood Drawn	12 ml	20 ml	108.5mL		215,5mL	264mL		355 ml

[1] Visit may be performed 1-3 days earlier
[2] Hemoglobin, hematocrit, platelets, white blood cell count + differentiation
[4] Creatinin, urea, sodium, potassium, bilirubin, AF, γGT, ASAT, ALAT, additional at screening: cholesterol + glucose
[5] Screening: HIV, HBV, HCV, *P. falciparum* (5.0ml). Inclusion: HIV, HBV, HCV (3.0ml)
[6] Immunization: prospective qPCR day 6-10, day 13, day 16, day 20 and day 23 (+/- 1 day)
[7] On indication.
[8] After one positive qPCR
[9] Group 5-8 only
[10] Taken from the same tubes as PBMCs
[11] Only if treated after positive qPCR
[12] 1 EDTA tube (2ml) and 1 serum tube (3,5mL)
[13] on day 13, 16, 20 and 23 after second immunization follow-up for prospective qPCR and adverse events
[14] Subjects of Cohort B will be treated presumptively on day 7 after the first immunization with A/L or on day 5 after the second immunization with A/P.

	CHMI							**End of study visit**
	Number of visits fixed		Number of visits depending on parasitemia	Number of visits fixed				
Visit Number	V26[1]	V27	V28-43	V44	V45	V46	V47	V48[1]
Trial timeline (D=day)	C-1	C	C+6 – C+21	TD[8]	TD[8]+1	TD[8]+2	TD[8]+3	End of Study
Number of visits	1x	1x	1x / day	1x	1x	1x	1x	1x
Eligibility criteria + informed consent								
Demographic data, medical history								
Physical examination and vital signs	X		X[7]	X[8]	X[7]	X[7]	X[7]	X[7]
ECG								
Temperature	X		X	X	X	X	X	X
Immunization with NF135.C10-infected mosquitoes								
Challenge with 5 infected mosquitoes		X						
Mefloquine prophylaxis[2]								
A/P treatment				X	X	X		
Haematology tests[3] (2.0ml)	X		X	X	X	X	X	X
Biochemistry tests[4]	3 ml		C+6: 3 ml	3 ml		3 ml		3 ml
HsTropT + LDH only (2.0ml)	X		X		X		X	
Serology[5]	X[9]							
Parasitology[6] (2.0ml)	X		X	X			X[6]	X
Exploratory immunology								
Citrate plasma[9]	X				X		X	X
PBMC for cellular studies	C-1: 80				TD:80 ml[11]		TD+3:80 ml[11]	80 ml[11]
PBMC for plasmablasts								
Whole-blood transcriptomics (2.5 ml)	X				X		X	X
Pregnancy and toxicology urine test	X							
Supply diary	X[9]							
Reviewing diaries	X		X	X	X	X	X	
Mefloquine blood levels[12]	X							
Blood Draw Estimate	95 ml		C+6: 7 ml rest: 6 ml	7 ml	88.5 ml	7 ml	88.5 ml	88.5 ml
Cumulative Blood Drawn	95 ml		192 ml	199 ml	287,5 ml	294,5 ml	383 ml	472.5 ml

[1] Visit may be performed 1-3 days earlier
[2] Groups 1-4 only
[3] Hemoglobin, hematocrit, platelets, white blood cell count + differentiation
[4] Creatinin, urea, sodium, potassium, bilirubin, AF, γGT, ASAT, ALAT, additional at screening: cholesterol + glucose
[5] Screening: HIV, HBV, HCV, *P. falciparum* (5.0ml). Inclusion: HIV, HBV, HCV (3.0ml)
[6] Immunization: retrospective qPCR day 6-10; Cohort A: thick smear day 7-9; Cohort B: thick smear day 10 (end of treatment); Challenge: prospective qPCR daily

[7] On indication.
[8] After one positive qPCR
[9] Group 5-8 only
[10] Taken from the same tubes as PBMCs
[11] Only if treated after positive qPCR
[12] Mefloquine will be collected prior to the challenge in all immunized volunteers: 1 EDTA tube (2ml) and 1 serum tube (3,5mL)

	CHMI2							
	Number of visits fixed		Number of visits depending on parasitemia	Number of visits fixed				
Visit Number	V49[1]	V50	V51-66	V67	V68	V69	V70	V71[1]
Trial timeline (D=day)	C-1 or 427	C or	C+6 – C+21 or	TD[8] or	TD[8]+1 or	TD[8]+2 or	TD[8]+3 or	463
Number of visits	1x	1x	1x / day	1x	1x	1x	1x	1x
Eligibility criteria + informed consent								
Demographic data, medical history								
Physical examination and vital signs	X		X	X[8]	X[7]	X[7]	X[7]	X[7]
ECG	X							
Temperature	X		X	X	X	X	X	X
Immunization with NF135.C10-infected mosquitoes								
Challenge with 5 infected mosquitoes		X						
Mefloquine prophylaxis[2]								
A/P treatment				X	X	X		
Haematology tests[3] (2.0ml)	X		X	X	X	X	X	X
Biochemistry tests[4]	3 ml		C+6: 3 ml	3 ml		3 ml		3 ml
HsTropT + LDH only (2.0ml)	X		X		X		X	
Serology[5]	X[9]							
Parasitology[6] (2.0ml)	X		X	X			X[6]	X
Exploratory immunology								
Citrate plasma[9]	X				X		X	X
PBMC for cellular studies	80 ml				80 ml[11]		80 ml[11]	80 ml
PBMC for plasmablasts								
Whole-blood transcriptomics (2.5 ml)	X				X		X	X
Pregnancy and toxicology urine test	X							
Supply diary	X[9]							
Reviewing diaries	X		X	X	X	X	X	
Blood Draw Estimate	89.5 ml		C+6: 7 ml rest: 6 ml	7 ml	88.5 ml	7 ml	88.5 ml	88.5 ml
Cumulative Blood Drawn	89.5 ml		186.5 ml	193.5 ml	282 ml	289 ml	377.5 ml	467 ml

[1] Visit may be performed 1-3 days earlier
[2] Groups 1-2 only
[3] Hemoglobin, hematocrit, platelets, white blood cell count + differentiation
[4] Creatinin, urea, sodium, potassium, bilirubin, AF, γGT, ASAT, ALAT, additional at screening: cholesterol + glucose
[5] Screening: HIV, HBV, HCV, *P. falciparum* (5.0ml). Inclusion: HIV, HBV, HCV (3.0ml)
[6] Immunization: retrospective qPCR day 6-10; Cohort A: thick smear day 7-9; Cohort B: thick smear day 10 (end of treatment); Challenge: prospective qPCR daily
[7] On indication.
[8] After one positive qPCR
[9] Group 5-8 only
[10] Taken from the same tubes as PBMCs
[11] Only if treated after positive qPCR

8.3 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences.

Safety criteria for Malarone treatment are listen in section 8.3.3. Volunteers can also be withdrawn from the study procedures at the discretion of the clinical investigator or the local safety monitor for the following reasons:

- Any serious adverse event
- Any adverse event that, according to clinical judgment of the investigator, is considered as a definite contraindication to proceeding with the study procedures.
- The use of concomitant, chronic medication active on the immune system (i.e. immunosuppressive agents) or against *P. falciparum*
- Pregnancy
- Withdrawal of informed consent by volunteer
- Completely lost to follow-up
- If, on balance, the investigator or safety monitor believes that continuation would be detrimental to the subject's well-being
- Any other protocol deviation that results in a significant risk to the subject's safety

If a subject withdrawal occurs for any reason, the investigator must make every effort to determine the primary reason for a subject's withdrawal from the study and record this information in the study file.

If it is felt that inclusion of the study subject's data for analysis is compromised, the subject will be terminated from the study and data will not be included in analysis. This does not preclude the ethical responsibility of the investigators to ensure the safety of the subject and offer curative therapy for a malaria episode, and to follow the subject for cardiac manifestations of disease. If the subject has already experienced a malaria episode and received curative therapy, continued inclusion in the study will be considered.

For subjects who are lost to follow-up (i.e. those subjects whose status is unclear because they fail to appear for study visits without stating an intention to withdraw), extensive effort (i.e. documented phone calls, letters and e-mails) will be undertaken to locate or recall him or at least to determine his health status. The investigator should show "due diligence" by documenting in the source documents steps taken to contact the subject, e.g. dates of telephone calls, registered letters, etc.

8.4 Replacement of individual subjects after withdrawal

If a subject withdraws before the first immunization (groups 1-4) or before the CHMI (group 5-8), he/she will be replaced with an alternate volunteer who passed screening, if possible.

8.5 Follow-up of subjects withdrawn from treatment

In the event that a volunteer discontinues the study for any reason, he/she will be required to complete all safety follow-up as appropriate, as determined by the principal investigator. All volunteers who have been exposed to the bites of infectious mosquitoes are required to complete adequate prophylaxis (in the case of subjects withdrawing during CPS immunization) or take a curative regimen of A/P or alternative effective anti-malarial treatment should A/P be contra-indicated (in the case of subjects withdrawing after CHMI, or subjects withdrawing during immunization, for who continued prophylaxis is contraindicated).

8.6 Premature termination of the study

The study may be discontinued by the sponsor:

- On advice of the safety monitor
- On advice of the Safety Monitoring Committee (SMC)
- On advice of the clinical investigator
- On advice of the CCMO

The safety monitor, SMC, CCMO or investigators may decide to put the study on hold based on adverse events, pending discussion with the Sponsor / SMC / CCMO / safety monitor / investigators. Following discussion, it may be decided to terminate the study. Safety reporting procedures are described in section 9.

9. SAFETY REPORTING

9.1 Temporary halt for reasons of subject safety

In accordance to section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardise subject health or safety. The sponsor will notify the accredited METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the accredited METC. The investigator will take care that all subjects are kept informed.
PATH will also be notified of any decisions to prematurely suspend or terminate the study.

9.2 AEs, SAEs and SUSARs

9.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to trial procedure or the experimental intervention. All adverse events reported spontaneously by the subject or observed by the investigator or his or her staff will be recorded.
Abnormal laboratory findings (e.g. clinical chemistry or haematology) or other abnormal assessments that are judged by the investigator to be clinically significant will be recorded as AEs (or SAEs if they meet the definition). The investigator will exercise his or her medical judgement in deciding whether an abnormal laboratory finding or other abnormal assessment is clinically significant.
If there are any severe complaints not typical for malaria infection, such as chest pain, the volunteer will be evaluated immediately by a qualified clinician using the appropriate clinical assessments (e.g. ECG or measurement of cardiac enzymes) according to standard hospital care.

9.2.2 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect; or

- any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been, based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a serious adverse event.

The investigator will report all SAEs to the sponsor without undue delay after obtaining knowledge of the events. All SAEs will be reported through the web portal *ToetsingOnline* to the CCMO, within 7 days for SAEs that result in death or are life threatening followed by a period of maximum of 8 days to complete the initial preliminary report. All other SAEs will be reported within a period of maximum 15 days after the sponsor has first knowledge of the serious adverse events.

9.3 Follow-up of adverse events

9.3.1 Adverse event data collection

Safety assessments will be performed and recorded by the investigators. All adverse events/reactions (solicited and unsolicited), noted by the investigators will be accurately documented in the case report form by the investigators. For each event/reaction the following details will be recorded:

1. Description of the event(s)/reaction(s)
2. Date and time of occurrence
3. Duration
4. Intensity
5. Relationship with the intervention
6. Action taken, including treatment
7. Outcome

In addition, symptoms will be ranked as (1) mild, (2) moderate, or (3) severe, depending on 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 or required bed rest

If an AE changes in intensity during the specified reporting period, a new description of the AE will be added.
When an AE/SAE occurs, it is the responsibility of the investigators to review all documentation (e.g. hospital progress notes, laboratory, and diagnostics reports) related to the event. The investigators will then record all relevant information regarding an AE/SAE on the CRF or SAE Report Form, respectively. The investigator will attempt to establish a diagnosis of the event based on signs, symptoms, and/or other clinical information. In such cases, the diagnosis should be documented as the AE/SAE and not the individual signs/symptoms.

The following signs and symptoms will be solicited at any visit:
Fever (by examination), headache, fatigue, malaise, chills, myalgia, dizziness, sweats, nausea, vomiting, abdominal pain, diarrhoea, chest pain.

The following signs and symptoms local to the site of mosquito bites will be solicited after every immunization and CHMI:
Tenderness, induration (assessed by palpation), erythema, swelling (assessed by direct or lateral visualization), and pain, pruritis (subjective symptoms).

9.3.2 Assessment of causality

The investigators are obligated to assess the relationship between study procedures and the occurrence of each AE/SAE. The investigators will use clinical judgment to determine the relationship. Alternative causes, such as natural history of the underlying diseases, concomitant therapy, other risk factors and the temporal relationship of the event to the challenge will be considered and investigated. The relationship of the adverse event with the study procedures will be categorized as:

Probable	An adverse event that follows a reasonable temporal sequence from the challenge procedure and cannot be reasonably explained by the known characteristics of the subject's clinical state.
Possible	An adverse event for which insufficient information exists to exclude that the event is related to the study procedure.
Not related	An event for which sufficient information exists to indicate that the aetiology is unrelated either because of the temporal sequence of events or because of the subject's clinical state or other therapies.

9.3.3 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the subject's general physician or a medical specialist.
AEs and SAEs will be reported until end of study within the Netherlands, defined as day 35 after the malaria challenge infection (see flowchart section 8.3.14).

9.4 Local Safety Monitor (LSM) and Safety Monitoring Committee (SMC)

9.4.1 Local safety monitor

For this study, a local safety monitor will be appointed, who is based in the Radboudumc and will be involved in the review of serious adverse events and volunteer safety. He/she is an experienced clinician qualified to evaluate safety data from clinical studies with malaria infections. He/she is independent of the sponsor and the investigators.

9.4.2 Safety Monitoring Committee (SMC)

An independent Safety Monitoring Committee (SMC) will be appointed, including 3 individuals. Their main responsibility will be assessing of any serious adverse events and halting further study procedures. A safety report including a list of all reported adverse events and any safety laboratory values outside the normal range will be prepared for review by the SMC at the end of the immunization period and after each CHMI.
The advice(s) of the SMC will only be sent to the sponsor of the study. Should the sponsor decide not to fully implement the advice of the SMC, the sponsor will send the advice to the CCMO, including a note to substantiate why (part of) the advice of the SMC will not be followed.

9.4.3 Review of safety data by the safety monitor and SMC

A safety report including a list of all reported adverse events and any safety laboratory values outside the normal range will be prepared after each immunization and on day 21 and 35 after each CHMI.
These reports will be prepared by a clinical investigator and sent to the safety monitor and all clinical investigators involved. The safety monitor will review the safety data within 2 workdays and if warranted instruct the site to take appropriate action.
In addition, safety data for all participants will be assessed by the SMC after the last immunization and after each CHMI. Responsibilities of the SMC are described in the SMC Charter. The

advice(s) of the SMC will only be communicated to the CCMO when the sponsor does not follow this. With this notification a statement will be included indicating whether the advice will be followed.

Any highly sensitive troponin T value greater than 60ng/l will be reported to the safety monitor within 24 hours. Any abnormal safety laboratory values that lead to immediate anti- malarial treatment will be reported to the safety monitor within 24 hours.

9.4.4 Safety stopping rules

The study may be placed on safety hold for the following reasons:

- On advice of the safety monitor
- On advice of the investigators
- On advice of the SMC
- On advice of the CCMO
- One or more participants experience an SAE that is determined to be related to a study intervention
- Two or more grade 3 adverse events after immunization, which are unexpected and possibly, probably or definitively related to the study intervention.
- Any clinical cardiac event that does not meet the criteria of SAE

The safety monitor, CCMO, or investigators may decide to put the study on hold based on adverse events, pending discussion with the safety monitor, SMC, CCMO and investigators. In addition, the PI can always decide based on characteristics, duration and severity of signs/symptoms to treat and stop the trial for individual cases. The PI will identify when stopping rule criteria are met and alert the appropriate parties. The safety monitor will review all available safety data on a pre-defined time point after the challenge period. If the CCMO has recommended safety hold, re-initiation of the study will require CCMO concurrence. The CCMO will be informed of a safety hold by the sponsor. Following discussion, it may be decided to terminate the study.

PATH will be informed of a safety hold by the sponsor.

10. STATISTICAL ANALYSIS

10.1 Primary study parameter

The primary endpoint is the number and intensity of adverse events (solicited and unsolicited combined) after NF135.C10 CPS immunization. Adverse events will be analyzed by calculating the proportion of volunteers in each group who reported mild, moderate or severe adverse events. Relatedness of events will be taken into account as well as the time point of the adverse events (e.g. during immunization phase or during challenge phase). The frequency of signs and symptoms (categorized by severity, relatedness and study phase) will be compared between groups with either the chi-square test or Fishers exact test (two-tailed) depending on definitive sample sizes. Adverse events will be categorized by ICD-10 coding. Assumptions of statistical tests will be evaluated prior to performing the analysis.

10.2 Secondary study parameter

The secondary endpoints are time to parasitemia, defined as the time to the first single positive qPCR, and sterile protection after each CHMI. Volunteers who remain negative until day 28 after each CHMI will be considered fully protected against malaria. Sterilely protected subjects will not be included in the time to parasitemia analysis. Comparison of mean time to first qPCR positivity between all groups will be assessed by Mann-Whitney U test (two- sided). Once daily qPCR (threshold 100 parasites/ml) has a 99 % sensitivity and a 100 % specificity [26], and in the 1% of cases where 100 Pf/mL is not detected, the qPCR test is always repeated.
The percentage of volunteers protected will be compared with either the chi-square test or Fishers exact test (two-tailed) depending on definitive sample sizes. Assumptions of statistical tests will be evaluated prior to performing the analysis.

10.3 Exploratory study parameters

In immunological analyses we will assess differences by comparing mean values between groups or time points using either a two-tailed student's t-test (if comparing two groups) or a one-way ANOVA (if comparing more than two groups), or non-parametric equivalents. Paired tests will be used if pre-intervention values are compared with post intervention values, unpaired tests will be used if comparisons are made between groups. For discrete variables (e.g. the number of positive assays), the chi-squared test or Fisher's exact test will be used (two-tailed). Assumptions of statistical tests will be evaluated prior to performing the analysis.

11. ETHICAL CONSIDERATIONS

11.1 Regulation statement

This study will be conducted in accordance with the latest Fortaleza revision of the Declaration of Helsinki (2013), the Medical Research Involving Human Subjects Act (WMO), the ICH Good Clinical Practice, and local regulatory requirements.

The investigators are responsible for obtaining Ethics Committee(s) approval (IRB) of the protocol and any subsequent amendments in compliance with local law before the start of the study.

This study will be reviewed by the Central Committee on Research Involving Human Subjects (CCMO) and the Western Institutional Review Board (WIRB).

11.2 Recruitment and consent

As soon as the study is approved, healthy volunteers will be recruited to participate in the study. Advertisements will be placed in prominent places on university campuses and other public places as well as on the intranet of the Radboud University. Furthermore, a Facebook page (link: http://www.facebook.com/malariavaccin) showing the advertisement text will be used to inform people about the trial. This brief advertisement will indicate a telephone number to call and an e-mail address for contact to request further information. It will furthermore indicate a website (www.malariavaccin.nl) which contains a form. This short questionnaire will be completed using the online form. When seemingly suitable volunteers contact investigators via e-mail, telephone or the online form, they will be invited to an information meeting during which the study will be explained to them by the study investigator. Directly after the meeting they will be provided with documents to review at home (the information sheet, the informed consent form, the application form and the insurance text). During and after the meeting there will be time for questions. After this free discussion with the investigator, and any follow-up discussion if necessary, the volunteer will be given sufficient time to consider participation.

Volunteers who are interested in participating will be asked to fill in the application form and will be invited to come for a screening visit. Eligible subjects may only be included in the study after providing written, CCMO-approved informed consent. Informed consent must be obtained before conducting any study-specific procedures (i.e. all of the procedures described in the protocol, including screening procedures). The process of obtaining informed consent should be documented in the subject source documents. During the screening visit, the questionnaire answers will be discussed and inclusion and exclusion criteria will be checked. Also, a letter for the general practitioner will be signed and sent after screening. Again, the investigator will answer any questions the volunteer has. The

possibility of withdrawal from the study, at any time, without penalty and without any declaration of the reason will be pointed out to the volunteers.

The investigators will be responsible for providing adequate verbal and written information regarding the objectives and procedures of the study, the potential risks involved and the obligations of the volunteers. Volunteers will be informed that they will not gain health benefits from this study. Trainees or other students who might be dependent on the investigators or the study group will not be included in the study.

11.3 Benefits and risks assessment, group relatedness

Two major areas of ethical concern are contained within this proposal, namely the use of blood from humans and the use of human volunteers for *P. falciparum* CHMI. All partners in this proposal are aware of and follow the relevant national and international rules and regulations as they pertain to access to material of human origin and clinical research. International agreements such as the Declaration of Helsinki will be observed and respected.

11.3.1 Ethical aspects concerning the production of *P. falciparum* infected mosquitoes

The human blood and serum used for parasite culture is obtained from screened healthy blood donors from Sanquin. Continuous culture of *P. falciparum* has been routine for the Central Animal Facility of the Radboudumc over the past 3 decades. All culture material is checked for bacterial contamination, including *Mycoplasma*, and for blood-borne pathogens (HIV, HBV, HCV, HTLV 1+2). The process has become highly standardized as described in standard operating procedures and was positively reviewed by an external auditor in 2014.

11.3.2 Ethical aspects concerning the use of human volunteers

Infection of humans with malaria has been carried out for nearly a century, including for therapeutic use as treatment for neurosyphilis and later for drug and vaccine evaluation. The ability to carry out this type of work is largely based on the relatively low morbidity and the lack of mortality seen in these studies since the advent of conducting human infections with mosquitoes fed on *P. falciparum* gametocyte cultures in 1986 [21]. The occurrence of four cardiac events in volunteers participating in phase I/IIa malaria vaccine trials in Nijmegen has raised discussion about the safety of malaria challenge trials with respect to cardiac events. Based on recommendations of the CCMO and an External Scientific Advisory Committee to the European Malaria Vaccine Development Association, this malaria challenge trial protocol has been adjusted (see also section 1.4 and 13). In 2016 the occurrence of mild, transient liver and kidney injury during CHMI was extensively evaluated. Based on recommendations

of the CCMO and external experts, the malaria challenge protocol was modified to include extra monitoring (see also section 1.4 and 13).

Testing in human subjects remains the only reliable and convincing way to obtain information on the immunological responses that are important for protection against malaria. Of course, the compelling need for a malaria vaccines and treatments needs to be balanced with the potential risks and discomforts of the volunteers. Explorative studies looking for new and complementary candidate malaria vaccines are of paramount importance with the potential of large-scale application in endemic countries.

The study will be undertaken in accordance with Good Clinical Practices (GCP), according to the standards defined in the EEC directive 91/507/EEC, and in the Directive on Good Clinical Practice in Clinical Trials (ICH GCP, 75/318/EEC, January 1997) and under the principles of the Declaration of Helsinki; ethical permission will be sought from the CCMO the Netherlands.

11.4 Compensation for injury

The sponsor/investigator has a liability insurance which is in accordance with article 7 of the WMO. The sponsor (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO). This insurance provides cover for damage to research subjects through injury or death caused by the study.

1. € 650.000,-- (i.e. six hundred and fifty thousand Euro) for death or injury for each subject who participates in the research;
2. € 5.000.000,-- (i.e. five million Euro) for death or injury for all subjects who participate in the research;
3. € 7.500.000,-- (i.e. seven million five hundred thousand Euro) for the total damage incurred by the organisation for all damage disclosed by scientific research for the sponsor as 'verrichter' in the meaning of said Act in each year of insurance coverage.

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

11.5 Incentives

Volunteers in group 1, 2, 3 and 4 will receive 2300 euros in compensation fee for the immunisations and the first CHMI. Volunteers participating in the second CHMI will receive an additional 800 euros. Volunteers in groups, 5-8 will receive 800 euros. These amounts are based on our predefined criteria, i.e. 300 euro reimbursement for each mosquito exposure (inconveniences associated with full day visit, mosquito bites and necessary

antimalarial prophylaxis/treatment), and a 20 euro reimbursement for each routine follow-up visit (maximally one hour, including venapuncture). Additionally, volunteers are compensated for completing trials with a long duration (20 euro per month). Travel expenses will not be additionally reimbursed. This amount of money is reasonable and in line with Dutch common practice. If a subject withdraws from the study prior to completion, they will receive reimbursement proportional to number of visits they attended. In case of unexpected medical complications related to participation in the study, there will be access to state-of-the-art medical treatment with full costs covered by the insurance of Radboudumc.

12. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1 Handling and storage of data and documents

12.1.1 Confidentiality

All parties agree to adhere to the principles of medical confidentiality in relation to clinical study subjects involved in this trial, and shall not disclose the identity of subjects to third parties without prior written consent of the subject.

All biological samples, with exception of those taken for safety diagnostics, will be labelled only with the volunteer study identification number. Samples taken for safety diagnostic (processed by the central clinical laboratory of the Radboudumc) will be labelled with part of the subject identification code, study identification name and a fictitious birth date (only using the subjects actual birth year). The samples will not be labelled with volunteer names or actual birth dates. The subject identification code will be kept by the principal investigator. All data will be stored for minimally 15 years. All biological samples will be stored for maximally 15 years.

12.1.2 Data collection

Designated trial staff will enter the data required by the protocol into the electronic CRF (eCRF).

12.1.3 Database management and quality control

All data should be recorded, handled and stored in a way that allows its accurate reporting, interpretation and verification.

A data monitor, contracted from an independent CRO, will review the data entered into the eCRFs by investigational staff for completeness and accuracy and instruct the site personnel to make any required corrections or additions. Queries are made during each monitor visit. Designated investigator site staff is required to respond to the query and confirm or correct the data. Medical history/current medical conditions and adverse events will be coded using the ICD-10 terminology.

12.2 Monitoring and Quality Assurance

Before study initiation, the protocol and eCRFs together with relevant SOPs will be reviewed by the sponsor, the investigators and their staff. During and after completion of the study, the data monitor will visit the site to check the completeness of records, the accuracy of entries on the eCRFs, the adherence to the protocol and to Good Clinical Practice, the progress of

enrolment, and to ensure that mefloquine and Malarone are being dispensed and accounted for according to protocol.

The investigator will maintain source documents for each subject in the study, consisting of case and visit notes containing demographic and medical information, laboratory data, electrocardiograms, and the results of any other tests or assessments. All information on eCRFs must be traceable to these source documents in the subject's file. The only exception is the data from the quantitative PCR, which is loaded from the PCR machine directly into the eCRF. As with all parts of the eCRF, there is an audit trail in place to register this data entry. The investigator will also keep the original informed consent form signed by the subject (a signed copy is given to the subject).

The investigator will give the monitor access to all relevant source documents to confirm their consistency with the eCRF entries. According to the NFU risk classification system, this clinical trial has been classified as 'middle risk'. The monitor will perform full verification for the presence of informed consent, adherence to the inclusion/exclusion criteria and documentation of SAEs. The recording of data that will be used for all primary and safety variables will be assessed for 25% of included subjects (i.e. 8 subjects from group 1, 2, 3 or 4 and 2 subjects from group 5, 6, 7 or 8).

12.3 Amendments

Amendments are changes made to the research after a favourable opinion by the CCMO has been given. All amendments will be notified to CCMO.

12.4 Annual progress report

The investigator will submit a summary of the progress of the trial to the CCMO once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed the trial, serious adverse events, serious adverse reactions, other problems and amendments.

12.5 Temporary halt and (prematurely) end of study report

The sponsor will notify the CCMO of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit.

The sponsor will notify the CCMO immediately of a temporary halt of the study, including the reason of such an action.

In case the study is ended prematurely, the sponsor will notify the CCMO within 15 days, including the reasons for the premature termination.

12.6 Public disclosure and publication policy

This trial will be registered in clinicaltrials.gov prior to study start. The final report will be prepared by the investigators at the Radboud university medical center. It will be signed by the project leader or the principle investigator. The investigators will make every effort to publish the results in a peer-reviewed journal.

13. STRUCTURED RISK ANALYSIS

13.1 Potential issues of concern

In this study volunteers will undergo a Controlled Human Malaria Infections by the bites of laboratory reared Anopheles mosquitoes infected with the NF135.C10 or NF54 strain of *Plasmodium falciparum*.

a.Level of knowledge about mechanism of action

The causative organisms of malaria, *Plasmodium* parasites, were first identified by Laveran in 1880 and their complete life-cycle in the mammalian host was elucidated by 1947. Extensive clinical experience of malaria infection has since been accumulated by the medical community. Nevertheless, certain aspects of the pathophysiology of (severe) malaria (e.g. cerebral malaria) remain incompletely understood. Such manifestations, however, do not occur during Controlled Human Malaria Infections due to the very early treatment of study subjects and hence the clearance of parasitemia at extremely low levels (see also below).

b.Previous exposure of human beings with the test product(s) and/or products with a similar biological mechanism

There is extensive clinical experience with controlled human *P. falciparum* malaria infections by the bite of infected mosquitoes. Since 1986 more than 3,500 volunteers have had CHMI by the bites of mosquitoes fed on cultures of P. falciparum gametocytes to produce *P. falciparum* sporozoites. Worldwide the majority of these infections have been performed with the laboratory NF54 strain or its daughter clone 3D7. This has proved to be a reproducible, predictable and safe method of inducing *P. falciparum* infections. The results of such studies were summarized in 1997 [22], in 2007 [23] and in 2011[33].

c.Can the primary or secondary mechanism be induced in animals and/or in ex-vivo human cell material?

In vitro assays cannot capture the complexity of the multi-stage life-cycle of *Plasmodium* parasites and their complex of interaction with the host. Although (parts of) the *Plasmodium* life-cycle can be reproduced in (rodent or primate) animal models, these either involve non- human *Plasmodium*-species or non-natural host-parasite combinations. As a result, extrapolation of pathophysiological and immunological aspects of infection to human disease are only possible to a limited extent [34, 35].

d.Selectivity of the mechanism to target tissue in animals and/or human beings

The life-cycle of malaria parasites follows a fixed and pre-determined course in the human host. *P. falciparum* sporozoites delivered by mosquito bite are only able to invade into and develop within hepatocytes. These developments are constrained by multiple parasite-host ligand interactions and the parasite's ability to manipulate the host cell's internal environment [3 6] .

e.Analysis of potential effect

Safety and tolerability of CHMI will be assessed by recording adverse events, reported at any time during the trial either at a visit or by writing in the diary. Temperature, will be recorded at every visit following the CHMI. Assessment of complete blood count, LDH and troponin-T will be performed daily starting on day 6 after the CHMI. Assessment of liver and kidney function will be performed at baseline, on day 6 post CHMI, at initiation of treatment, on day 2 post treatment and at the end of study visit.

The gold-standard for the detection of blood stage malaria infections is thick blood smear microscopy (detection limit 4 parasites/ul). Previously, we have used qPCR as the main tool to detect blood-stage malaria parasites [25, 32], which has an even greater sensitivity.

When treatment is initiated based on thick smear, parasitemia in subjects undergoing CHMIs should never exceed ±0.001% [24]. In contrast, manifestations of severe malaria generally only occur above 5% parasitemia and never below 1% parasitemia (WHO Guideline Severe Malaria, SWAB Richtlijn).

In two previous CHMI trials (NL48301.091.14; NL48732.091.14) qPCR was performed daily during follow-up and volunteers were treated with anti-malarial drugs after a single positive qPCR. During these two studies, 39 volunteers developed patent parasitemia. Of these volunteers 26% developed no malaria symptoms, 38% had only grade 1 symptoms (no influence on daily activities), 28% had grade 2 symptoms (some influence on daily activities) and only 13% had grade 3 symptoms (requiring bedrest). All grade 3 symptoms lasted for less than one day.

f.Pharmacokinetic considerations

Not applicable.

g.Study population

Included subjects are healthy young adult volunteers, who have been extensively screened for any evidence of co-morbidity, in particular cardiovascular risk factors. Female subjects of child-bearing age are screened for pregnancy by urine test and are required to use contraception throughout the study period, as described in section 8.

h.Interaction with other products

Concurrent use of drugs potentially interacting with atovaquone-proguanil (e.g. artemether-lumefantrine, rifampicin, metoclopramide, oral anti-coagulants and certain anti-retrovirals) are contra-indicated.

i.Predictability of effect

In this trial at least some volunteers will experience blood stage malaria infection. Blood stage malaria infection has been seen after CHMI in over 3,500 volunteers. The progression and symptoms of this parasitemia has been demonstrated to be reproducible and predictable [22-24]. However, the occurrence of cardiac serious adverse events in Radboudumc CHMI trials has led to an increase in safety measures, discussed in a separate section below.

j. Can effects be managed?

Subjects are followed up intensively at Radboudumc on an outpatient basis for clinical and parasitological assessment to ensure treatment is started at the earliest possible time point. The NF135.C10 andNF54 *P. falciparum* strains have been tested for susceptibility to atovaquone-proguanil in vitro. Should treatment with this drug need to be discontinued prematurely in any subject for whatever other reason (e.g. intolerability), various other anti-malarial drugs are available, both oral and intravenous, to which this strain is also susceptible.

13.2 Cardiac events following Controlled Human Malaria Infections

In the twenty-three CHMI studies conducted at Radboudumc prior to CPS135* (involving 376 healthy volunteers), three cardiac events have occurred after infection with the NF54 strain with the confirmed or differential diagnosis of myocarditis. The occurrence of myocarditis in both natural uncomplicated malaria and CHMI has never been described elsewhere. However, the three cardiac events share no specific factor other than malaria infection.

With regards to the malaria infection, these myocarditis cases do share a number characteristics: i) they took place 1-5 days after start of antimalarial treatment but different anti-malarial drugs were used in all cases ii) there was no parasitemia detectable at the time of the event, and iii) next to the P. falciparum infection, other and known possibly triggering factors (e.g. preceding vaccinations, concomitant infections, cannabis use) were present during or preceding the event. The three cases are discussed in detail below.

*Additionally, after the first immunization in cohort B of the CPS135 trial, a fourth SAE occurred, also described below.

Case 1

In 2007 a 20 year old female was immunized with the candidate malaria vaccine, LSA3 (NL14715.000.06), underwent a CHMI and was treated with artemether/lumefantrine (Riamet®) after a positive thick smear. She presented with retrosternal chest pain three days after treatment, blood slide was negative. Elevated troponin-I (maximally 11.80ug/l) and ECG abnormalities were suggestive for a cardiac event with minimal cardiac damage and an MRI showed no abnormalities. Risk factors for cardiac disease in this volunteer included an acute myocardial infarction in a paternal grandfather at age 43 and a paternal family history of dyslipidemia. The final diagnosis was acute coronary syndrome or myocarditis. This case is reported in [27], attachment K4a. Following this event, recommendations of the European Malaria Vaccine Development Association and the CCMO followed for improved safety of participating volunteers:

- A/P replaced Riamet® as treatment during CHMIs at Radboudumc. it.
- Individuals will be excluded from participation if they have 1st or 2nd degree relatives who had cardiac events when less than 50 years of age.
- Volunteers will be required to stay very close to the Radboudumc to ensure maximal safety from day 5 after CHMI until treatment has been finished (maximum 12 days).
- Monitoring of highly sensitive troponin T (hsTropT), D-dimer, lactate dehydrogenase (LDH) and thrombocytes.

Case 2

In 2013, a healthy male, taking part in the TIP5 clinical trial (NL39541.091.12) underwent a CHMI challenge after immunization with cyropreserved sporozoites under chloroquine prophylaxis (CPS protocol). The volunteer became thick-smear positive after challenge infection and the volunteer was treated with A/P. Per protocol blood examination revealed an elevated troponin-T (maximally 1115ng/l) on the second day of Malarone treatment, while the volunteer was asymptomatic. He was admitted to cardiology and troponin-T values, ECG abnormalities and a cardiac MRI confirmed the diagnosis acute myocarditis. During admission he experienced a 20 minute episode of retrosternal chest pain. Further diagnostic tests and patient history revealed a concurrent rhinovirus infection and a boosting regime of standard travel vaccines 46 days preceding the CHMI, with a booster 20 days before the CHMI. This case has been reported in [28], attachment K4b. Due to this SAE, new safety recommendations were integrated into our procedures for CHMI. These include:

- Exclusion of volunteers who took standard vaccinations within 3 months before the start of the trial or are planning to take standard vaccinations during the trial period up to 8 weeks after CHMI.
- Increased control of hs troponin T as a marker of cardiac damage; initiation of treatment with Malarone when hs troponin T > 0.1 µg/ml or on recommendation of the cardiologist.
- Continuation of daily LDH measurements starting from day 5 after CHMI; Malarone treatment will be initiated if LDH values are above 1000 U/L.
- Daily measurements of thrombocytes; volunteers will be treated with Malarone when thrombocyte levels are < 120x 10^9/L.
- After CHMI we will rely on real-time qPCR for diagnosis of malaria and initiation of treatment, using the following criteria:
    - Two consecutive positive qPCR results in a volunteer with temperature <38.0^{0}C
    - One positive qPCR result in a volunteer with temperature ≥38.0^{0}C
    - One positive thick smear (which will be made if a volunteers attends for evening follow-up with temperature ≥38.0^{0}C or on decision of the trial clinician)

Case 3

In 2014, a healthy, 23 year old, male volunteer underwent a CHMI under chloroquine prophylaxis (CPS-immunization). On day 10 after exposure to bites of 15 malaria infected mosquitoes, routine per protocol blood examinations revealed an elevated troponin-T (maximally 168ng/l) though the volunteer was asymptomatic. This volunteer had no obvious risk factors for cardiac disease other than cigarette smoking and recent cannabis use. This volunteer was negative by thick smear but positive by PCR with maximum parasitemia was 1265Pf/ml while under chloroquine prophylaxis. Following this cardiac event, further safety measures have been integrated into our CHMI protocols:

- Volunteers will be treated with antimalarial after a single positive qPCR after malaria challenge infection
- Use of cannabis will be added as an exclusion criterion
- Excessive physical exercise around immunization and during challenge period will be prohibited

Case 4

In 2020, a 23 year old healthy female volunteer underwent a first immunization with 15 NF135 infected mosquitoes. On day 7 after exposure she started with presumptive A/L treatment, per protocol. On this same day she had parasitaemia, as detected by qPCR, with a density above the theoretical detection limit of thick blood smear microscopy. By day 9, her qPCR was again

negative. On day 10 after exposure she developed chest pain, a dynamic ECG and elevated cardiac biomarkers occurring 1 day after completion of presumptive artemether-lumefantrine treatment. Chest pain decreased spontaneously and troponin levels decreased. MRI, Coronary CT-A, CAG and echocardiography showed no evidence of myocarditis, Tako Tsubo or coronary occlusion / dissection. The presumptive diagnosis was an inflammatory response with no evidence of myocarditis on MRI.

Considerations regarding presumptive A/L treatment during the first immunization phase of cohort B

Both A/L and A/P are recommended in the Dutch national treatment guidelines for uncomplicated *P. falciparum* malaria. After the first cardiac SAE in the LSA-3 trial in 2007, as described in Case 1, it was decided in consultation with CCMO to favor treatment with A/P over A/L. Although at the time of this serious adverse event, A/L treatment was considered unlikely to be a cause/risk factor and there was no direct evidence of A/L cardiotoxicity, an alternative treatment was preferred based on:

1. The hypothesis that due to the rapid killing of parasites, A/L would induce an inflammatory response with possible predisposition to myocarditis. Further clinical or other evidence to support this assumption remains lacking, however.
2. A specific case report of vasospasms after A/L use, where the patient's urine was also tested positive for tetrahydrocannabidol [37].

Since both drugs are registered for malaria treatment, a preference was expressed at that time for A/P, on the basis of the above considerations. After the switch to A/P, two other cardio-ischemic events occurred in CHMI volunteers, which does not support A/L as a possible etiology. All things considered, therefore, from the perspective of the safety of volunteers in this study, the substitution of A/P by A/L as presumptive treatment during the first immunization of cohort B is considered justifiable.

Considerations regarding presumptive A/P treatment during the second immunization phase of cohort B

After the cardiac SAE in cohort B, immunization 1, an alternative presumptive treatment based on A/P was chosen, starting on day 5 after immunization2. A/P treatment is preferred over A/L because of i) risk of treatment failure after A/L treatment, and ii) the higher inflammatory responses after A/L treatment (potentially due to more rapid-acting mechanism) as is also illustrated by the higher number and severity of adverse events following cohort B compared to the 15 mosquito-bite dose arm in cohort A, despite similar parasite densities. Treatment will be initiated on day 5 to potentially block or reduce release of merozoites from the liver, thus further reducing inflammation, whilst still allowing late liver-stage development and the consequent development of pre-erythrocytic immunity. Even if merozoite release is not fully blocked, the presence of circulating drug concentrations upon merozoite release will help to ensure that intra-

erythrocytic development is suppressed from the start.

13.3 Transient liver function test elevations

Transient, asymptomatic liver function derangements have been reported in volunteers in previous CHMI studies, and are likely to be related to the challenge infection (*Reuling et al. manuscript in preparation*). A retrospective analysis of 13 CHMI studies conducted in the Radboudumc showed that 72/120 (60%) of the volunteers that were treated at thick smear parasitaemia levels have mild (38%, <2.5xULN), moderate (10%, ≥2.5-5.0xULN) or severe (12%, ≥5.0xULN) increases of liver transaminases (ALT/AST). Among these volunteers the median increase of ALT was 71.5 U/l (range 19-870 U/l, Q25 of 48 U/l, Q75 of 116 U/l), and median increase of AST was 57 U/l (range 31-723 U/l, Q25 of 45 U/l, Q75 of 97 U/l). The liver function test (LFT) elevations remained asymptomatic in all volunteers, and the typical pattern is limited to ALT/AST elevations without change in bilirubin. The LFT elevations exceeded the upper limit of normal (ULN) at the start up to two days after the start of malaria treatment. LFT monitoring before treatment was only assessed in 1 CHMI study (n=18), showing that LFT elevations typically arise after treatment. The LFTs peak between 2-14 days post treatment with values normalized at study end (35–42 days after challenge infection).

Volunteers treated earlier with positive qPCR rather than thick smear, showed a lower percentage, and severity of LFT abnormalities, 13/58 volunteers (22%) (11/58 (19%) mild, 1/58 (2%) moderate, and 1/58 (2%) severe). All CHMI-volunteers showed normalized values at study end.

A clear explanation for the transient elevated transaminases is not obvious. Although higher parasitaemia associates with higher LFT at group level, there is no clear relationship on individual levels. It also seems unlikely related to choice of anti-malarials, given the diversity of drug regimens used. Similarly, the timing, different, and limited use of paracetamol does not support a clear relationship. Rather a combination of the above mentioned factors, and individual susceptibility may have triggered these abnormalities.

According to an independent safety monitoring committee and an independent hepatologist, these transient asymptomatic and severe LFT elevations are most likely a direct consequence of the malaria infection and subsequent treatment, rather than antimalarial drugs only or another liver nox. Given the rapid and spontaneous resolution and the absence of clearly elevated bilirubin levels, this damage was considered transient, and to permit further studies utilising Plasmodium falciparum challenge.

Consequently, the CHMI protocol has been adapted to include additional safety measures.

- Volunteers will undergo regular safety monitoring to assess asymptomatic liver function test abnormalities

- Volunteers must avoid additional triggers that may cause elevations in liver enzymes including alcohol from baseline up to 1 week post treatment.
- The maximum dose of paracetamol/acetaminophen is 3000 mg per day
- Curative anti-malarial treatment will be initiation of a at ALT/AST levels >5x ULN

13.4 Transient serum creatinine elevations

Transient, asymptomatic increases in serum creatinine compared to baseline (though within normal limits) have also been found during CHMI in the Radboudumc. A retrospective analysis of 5 CHMI studies showed a small increase of creatinin, relative to the subject's own baseline (but within the accepted range of normal), in 70/80 (88%) of the subjects. Only 5/80 (5%) showed mild elevations above the upper limit of normal 2 days after treatment. No moderate or severe elevations have been found. No difference in serum creatinine was found between subjects that were treated based on thick smear threshold or qPCR threshold. All subjects showed normalized values at the end of the study. An independent nephrology expert did not consider the transient decrease in renal function as a particular risk for permanent kidney damage.

Based on these findings the CHMI protocol has been adapted to:

- Exclude subjects with abnormal renal function at baseline.
- Avoid additional triggers that may cause renal dysfunction, such as:
  - Alcohol intake from baseline up to 1 week post-treatment
  - Heavy physical exercise
  - Use of recreational drugs
- Add standard monitoring of renal function at specific time points after CHMI

13.5 Synthesis

There is a large clinical experience with infecting humans by the bite of P. falciparum-infected mosquitoes. Since 1986 more than 3,500 volunteers have had CHMI by the bites of mosquitoes fed on cultures of *P. falciparum* gametocytes to produce sporozoites [21]. This has proved to be a reproducible, predictable and safe method of inducing *P. falciparum* malaria. The results of such studies were summarized in 1997 [22], in 2007 [23] and in 2011 [24]. Recently, treatment based on qPCR has allowed for a significant decrease in intensity and duration of adverse events [26].

Following four cardiac events after blood stage parasitemia, our safety procedures for CHMI remain strongly intensified. In the current trial, we will adhere to those stringent procedures that are relevant, see section 1.4.

14. REFERENCES

1. WHO, *WHO World Malaria Report 2016.* 2016.
2. Dondorp, A.M., et al., *Artemisinin Resistance in Plasmodium falciparum Malaria.* New England Journal of Medicine, 2009. **361**(5): p. 455-467.
3. Noedl, H., et al., *Evidence of artemisinin-resistant malaria in western Cambodia.* N Engl J Med, 2008. **359**(24): p. 2619-20.
4. Crompton, P.D., S.K. Pierce, and L.H. Miller, *Advances and challenges in malaria vaccine development.* J Clin Invest, 2010. **120**(12): p. 4168-78.
5. Moorthy, V.S. and J.M. Okwo-Bele, *Final results from a pivotal phase 3 malaria vaccine trial.* Lancet, 2015. **386**(9988): p. 5-7.
6. Bijker, E.M., et al., *Protection against malaria after immunization by chloroquine prophylaxis and sporozoites is mediated by preerythrocytic immunity.* Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(19): p. 7862-7867.
7. Bijker, E.M., et al., *Cytotoxic markers associate with protection against malaria in human volunteers immunized with Plasmodium falciparum sporozoites.* The Journal of infectious diseases, 2014. **210**(10): p. 1605-1615.
8. Roestenberg, M., et al., *Protection against a malaria challenge by sporozoite inoculation.* N Engl J Med, 2009. **361**(5): p. 468-77.
9. Roestenberg, M., et al., *Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study.* Lancet, 2011. **377**(9779): p. 1770-6.
10. Hickey, B.W., et al., *Mosquito bite immunization with radiation-attenuated Plasmodium falciparum sporozoites: safety, tolerability, protective efficacy and humoral immunogenicity.* Malaria Journal, 2016. **15**(1): p. 377.
11. Hoffman, S.L., et al., *Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites.* J Infect Dis, 2002. **185**(8): p. 1155-64.
12. Mordmüller, B., et al., *Sterile protection against human malaria by chemoattenuated PfSPZ vaccine.* Nature, 2017. **542**(7642): p. 445-449.
13. Walk, J., et al., *Modest heterologous protection after Plasmodium falciparum sporozoite immunization: a double-blind randomized controlled clinical trial.* BMC Med, 2017. **15**(1): p. 168.
14. McCall, M.B.B., et al., *Infectivity of Plasmodium falciparum sporozoites determines emerging parasitemia in infected volunteers.* Sci Transl Med, 2017. **9**(395).
15. Ishizuka, A.S., et al., *Protection against malaria at 1 year and immune correlates following PfSPZ vaccination.* Nat Med, 2016. **22**(6): p. 614-23.
16. Lyke, K.E., et al., *Attenuated PfSPZ Vaccine induces strain-transcending T cells and durable protection against heterologous controlled human malaria infection.* Proc Natl Acad Sci U S A, 2017. **114**(10): p. 2711-2716.
17. Teirlinck, A.C., et al., *NF135.C10: a new Plasmodium falciparum clone for controlled human malaria infections.* J Infect Dis, 2013. **207**(4): p. 656-60.
18. Bijker, E.M., et al., *Sporozoite immunization of human volunteers under mefloquine prophylaxis is safe, immunogenic and protective: a double-blind randomized controlled clinical trial.* PLoS One, 2014. **9**(11): p. e112910.
19. Laurens, M.B., et al., *A consultation on the optimization of controlled human malaria infection by mosquito bite for evaluation of candidate malaria vaccines.* Vaccine, 2012. **30**(36): p. 5302-4.
20. Sauerwein, R.W., M. Roestenberg, and V.S. Moorthy, *Experimental human challenge infections can accelerate clinical malaria vaccine development.* Nat Rev Immunol, 2011. **11**(1): p. 57-64.
21. Chulay, J.D., et al., *Malaria transmitted to humans by mosquitoes infected from cultured Plasmodium falciparum.* Am J Trop Med Hyg, 1986. **35**(1): p. 66-8.
22. Church, L.W., et al., *Clinical manifestations of Plasmodium falciparum malaria*

experimentally induced by mosquito challenge. J Infect Dis, 1997. **175**(4): p. 915-20.
23. Epstein, J.E., et al., *Malaria vaccines: are we getting closer?* Curr Opin Mol Ther, 2007. **9**(1): p. 12-24.
24. Roestenberg, M., et al., *Comparison of Clinical and Parasitological Data from Controlled Human Malaria Infection Trials.* PLOS ONE, 2012. **7**(6): p. e38434.
25. Hermsen, C.C., et al., *Testing vaccines in human experimental malaria: statistical analysis of parasitemia measured by a quantitative real-time polymerase chain reaction.* Am J Trop Med Hyg, 2004. **71**(2): p. 196-201.
26. Walk, J., et al., *Diagnosis and treatment based on quantitative PCR after controlled human malaria infection.* Malaria journal, 2016. **15**(1): p. 398-398.
27. Nieman, A.E., et al., *Cardiac complication after experimental human malaria infection: a case report.* Malar J, 2009. **8**: p. 277.
28. van Meer, M.P., et al., *Idiopathic acute myocarditis during treatment for controlled human malaria infection: a case report.* Malar J, 2014. **13**: p. 38.
29. Reuling, I.J., et al., *Liver Injury in Uncomplicated Malaria is an Overlooked Phenomenon: An Observational Study.* EBioMedicine, 2018. **36**: p. 131-139.
30. Tickell-Painter, M., et al., *Mefloquine for preventing malaria during travel to endemic areas.* Cochrane Database Syst Rev, 2017. **10**: p. Cd006491.
31. Sondén, K., et al., *High Rate of Treatment Failures in Nonimmune Travelers Treated With Artemether-Lumefantrine for Uncomplicated Plasmodium falciparum Malaria in Sweden: Retrospective Comparative Analysis of Effectiveness and Case Series.* Clinical Infectious Diseases, 2016. **64**(2): p. 199-206.
32. Hermsen, C.C., et al., *Detection of Plasmodium falciparum malaria parasites in vivo by real-time quantitative PCR.* Mol Biochem Parasitol, 2001. **118**(2): p. 247-51.
33. Roestenberg, M., et al., *Efficacy of preerythrocytic and blood-stage malaria vaccines can be assessed in small sporozoite challenge trials in human volunteers.* J Infect Dis, 2012. **206**(3): p. 319-23.
34. Craig, A.G., et al., *The role of animal models for research on severe malaria.* PLoS Pathog, 2012. **8**(2): p. e1002401.
35. Langhorne, J., et al., *The relevance of non-human primate and rodent malaria models for humans.* Malar J, 2011. **10**: p. 23.
36. Gaur, D. and C.E. Chitnis, *Molecular interactions and signaling mechanisms during erythrocyte invasion by malaria parasites.* Curr Opin Microbiol, 2011. **14**(4): p. 422-8.
37. Database, N., *Coronary artery vasospasm in 17 y/o after Riamet.* http://www.novartis.com, 2004.