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

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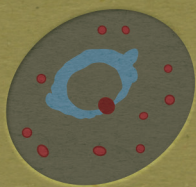


Chad. June 2005. Two refugee women from Darfur presenting a Teletubby.



CHAPTER 5

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



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ABSTRACT

Background

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

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

Methods

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

Results

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

Conclusion

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Study design and subjects

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

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

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

Safety monitoring during immunizations and after challenge

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

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

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

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

Parasitological monitoring

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

Parasitaemia was retrospectively quantified by qPCR on samples as described previously [7, 8, 11]. The qPCR was considered positive if both in duplex performed samples were found positive for *Pf*. A cycle threshold (Ct) value of ≥ 40 was considered negative for *Pf*. The detection limit for qPCR was either 20 or 35 parasites per mL [11] depending on the study. If CT values were ≥ 40 the parasite density was set at half the detection threshold at respectively 10 or 17.5 parasites per mL.

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

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

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

Statistical analysis

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

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

Definitions

Mean AEs

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

Total number of AEs

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

Cumulative number of parasites

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

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

RESULTS

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

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

Adverse events and parasitaemia during immunization

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

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

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

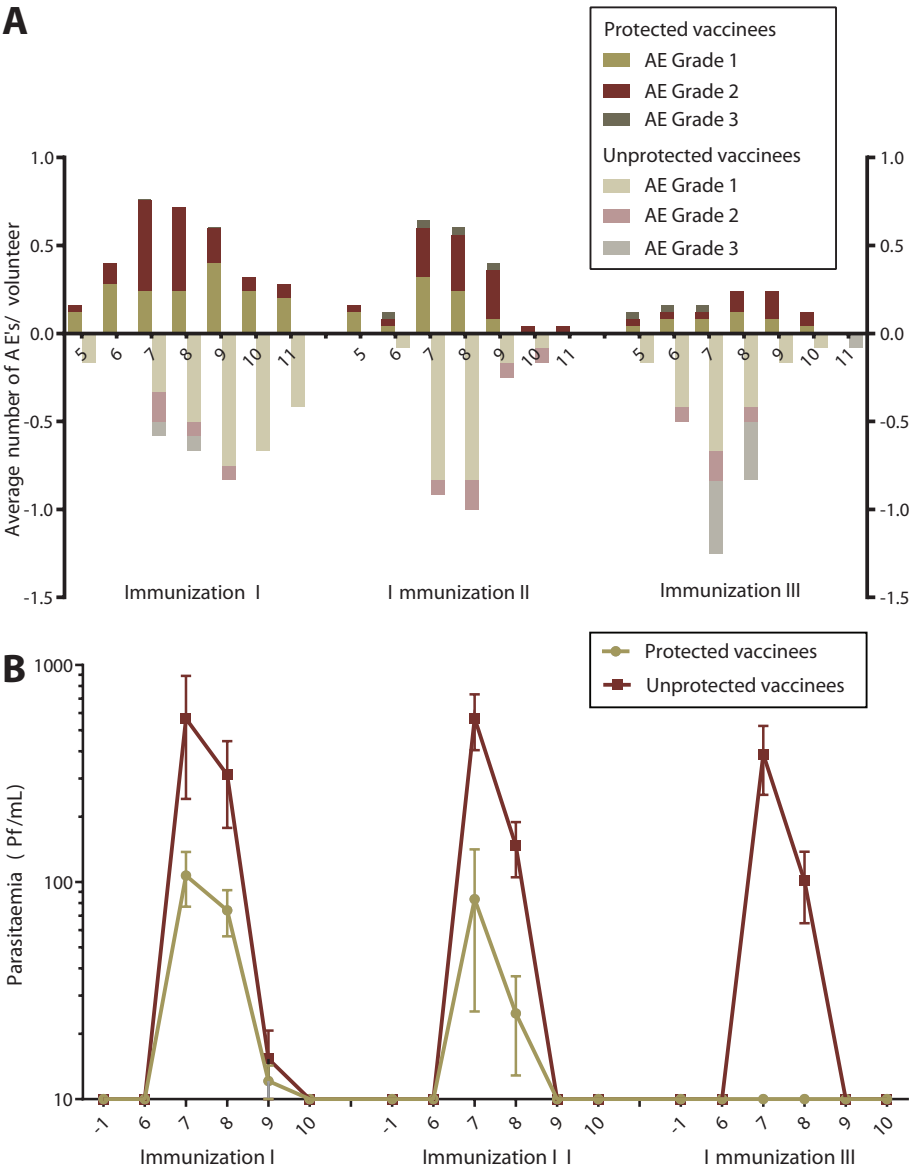


Figure 5.1 Mean number of adverse events and parasitaemia during immunization in protected and unprotected vaccinees

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

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

Adverse events and parasitaemia after challenge

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

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

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

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

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

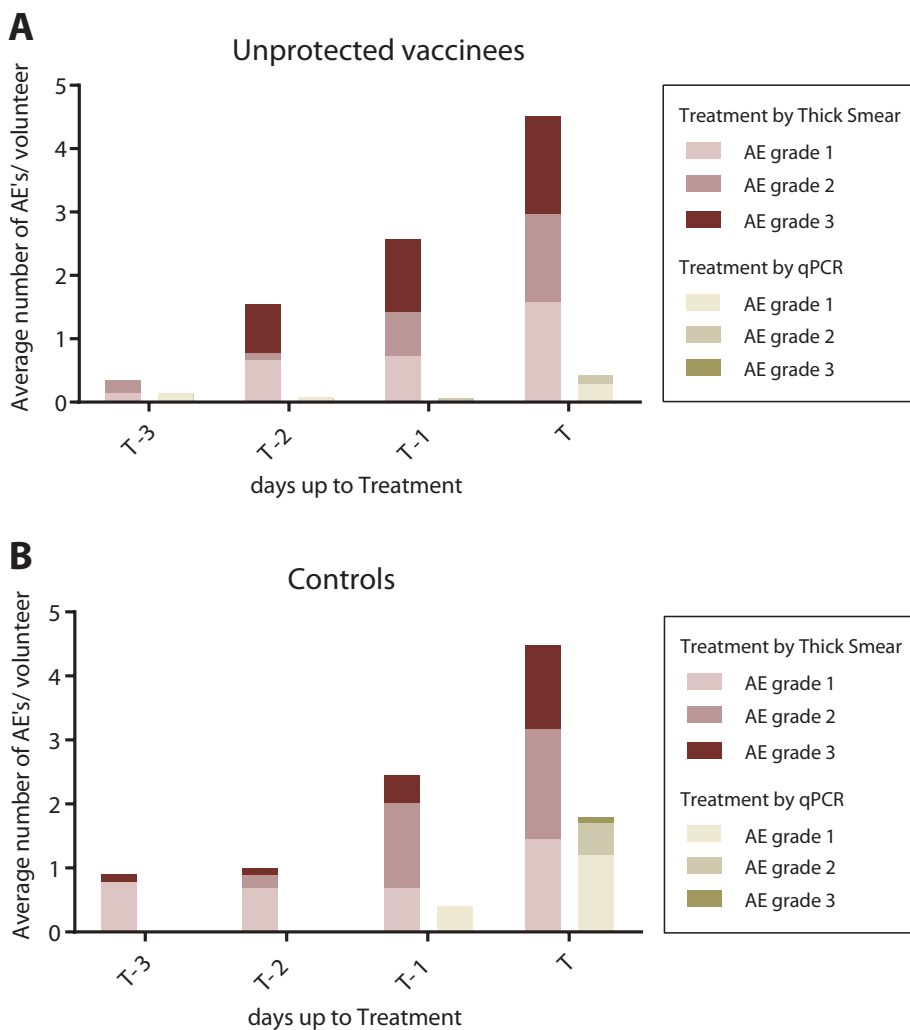


Figure 5.2 Reduction of AEs in unprotected vaccinees and controls following *in silico* application after second consecutive qPCR-based treatment.

2(A) AEs in unprotected vaccinees by thick smear-based (red bars) and second consecutive qPCR-based (brown bars) initiation of treatment. Cumulative reduction of AEs/per subject of 92% from T-3 to T.

2(B) AEs in controls by thick smear-based (red bars) and second consecutive qPCR-based (brown bars) initiation of treatment. Cumulative reduction of AEs/per subject of 75% from T-3 to T. T=day of treatment, T-1= one day before day of treatment.

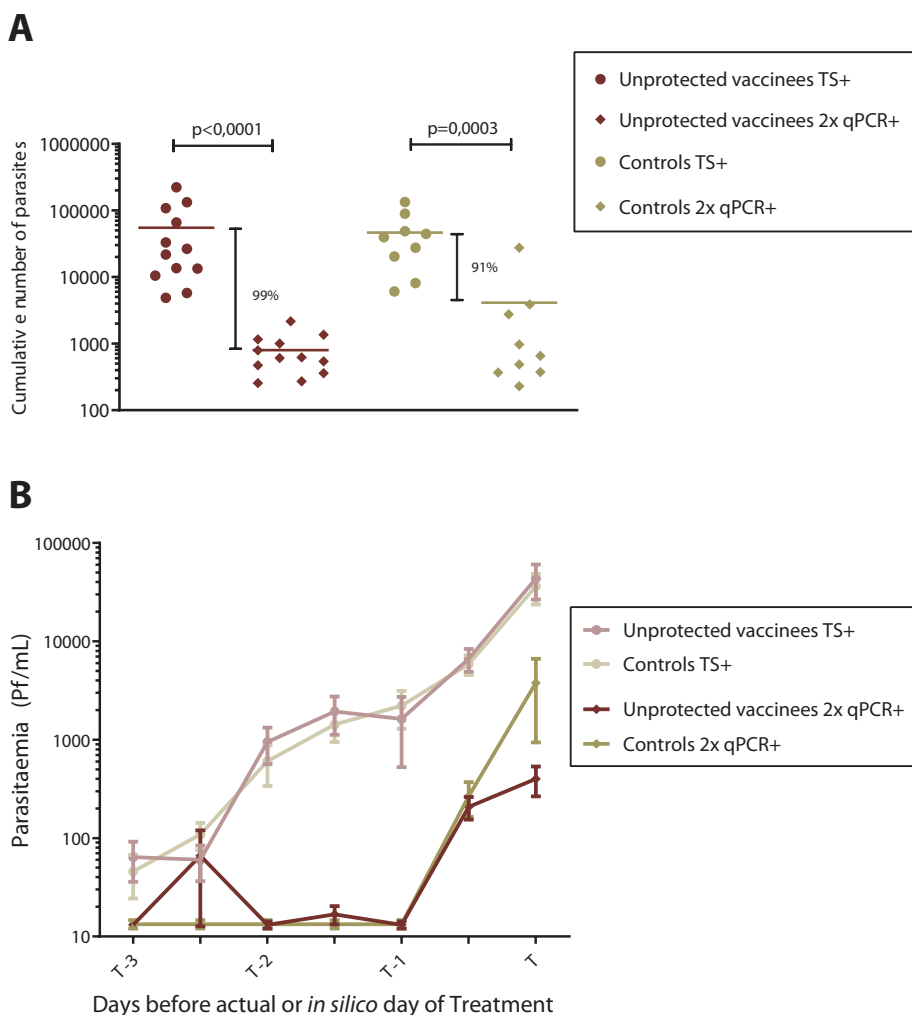


Figure 5.3 Significant reduction of cumulative number of parasites after challenge when applying the new treatment in unprotected vaccinees and controls

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

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

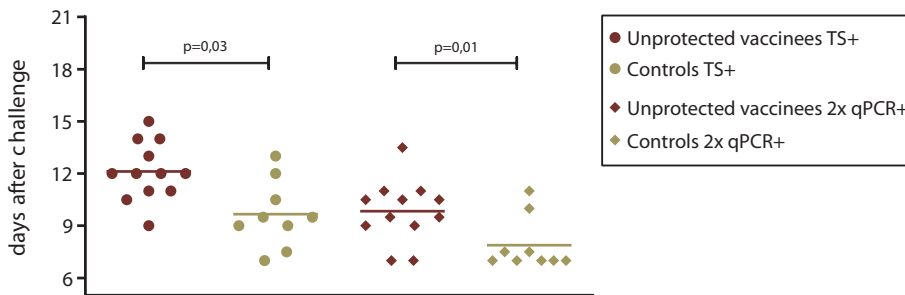


Figure 5.4 Discrimination between unprotected vaccinees and controls remains achievable using second consecutive qPCR-based initiation of treatment.

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

Conclusion

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

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