RESEARCH ARTICLE

REVISED Functional antibodies against *Plasmodium falciparum* sporozoites are associated with a longer time to qPCR-detected infection among schoolchildren in Burkina Faso [version 2; peer review: 3 approved]

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Abstract

Background: Individuals living in malaria-endemic regions develop immunity against severe malaria, but it is unclear whether immunity against pre-erythrocytic stages that blocks initiation of blood-stage infection after parasite inoculation develops following continuous natural exposure.
Methods: We cleared schoolchildren living in an area (health district of Saponé, Burkina Faso) with highly endemic seasonal malaria of possible sub-patent infections and examined them weekly for incident infections by nested PCR. Plasma samples collected at enrolment were used to quantify antibodies to the pre-eryhrocytic-stage antigens circumsporozoite protein (CSP) and Liver stage antigen 1 (LSA-1). *In vitro* sporozoite gliding inhibition and hepatocyte invasion inhibition by naturally acquired antibodies were assessed using *Plasmodium falciparum* NF54 sporozoites. Associations between antibody responses, functional pre-erythrocytic immunity phenotypes and time to infection detected by *18S* quantitative PCR were studied.
Results: A total of 51 children were monitored. Anti-CSP antibody titres showed a positive association with sporozoite gliding motility inhibition (P<0.0001, Spearman's ρ=0.76). *In vitro* hepatocyte invasion was inhibited by naturally acquired antibodies (median inhibition, 19.4% [IQR 15.2-40.9%]), and there were positive correlations between invasion inhibition and gliding inhibition (P=0.005, Spearman's ρ=0.67) and between invasion inhibition and CSP-specific antibodies (P=0.002, Spearman's

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ρ=0.76). Survival analysis indicated longer time to infection in individuals displaying higher-than-median sporozoite gliding inhibition activity (P=0.01), although this association became non-significant after adjustment for blood-stage immunity (P = 0.06).
Conclusions: In summary, functional antibodies against the pre-erythrocytic stages of malaria infection are acquired in children who are repeatedly exposed to *Plasmodium* parasites. This immune response does not prevent them from becoming infected during a malaria transmission season, but might delay the appearance of blood stage parasitaemia. Our approach could not fully separate the effects of pre-erythrocytic-specific and blood-stage-specific antibody-mediated immune responses *in vivo*; epidemiological studies powered and designed to address this important question should become a research priority.

Keywords
malaria, sporozoites, antibodies, immunity, sterilizing, pre-erythrocytic, liver-stage

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REVISED **Amendments from Version 1**

The manuscript has been modified to address the comments made by the three reviewers. The main modifications in this version are related to the discussion and interpretation of the results. We now mention that our approach might not have fully addressed the correlation between immune responses against asexual stage antigens and pre-erythrocytic immunity, and that the multivariate analysis performed that included both types of immunity revealed a non-significant association between infection risk and functional pre-erythrocytic-stage immune responses – these modifications can be seen in the Abstract, Results and Discussion section. In this version, we have also removed the term 'sporozoite-specific', that was used in the first version to describe the assay that assessed antibody responses against whole sporozoites, since we did not show the specificity of these responses. Additionally, we have included descriptive analysis on the relationship between sporozoite gliding and hepatocyte invasion assays. We have also included a few sentences that discuss the potential role of mosquito saliva as a factor influencing our results.

See referee reports

Introduction

The most advanced malaria vaccine, RTS,S (trade name, Mosquirix), induces immune responses that target *P. falciparum* circumsporozoite protein (CSP), and thereby the pre-erythrocytic stages of malaria, and has been shown to be partially effective in delaying the time to clinical malaria episodes[1]. Alongside the RTS,S subunit vaccine, several other pre-erythrocytic stage vaccines are under development, based on subunit or whole-parasite vaccination[2–5]. Vaccination with the attenuated sporozoite vaccine PfSPZ resulted in a protective efficacy of ~48%, as quantified by differences in time to first positive blood smears in malaria-experienced adults in Mali[6]. The results of this and other vaccine trials and the efficient immunisation of malaria-naive individuals with multiple infected mosquito bites while receiving chloroquine[7] contrast with the limited epidemiological evidence of naturally acquired functional immunity to *Plasmodium* pre-erythrocytic stages, which could be possibly linked to the lower number of sporozoites in natural parasite inoculations or to the frequency of host-vector contacts. Individuals living in malaria-endemic regions can develop naturally acquired immunity against severe malaria disease and death[8,9], but it is unclear whether immunity that reduces, entirely or partially, the probability of blood-stage infection after parasite inoculation develops following natural exposure[9]. The high incidence of blood-stage re-infection after effective anti-malarial treatment in adults living in malaria-endemic regions suggests that sterilizing immunity does not develop even after years of repeated infection[10,11]. Similarly, cohort studies that have analysed the relationship between age and risk of *P. falciparum* infection showed no evidence for complete protection against infection and conflicting evidence on whether naturally acquired immunity can result in a different time to patency[12,13]. One of the most detailed studies on this topic reported clear negative associations between age and the risk of clinical malaria or microscopy-detected malaria infection, but similar times to PCR-detected infection for all age groups. The study concluded no or very limited evidence for an age-dependent acquisition of immunity that protects from infection[13].

Given the interest in pre-erythrocytic vaccines, studies are needed to understand natural protective immune responses that target sporozoite and liver-stages of malaria infection. Here, we determine the associations between responses affecting sporozoite gliding motility, hepatocyte invasion and malaria infection risk assessed by weekly quantitative PCR (qPCR) in a cohort of schoolchildren from Burkina Faso exposed to intense malaria transmission.

Results

Study population and follow-up

Of the 58 school-aged children who were recruited and received treatment at enrolment, 6 were PCR-positive 3 weeks after dihydroartemisinin-piperaquine (DHA-PQ) administration and were not eligible to continue follow-up. One child who was only followed for one routine visit, when no infection was detected, and who withdrew from the study, did not have immune responses quantified and was not included in this analysis.

Parasitological and immunological data from the remaining 51 children followed intensively were analysed (Table 1). Every week these study participants were screened for incident infections. All but one study participant had *P. falciparum* parasites detected by *18S* qPCR during follow-up. Malaria infection caused clinical disease in 43/50 children. One child developed symptomatology suggestive of malaria, but no parasites were detected in samples collected before and during the clinical episode; data from this child were censored after the onset of

Table 1. Study population.

Variable	Value
Number of individuals	
Screened and parasite-free by microscopy	58
Presence of parasites post-treatment	6
Consent withdrawn	1
Monitored	51
Age in years, median (IQR)	7.1 (5.7–8.1)
Gender	
Female, % (N)	37.2 (N = 19)
Reported bed net use, % (N)	51.0 (26)
Haemoglobin levels at the beginning of follow-up*, median (IQR)	11.9 (11.3–12.5)
Haemoglobin types	
AA	70.6 (36)
AC	21.6 (11)
AS	5.9 (3)
SS	2.0 (1)
Total number of weekly surveillance visits	222
Weekly visits/participant, median (IQR)	4 (2–6)

*First weekly visit. IQR, interquartile range.

symptoms. The median time from confirmation of the absence of parasites (i.e. 3 weeks after anti-malarial treatment) to infection detection by nested PCR or onset of symptoms was 28 days; one child who did not have parasites detected by nested PCR was not included in this calculation. Similarly, the median time to parasite detection by *18S* qPCR was 30 days. In Figure 1, both the times of first *18S* qPCR positive result and, if applicable, of development of clinical disease are presented for all study participants.

Naturally acquired IgG and IgM antibodies targeting sporozoites

Malaria antigen-specific antibodies to pre-erythrocytic antigens CSP, liver stage antigen (LSA-1) and to asexual lysate were determined in naturally exposed children and malaria-naive European donors by ELISAs. Antibody titres to the CSP pre-erythrocytic antigen were on average low in naturally exposed children and not significantly different from malaria-naive donors (Figure 2A, P=0.11; non-parametric tests were used for all comparisons), while LSA-1 antibody levels were significantly higher in malaria-exposed children compared to malaria-naive donors (Figure 2B, P=0.006). As expected, asexual blood stage-specific antibody titers in naturally exposed children were higher compared to malaria-naive donors (Figure 2C, P<0.0001). In addition to antigen-specific assays, IgG and IgM antibodies recognizing *Plasmodium* sporozoites were quantified using fluorescently labelled anti-IgG and anti-IgM antibodies by flow cytometric analysis. Levels of antibodies targeting whole sporozoites were significantly higher in the study participants compared to malaria-naive donors (Figure 2D, E, P=0.01 and 0.04 for IgG and IgM, respectively). Strong correlations between IgG and IgM antibodies targeting whole sporozoites (Figure S1A in Extended data[14], P=0.0005, Spearman's ρ=0.75), and between CSP-IgG antibody levels and whole sporozoite IgG antibodies (Figure S1B in Extended data[14], Spearman's ρ=0.83, P<0.0001) were also observed.

Naturally acquired antibodies in children neutralize *in vitro* sporozoite gliding motility

The neutralizing activity of naturally acquired antibodies against sporozoite motility was determined in an *in vitro* assay. Sporozoites pre-treated with PBS showed an average gliding trail surface of 7,392 (95% confidence interval [CI] 4,151-9,388) pixels. To characterize the effect of human plasma on sporozoite gliding motility that is independent of naturally acquired immunity, sporozoites were incubated in the presence of plasma from malaria-naive individuals (n=5) and showed an average gliding trail surface of 1,427(95% CI 646.6-2,207) pixels, significantly greater than the gliding trail surface of sporozoites incubated with 30 µg/ml of a monoclonal anti-CSP antibody, our positive control, 141 (95% CI 71.9-225.4) pixels.

Plasma from the majority of cohort participants reduced *in vitro* sporozoite gliding motility with a median gliding inhibition of 59.6% (IQR 27.8-77.0%); incubation of sporozoites with plasma from three participants resulted in lower gliding trail surfaces than the positive control. We defined two groups: one with poor gliding inhibition (individuals whose plasma inhibited less than 20% of sporozoite gliding motility, N=8) and the other with strong gliding inhibition (more than 80% gliding inhibition, N=8). The first group had similar gliding trail surface (median, 1,353; IQR, 1,311.7-1,639.2) compared to malaria-naive donors (median 1605.7,

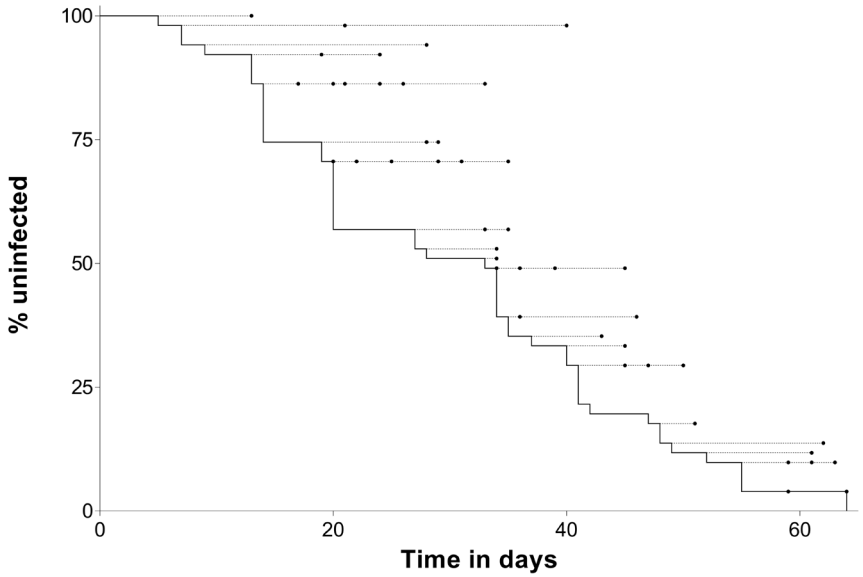

Figure 1. Time to malaria infection detection. Time-to-infection data from all study participants (N=51) are shown. The black line represents the percentage of the study population that remained uninfected at different time-points (y-axis). Circles indicate when individuals with first parasite detection at the start of the corresponding dashed line developed clinical symptoms. Time, x-axis, is relative to the confirmation of parasite clearance, 3 weeks after anti-malarial administration.

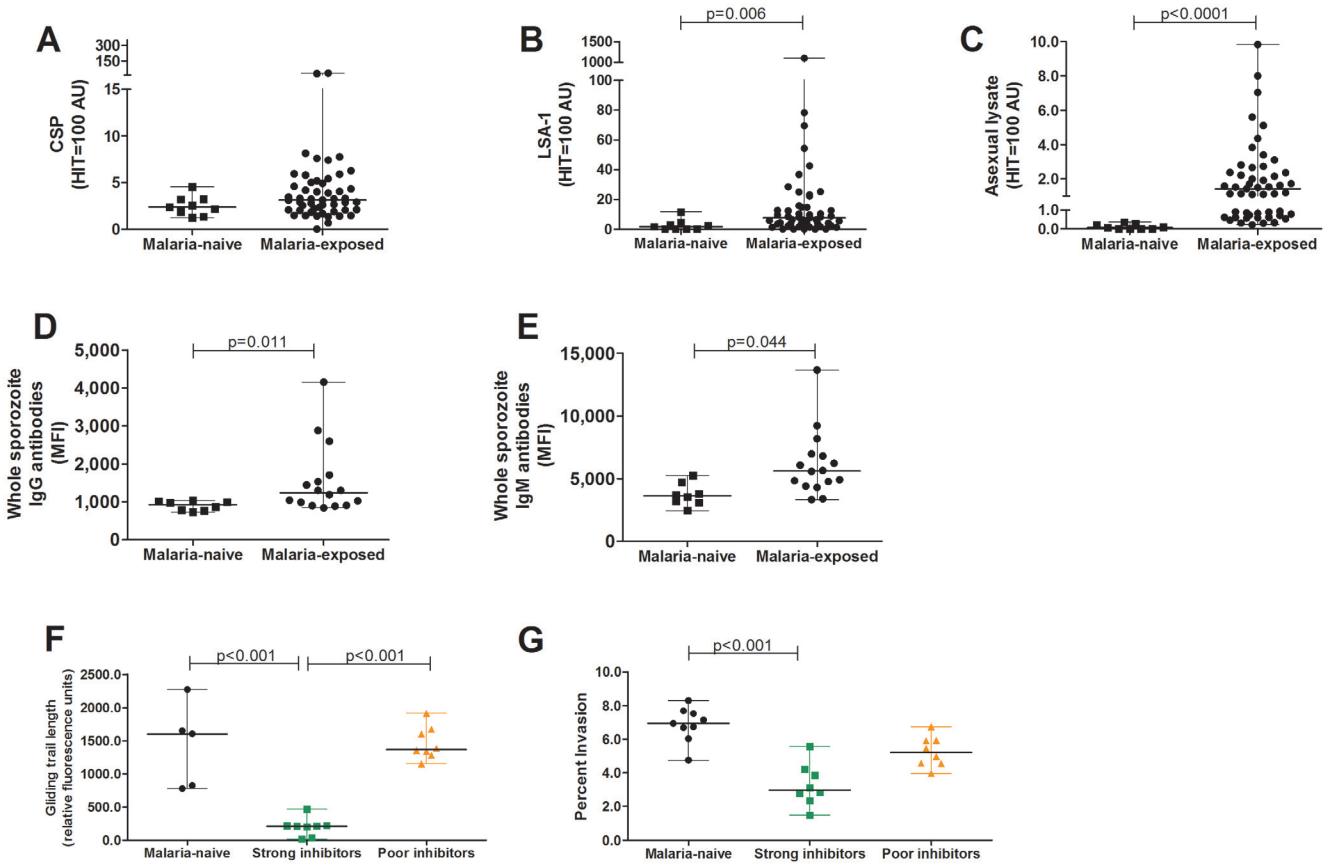

Figure 2. Naturally acquired pre-erythrocytic antibody levels and their functionality against sporozoite infectivity *in vitro*. Malaria antigen-specific antibody levels in children from Burkina Faso (n=51) and European malaria-naive adults (n=9) to the pre-erythrocytic antigens (**A**) circumsporozoite protein (CSP), (**B**) liver-stage antigen-1 (LSA-1), and (**C**) asexual lysate as an internal control were determined by ELISAs and expressed as arbitrary units (AU). The amount of (**D**) IgG and (**E**) IgM antibodies recognizing sporozoites was determined by flow cytometry and shown as the geometric mean fluorescence intensity (MFI). To this end, *P. falciparum* NF54 sporozoites were pre-treated with 10% heat-inactivated plasma from children from Burkina Faso (N=16) and malaria-naive adults (N=8) and stained with fluorescently labelled antibodies against IgG and IgM antibodies. (**F**) The gliding motility of *P. falciparum* NF54 sporozoites, pre-treated with plasma from children from Burkina Faso (N=51) and malaria-naive adults (N=5), was determined by *in vitro* gliding motility assays. Gliding trail length of sporozoites incubated with plasma from malaria-naive donors (N=5) or naturally exposed children who were poor (N=8) or strong (N=8) inhibitors of gliding motility are shown. (**G**) The percent hepatocytes invaded by *P. falciparum* NF54 sporozoites pre-treated with plasma from children from Burkina Faso (N=16) who were shown to be either poor (n=8) or strong (n=8) gliding inhibitors and malaria-naive adults (N=9) was determined by *in vitro* invasion assays in human hepatoma cells. Comparisons between multiple groups were tested with Kruskal-Wallis test with Dunn's multiple comparison post hoc test.

IQR 824.3 - 1,652) (Figure 2F), whilst strong gliding inhibitors had a median trail surface of 186.2 (IQR 9.4 - 208.6), which was significantly lower compared to malaria-naive donors (Figure 2F, P<0.01). *In vitro* gliding inhibition did not correlate with LSA-1 IgG antibody levels (P=0.11, Spearman's ρ=0.23), but correlated with CSP-specific IgG antibodies (Figure S2A in Extended data[14], P<0.0001, Spearman's ρ=0.76), whole sporozoite IgG (Figure S2B in Extended data[14], P<0.0001, Spearman's ρ=0.81) and IgM antibodies (Figure S2C in Extended data[14], P=0.01, Spearman's ρ=0.61). While levels of IgG and IgM antibodies targeting whole sporozoite did not significantly differ between poor gliding inhibitors and malaria-naive adults, whole sporozoite antibody levels of strong gliding inhibitors were significantly higher compared to poor gliding inhibitors (Figure S2D in Extended data[14], P<0.0001 and P=0.005 for IgG and IgM antibodies, respectively) and malaria-naive adults (Figure S2D, E in Extended data[14], P<0.0001 and P=0.0006 for IgG and IgM antibodies, respectively). This suggests that quantitative differences in measured immune responses might explain variation in these functional phenotypes.

Antibodies in malaria-exposed children neutralize *in vitro* sporozoite infectivity of hepatocytes

The inhibitory effect of naturally acquired antibodies on *in vitro* sporozoite invasion of hepatocytes was also assessed in a selected number of samples shown to inhibit gliding motility strongly (n=8) or poorly (n=8). *In vitro* invasion was inhibited by naturally acquired antibodies (median invasion inhibition, 19.4% [IQR, 15.2-40.9%]), and plasma from children categorized as strong gliding inhibitors (see previous section) also prevented hepatocyte invasion more effectively compared to malaria-naive donors (Figure 2G, P<0.001). 7/8 plasma samples from strong gliding

inhibitors reduced hepatocyte invasion by 20% or more, whereas only 2/8 poor gliding inhibitors inhibited at least 20% of hepatocyte invasion. There was a positive correlation between gliding and invasion inhibition (Figure S3A in Extended data[14], P=0.005, Spearman's ρ=0.67), suggesting that *in vitro* gliding inhibition by naturally acquired antibodies might serve as a good surrogate for *in vitro* hepatocyte invasion inhibition. Sporozoite invasion inhibition correlated with IgG targeting whole sporozoites (Figure S3B in Extended data[14], P=0.004, Spearman's ρ=0.67), but not with whole sporozoite IgM antibody levels (Figure S3C in Extended data[14], P=0.13, Spearman's ρ=0.38). There was a correlation of hepatocyte invasion inhibition with CSP-specific IgG antibodies (Figure S3D in Extended data[14], P=0.002, Spearman's ρ=0.76), but not with LSA-1-specific IgG antibodies (P=0.08, Spearman's ρ=0.48).

Evidence of natural risk-modifying pre-erythrocytic immunity

For each immunological assay, ELISA or sporozoite gliding motility assays, children were categorized in two groups: participants with high antibody responses (or high sporozoite gliding inhibition activity) were those with assay values higher than the study population median (Figure 3A); children considered to have low antibody responses or low sporozoite gliding inhibition capacity had values lower than the median. Based on this categorization, study subjects with high CSP responses and those whose plasma inhibited sporozoite gliding movement acquired blood-stage *P. falciparum* infection (qPCR-based parasitaemia ≥ 0.1 parasites/µl) later compared to children with lower CSP responses and less efficient gliding inhibitory activity (Figure 3B; P=0.05 and P=0.01 for CSP responses and sporozoite gliding inhibition, respectively). High anti-LSA-1 antibody levels, on the other hand, did not influence time to infection (P=0.31). Blood-stage immunity, i.e. high response in the asexual stage lysate assay, was also associated with longer time to PCR-detected infection (Figure 3C, P=0.005). We repeated these analyses excluding children with haemoglobin S and haemoglobin C mutations, as these conditions might influence both immunity[15] and parasite carriage[16,17]. Despite the limited number of individuals included in this analysis (n=36), similar results were obtained (P=0.01 for both CSP and asexual stage lysate assays and P=0.05 for the sporozoite gliding inhibition

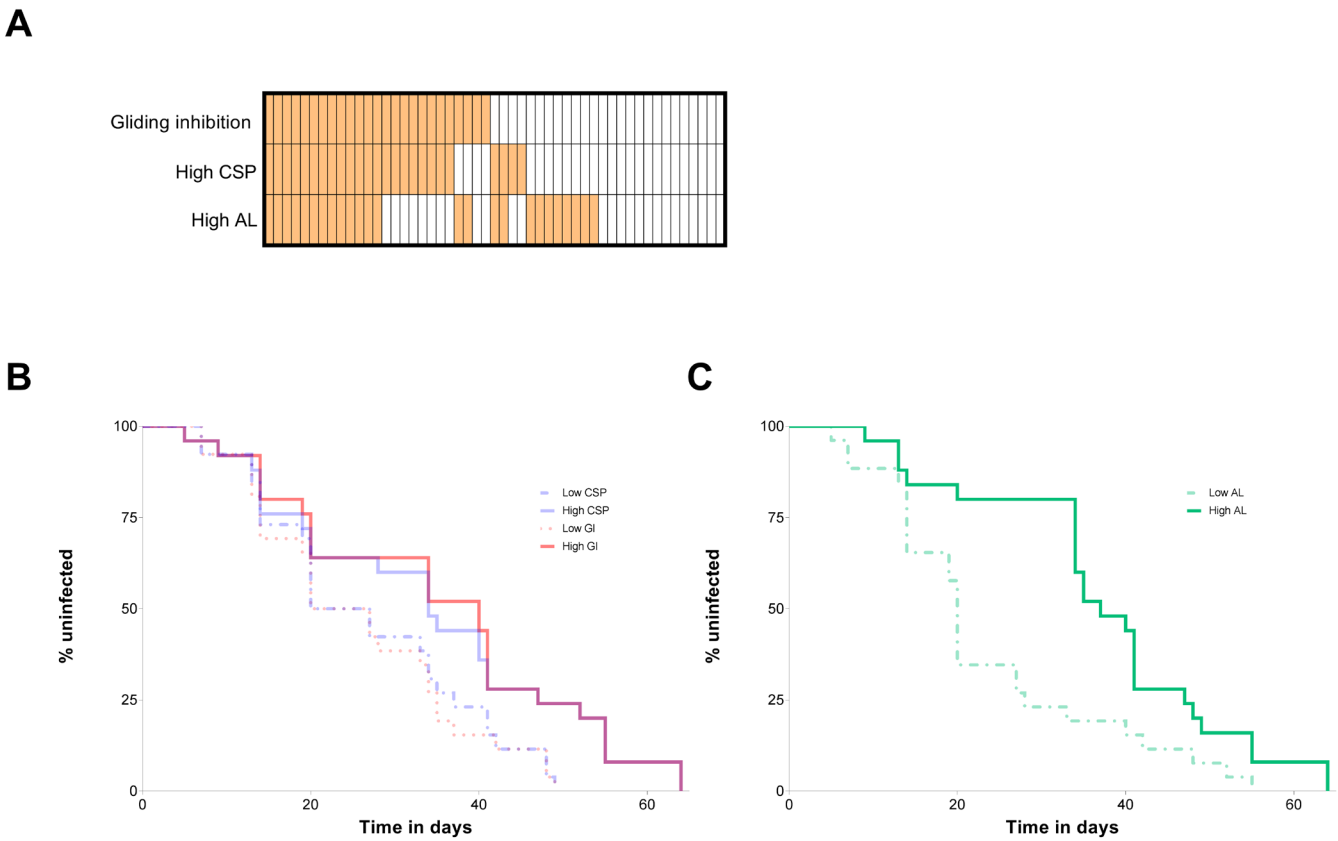

Figure 3. Effects of immune responses against liver- and blood-stage antigens on malaria infection risk. To assess the effect of immune responses on infection risk, children were classified based on whether the results of their assays were higher or lower than the study population median (see Results): in (**A**), vertically aligned cells represent the same participant, and orange cells indicate that assay results are higher than the median (see also Figure S4, that shows a scatter plot of circumsporozoite protein (CSP) antibody levels and immunity against asexual lysate). In (**B**), Kaplan-Meier curves for children with high and low circumsporozoite protein CSP responses and gliding inhibition (GI) phenotypes are presented (N = 51); in (**C**), curves for participants categorized based on their responses to asexual stage antigens (AL) are shown (N = 51). In both panels (**B**) and (**C**), the y-axis corresponds to the percentage of the population uninfected at different time points.

assay). Additional analyses (Extended data[14]) show that functional responses against pre-erythrocytic stages were also associated with infection risk when different parasite density thresholds are used to define infection.

Since children with high CSP responses were more likely to have high antibody responses to asexual blood stage parasite lysate compared to low CSP responders (60% versus 38.5%, respectively; P=0.12), multivariate Cox proportional hazards models were fit to assess the mutually adjusted effects of these immune responses on time to infection detection (Table 2). In a model that also included the results of the asexual stage lysate assay, the relationship between anti-CSP responses and time to malaria infection was not statistically significant. Despite a clear trend for a protective effect, the association between inhibition of *in vitro* sporozoite gliding motility and time to falciparum infection also did not reach statistical significance after adjustment for blood-stage immunity (hazard ratio, 0.55; 95% CI, 0.29–1.01).

Discussion

In this longitudinal study in Burkina Faso, we analysed the associations between functional immune responses against pre-erythrocytic stages of malaria and the incidence of qPCR-detected infections in children aged 5–10 years. We observed that (i) schoolchildren develop antibody responses that can interfere with sporozoite motility and infectivity *in vitro*, and (ii) those children with antibodies that more efficiently reduced *in vitro* sporozoite gliding motility remained uninfected for longer periods of time. Our epidemiological data together with the *in vitro* data provide evidence that there is partially effective pre-erythrocytic immunity that influences individual level infection incidence, although we cannot exclude the contribution of immunity against asexual blood-stage antigens to this observation.

Sterile protection against malaria can be readily demonstrated in human and animal experimental models[7,18,19], but not following natural malaria exposure[13]. Field studies showed conflicting evidence on whether naturally acquired immunity can result in delays to patency and showed no evidence for sterile immunity completely preventing the appearance of parasites in the blood stream of exposed individuals[11,13]. We selected children aged 5–10, who have lower blood stage immune responses (a potential confounder when studying pre-erythrocytic immunity) compared to semi-immune adults, and who allow repeated blood sampling compared to toddlers. Since submicroscopic infections are prevalent in the area[20], we used a curative dose of antimalarials to clear possible sub-patent infections and, upon confirmation that children were parasite-free at the start of the transmission season, assessed the infection incidence by sensitive molecular assays. Our finding that 98% of the cohort became infected with *P. falciparum* within 3 months confirms the high force of infection in the area.

Consistent with previous sero-epidemiological studies[21–24], our results indicate that antibodies to *P. falciparum* sporozoite and liver-stage antigens are acquired following natural exposure. Antibody titres to the CSP antigen were on average low in our cohort and not significantly different from malaria-naive donors. These low titres could be related to the timing of the study recruitment (prior to the transmission season after ~7 months of very low malaria exposure), which would imply that responses to CSP are short lived. The antibody levels could also have been influenced by repeated blood-stage infections in our cohort, which may have suppressed immune responses against the pre-erythrocytic stages[25], although, in theory, responses to both LSA-1 and CSP would be affected. To assess the contribution of pre-erythrocytic humoral immunity to protection, previous studies have related antibodies at baseline with time-to-infection after parasite clearance with antimalarials[12]. A significant challenge in these studies is that both protective immunity and cumulative exposure increase with age, and so it is often unclear whether measured responses mediate protection or are merely a marker of past exposure[26,27]. In an attempt to move beyond indirect epidemiological associations, we explored functional anti-sporozoite immunity by assessing the ability of plasma to inhibit sporozoite gliding motility[28] and hepatocyte invasion[29] and related these *in vitro* phenotypes to field findings. We observed that CSP IgG antibodies showed a strong positive association with sporozoite gliding motility inhibition. It has been demonstrated that *Plasmodium* parasites use the system of adhesion-based motility, gliding, to actively penetrate host cells[30]; and that the invasive ability of sporozoites is associated with their motility[31]. In agreement with these findings, the data presented here suggest that *in vitro* gliding inhibition by naturally acquired antibodies may be a useful surrogate marker for *in vitro* hepatocyte invasion. In an immuno-epidemiological

Table 2. Multivariate Cox proportional hazards models for time-to-infection outcome.

Variable	Hazard ratio	95% CI	P-value
Model I			
High CSP response	0.62	0.34–1.15	0.13
High response to asexual stage lysate	0.49	0.27–0.89	0.02
Model II			
High gliding inhibition activity	0.55	0.29–1.01	0.06
High response to asexual stage lysate	0.52	0.29–0.94	0.03

CI, confidence interval; CSP, circumsporozoite protein.

study undertaken in Indonesia, hepatocyte invasion inhibition was associated with higher anti-CSP antibody titres[32]. In our cohort, subjects with high CSP responses (i.e. higher than the study population median) and those whose plasma more efficiently inhibited sporozoite gliding movement developed *P. falciparum* infection later compared to children with lower responses. To our knowledge, this is the first study to show that there are functional antibodies against pre-erythrocytic malaria stages in malaria-exposed children.

Our study has several limitations. We observed that high responses in the asexual stage lysate assay with unknown functionality were also associated with longer time to infection. We thus cannot rule out a supportive role for asexual antibody responses in the observed associations[33]. Indeed, as pointed out by one of the reviewers of the first version of this manuscript, 17/29 (58.6%) children with high anti-sporozoite responses in either CSP-specific or sporozoite gliding assays had above-median asexual lysate responses versus 8/22 (36.4%) children with below-median anti-sporozoite activities. We believe it is likely that the functionally important pre-erythrocytic antibody responses that we quantified here are acquired alongside anti-blood-stage antibodies. In addition, a recent study in the same geographical area demonstrated that heterogeneity in mosquito exposure contributes considerably to heterogeneity in parasite inoculation risk[34]. In the current study, we used delayed time to blood-stage infection as a simplistic indicator of partial protection, which fails to take into account variation in exposure. In an ideal approach, we would have been able to quantify malaria exposure at an individual level, which may involve linking of blood meals in household-caught mosquitoes to household occupants and determining sporozoites in the salivary glands of these mosquitoes[35]. Measuring exposure at individual level in such an approach will help to shed further light on pre-erythrocytic immunity in naturally exposed individuals. With respect to discriminating between pre-erythrocytic and blood-stage immunity, a valuable but laborious approach would be to examine the observed relationships in a larger cohort that allows stratification based on similar blood stage immunity but different levels of pre-erythrocytic immunity at baseline and that provides sufficient power to detect weaker associations. Another limitation of our study is that we were only able to determine humoral responses. Cellular responses to pre-erythrocytic stages have been implicated in malaria protection in multiple studies[36,37]. It is conceivable that by quantifying both antibody and cellular responses we would be able to better define natural immunological phenotypes associated with differential malaria risk[38]. Immunity to mosquito saliva might have also influenced sporozoite invasion although the magnitude of such an effect compared to the established impact of anti-sporozoite responses is currently unknown and it is currently unknown whether this would require responses to antigens that are conserved between *A. stephensi* mosquitoes used for sporozoite production and vectors that study participants were naturally exposed to 39.

In summary, in our cohort of children, anti-CSP antibodies were strongly associated with *in vitro* sporozoite gliding inhibition and hepatocyte invasion inhibition. Children with functional anti-sporozoite antibody responses had a longer time to *P. falciparum* infection compared to children with lower functional responses, suggesting that these *in vitro* assays are relevant to understand natural protection. The partial protection (i.e., delay in infection) observed in our study does not prevent individuals from becoming infected during an entire transmission season, but reduces infection incidence and consequently needs to be considered in epidemiological studies aiming to understand malaria risk heterogeneity and in malaria vaccines trials. Identifying host or parasite factors linked to these functional immunological phenotypes and characterizing how these phenotypes change with cumulative exposure to malaria parasites will help the understanding of why natural immunity against pre-erythrocytic stages is incomplete.

Methods

Study design

This study was performed from June to December 2015 in the village of Balonghin in the Saponé health district, Burkina Faso, which is exposed to intense and seasonal *P. falciparum* transmission[20]. Written informed consent was provided by the parent or guardian of each child. The study was approved by the ethics committees of the London School of Hygiene and Tropical Medicine (reference number 9008) and the Ministry of Health in Burkina Faso (reference number 2015-3-033). Children aged 5–10 years with haemoglobin levels above 8 g/dl and no *Plasmodium* parasites detected by microscopy were eligible. DHA-PQ was used to clear sub-microscopic infections. At 3 weeks (20–22 days) after treatment, finger-prick blood samples were collected to ensure parasite negativity by nested PCR[40] prior to formal enrolment into the cohort. Citrated plasma samples were collected before treatment using citrated vacutainer cell preparation tubes (CPT vacutainers, Becton Dickinson), stored at -80°C and used for malaria-antigen-specific IgG ELISAs and sporozoite assays. Peripheral blood mononuclear cells (PBMC) were also collected, but were lost due to the inability to maintain liquid nitrogen supplies during civil unrest in Ouagadougou. Following enrolment, participants were examined during weekly visits, when finger-prick samples were collected for *P. falciparum* nested PCR that was performed within 48 hours. Following parasite detection, finger-prick blood samples were collected every day for 1 week, and every week afterwards, up to 35 days after parasite detection. Study participants were closely monitored for the development of malaria symptoms. Artemether-lumefantrine was given upon the detection of symptoms or 35 days after initial detection of infection by nested PCR, whichever came first. For the current analyses, only the time to first infection detection was used and related to baseline immunological assays.

Molecular analyses

Nucleic acids from 100 µl whole-blood samples stored in RNAprotect Cell Reagent were extracted using MagNAPure LC automatic extractor (Total Nucleic Acid Isolation Kit—High Performance, Roche Applied Science) and used for qPCR targeting *18S* rRNA[41]. Genomic DNA from the same extraction was used to test for human haemoglobinopathies haemoglobin S and C[42].

Parasite culture and generation of P. falciparum-infected mosquitoes

As source of sporozoites, *Anopheles stephensi* mosquitoes were infected by standard membrane feeding on *P. falciparum* NF54 gametocyte cultures[43]. Salivary glands from infected mosquitoes

were dissected, collected in Leibovitz culture medium (Lonza) without serum (supplemented with 1% penicillin-streptomycin and 1% L-glutamine for *in vitro* gliding motility assays), and homogenized in a homemade glass grinder. The number of sporozoites was counted in a Bürker-Türk counting chamber using phase contrast microscopy[18].

Human hepatoma HC-04 cell line

The HC-04 human hepatoma cell line[29] was acquired through MR4 as part of the Biodefense and Emerging Infections Research Resources Repository (BEI Resources). Hepatoma cells (referred to as hepatocytes) were cultured in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 nutrient mixture medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO), 1% glutamine and 1% penicillin/streptomycin (GIBCO) at 37°C in an atmosphere of 5% CO_2.

Enzyme-linked immunosorbent assays and sporozoite opsonization assays

Levels of antibodies were determined to circumsporozoite protein (CSP: full-length *P. falciparum* NF54 CSP with repeats, produced in *E. coli* by Gennova Biopharmaceuticals Ltd., Pune, India), LSA-1 (LSA-NRC construct expressed in *E. coli*) and asexual lysate using previously reported standardized enzyme-linked immunosorbent assays (ELISAs)[44,45] in naturally exposed children (n=51) and malaria-naive European donors (n=9). Antibody levels were calculated in relation to the positive control (hyperimmune plasma pool from Tanzania) that was set at 100 arbitrary units (AU) using Auditable Data Analysis and Management System (ADAMSEL, version 1.1)[19].

Recognition of whole sporozoites by naturally acquired IgG and IgM antibodies was determined by an *in vitro* flow-cytometry-based antibody opsonization assay that was presented in detail elsewhere[46]. Due to limited plasma availability and available sporozoite numbers, 16 naturally exposed children with highest (8) and lowest (8) gliding activity were selected for sporozoite opsonization assays and invasion assays, allowing us to investigate potential correlations. Flow cytometric analysis was performed with a LSRII flow cytometer (BD BioSciences); data analysis by FlowJo software (version 10.0.8, Tree Star).

Malaria-naive donors are healthy malaria-naive European volunteers who participated in CPS-immunization trial (immunization of malaria-naive human volunteers under chloroquine prophylaxis with sporozoites delivered by mosquito bites) at the Radboud University Medical Center (Nijmegen, The Netherlands)[47]. Written informed consent was obtained from these individuals including for their samples to be stored and used in additional immunological experiments. Pre-immunization samples collected before the CPS-immunization were used for analysis of malaria antigen-specific antibody levels.

In vitro sporozoite gliding motility assay

Prior to *in vitro* sporozoite assays, plasma aliquots were heat-inactivated for 30 minutes at 56°C, centrifuged at 13,000 rpm for 5 minutes at room temperature and kept at 4°C. Flat-bottom optical-bottom 96-well plates with cover glass base were incubated overnight at 4°C with an anti-CSP monoclonal antibody (produced at Radboudumc Nijmegen, Netherlands[48]) 3SP2; 5 µg/ml in PBS). Following incubation, wells were washed twice with 150 µl/well PBS, blocked for 20 minutes at room temperature with 100 µl/well Leibovitz medium (Lonza) supplemented with 1% penicillin-streptomycin (GIBCO), 1% L-glutamine (GIBCO) and 10% heat-inactivated FBS(GIBCO). *P. falciparum* NF54 sporozoites (100 µl) were pre-incubated with citrated samples (70 µl; 40% final concentration) for 30 minutes at room temperature and added to each well in triplicate (50 µl/well) at a concentration of 10,000 sporozoites/well. Sporozoites were allowed to glide for 90 minutes at 37°C, 98% humidity, 93% N_2, 4% CO_2 and 3% O_2. Wells were washed thrice with 100 µl/well PBS and gliding trails were fixed for 15 minutes at room temperature with 4% paraformaldehyde (Affymetrix). Following fixation, wells were washed thrice with 100 µl/well PBS and blocked with 150 µl/well 10% FBS/PBS for 20 minutes at room temperature. Subsequently, gliding trails were stained for 1 hour at room temperature with 50 µl/well 5 µg/ml biotinylated anti-CSP monoclonal antibody (anti-CSP 3SP2 antibodies were produced at Radboudumc, Nijmegen, the Netherlands[48]), followed by a wash step (thrice with PBS) and a 1 hour incubation at room temperature with 50µl/well 10 µg/ml streptavidin-Alexa Fluor-594 (Life Technologies) diluted in 10% FBS in PBS. Subsequently, wells were washed thrice with 100 µl/well PBS and stored in 150 µl/well PBS at 4°C in the dark until analysis. Gliding trails were imaged automatically with the BioTek Cytation cell imager (25 images per well at 200x magnification) and images were analysed automatically by FIJI software (under ImageJ version 2.0.0-rc-68/1.52h) with Otsu's thresholding[28]. Results were plotted in GraphPad Prism version 5.03.The number of pixels present on a stitched image made from 25 individual pictures taken per well is a measure of the amount of shed CSP in that particular well and therefore, differences in the number of pixels can be interpreted as differences in sporozoite gliding trail surface[28].

In vitro sporozoite infectivity assay of a human hepatoma cell line

Neutralization of *P. falciparum* sporozoite hepatocyte invasion by naturally acquired antibodies was assessed in a flow-cytometry-based *in vitro* invasion assay, as previously described with small adaptations[46]. Briefly, freshly dissected *P. falciparum* NF54 sporozoites were added to heat-inactivated plasma samples (10% final concentration) from malaria-naive or malaria-exposed individuals and pre-incubated for 30 minutes at 4°C. Subsequently, the sporozoite-plasma mixtures (5.10^4 sporozoites in the presence of 10% plasma) were added to HC-04 hepatocytes in 96-well plates. Following 3 hours of incubation at 37°C in 5% CO2, invaded and intracellular sporozoites were stained with an Alexa Fluor 488-conjugated anti-CSP antibody. Flow cytometric analysis was performed with a Gallios (Beckman Coulter) flow cytometer and data were analysed with FlowJo software (version 10.0.8, Tree Star). The percentage of CSP-positive hepatocytes was first corrected for background reactivity by subtracting the

background (uninfected HC-04 cells in the presence of 3SP2-Alexa Fluor-488 antibody). The percent invasion inhibition was expressed relative to control IgG.

Statistical analysis

For analysis of *in vitro* sporozoite data, comparisons between two (controls versus field samples) or multiple groups were performed using Mann-Whitney U-test and Kruskal-Wallis test followed by Dunn's test between two groups, respectively. The associations between immune responses and malaria infection risk were assessed using survival analysis methods. Log-rank test was used to compare times to infection incidence for individuals with different values of immune phenotypes. Cox survival models were fit to assess the effect of pre-erythrocytic immunity after adjustment for blood-stage immunity; the proportional hazards assumption was tested using Schoenfeld residuals. In these analyses, study participants were considered to have high or low responses (binary explanatory variables) based on the study population median (see Figure S5 in Extended data[14]). The first scheduled weekly visit or intensive follow-up visit when parasitaemia of at least 0.1 parasites per µl was detected by *18S* qPCR was considered the time of infection incidence. Using this criterion, the median 18S qPCR-based parasitaemia at infection detection was 2.2 parasites per µl and the interquartile range was 0.4 – 80.4. This threshold of parasitaemia was chosen to minimise false-positive results. In the supplemental material, sensitivity analyses were included that used different cut-offs of *18S* qPCR-based density to determine infection positivity. Stata 14 (StataCorp LP, Texas, USA) and GraphPad Prism software (version 5, GraphPad Software Inc., California, USA) were used for statistical analysis. P<0.05 was considered statistically significant.

Data availability

Underlying data

The main dataset relating to the field study contains individual level data and identifying information on participants; as such, this dataset is stored under restricted access and not available through an open-access repository. Requests from researchers to access these data for pooled or meta-analysis should be addressed to the corresponding author (teun.bousema@radboudumc.nl). However, the dataset used in the survival analysis has been de-identified and is available from the Dryad repository, along with raw ELISA results and sporozoite gliding data. DOI: https://dx.doi.org/10.5061/dryad.n1m33qq[14].

Extended data

The results of sensitivity analyses (see Results section) and Figure S1–Figure S5 are available from the Dryad repository.

Figure S1. Correlation analyses of naturally acquired whole sporozoite IgG and IgM antibodies. (A) Scatter plot of whole sporozoite IgG and IgM antibodies targeting *P. falciparum* NF54 sporozoites is shown (n=17). (B) Correlation analysis for *P. falciparum* NF54 sporozoite and CSP-specific IgG antibodies as determined by ELISAs is shown.

Figure S2. Antibody specificity and *in vitro* gliding inhibition by naturally acquired antibodies. (A). Scatter plot of *in vitro* gliding inhibition and CSP-specific IgG antibodies as determined by ELISAs is shown (n=51). Samples selected for additional invasion experiments had either poor (orange) or strong (green) gliding inhibitory activity. Correlation analysis for whole sporozoite (B) IgG or (C) IgM antibodies and *in vitro* gliding inhibition by naturally acquired antibodies was conducted with samples from 16 children. Recognition of *P. falciparum* NF54 sporozoites by whole sporozoite **(D)** IgG and (E) IgM antibodies from naturally exposed children (n= 16) or malaria-naive adults (n=8) was shown as the geometric mean fluorescent intensity (MFI) and divided in subgroups: malaria-naive adults (black), poor (orange) *versus* strong gliding inhibitors (green). Correlation analyses were conducted with Spearman correlation analysis. Comparisons between multiple groups were tested by Kruskal Wallis test.

Figure S3. Inhibition of *in vitro* sporozoite invasion of hepatocytes by antibodies from children in Burkina Faso. Gliding motility and invasion of *P. falciparum* NF54 sporozoites pre-treated with plasma from children from Burkina Faso and malaria-naive adults was determined by *in vitro* gliding motility and invasion assays in human hepatoma cells. (A) Scatter plot of *in vitro* gliding and invasion inhibition by naturally acquired antibodies is shown. Additionally, correlation analyses of the percent invasion inhibition with whole sporozoite (B) IgG, (C) IgM antibodies or (D) CSP-specific IgG antibodies is shown. Children whom had poor or strong neutralizing effect on sporozoite infectivity are shown in orange and green circles, respectively.

Figure S4. CSP antibody levels versus immune responses against asexual stage antigens. In this figure, the x-axis shows CSP antibody levels; the y-axis represents antibody responses against asexual stage parasites lysate. Both axes are in log-scale; one child with undetectable CSP response is not included in this graph.

Figure S5. Distribution of immune phenotypes. The distributions of $\log_{10}$-transformed antibody responses (x-axes) against CSP, LSA-1 and asexual blood stage lysate (AL) are presented in panels (A), (B) and (C). The y-axes in these panels represent the percentages of study population with various levels of responses. In (A) and (B), one and seven individuals had undetectable responses and, to be included in this figure, were assigned response values

equivalent to half of the lower limit of detection. In (D), the results of gliding assays are presented: the left plot presents the distribution of $\log_{10}$-transformed sporozoite gliding surface; the right plot shows gliding inhibition (y-axis) for each study participant (different bars; x-axis). The median gliding inhibition (59.6%) was used to define high and low inhibition in the survival analysis.

DOI: https://doi.org/10.5061/dryad.n1m33qq.2[14].

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Version 2

Reviewer Report 15 May 2019

https://doi.org/10.21956/wellcomeopenres.16607.r35410

✔ **Carlota Dobaño**
ISGlobal, Hospital Clínic, University of Barcelona, Barcelona, Spain

The authors have addressed the points raised in the review.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 13 May 2019

https://doi.org/10.21956/wellcomeopenres.16607.r35411

✔ **Silvia Portugal**
Malaria Infection Biology and Immunity Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA

The manuscript version following the reviewer's suggestions and comments has improved and I have no problems with the current version.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Malaria, immunoparasitology.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 19 February 2019

https://doi.org/10.21956/wellcomeopenres.16280.r34757

?

Carlota Dobaño
ISGlobal, Hospital Clínic, University of Barcelona, Barcelona, Spain

The study by Barry and colleagues is important to shed more light into the efficiency of naturally-acquired sporozoite immunity to interfere with the parasite development at the pre-erythrocytic stages. Functional antibodies are assessed *in vitro* in plasma from *P. falciparum* exposed children for their capacity to interfere with sporozoite gliding and hepatocyte invasion which are required for progress with their life cycle to liver and blood stages, and these are related to titers. The outcome measured is blood stage parasitemia (delay of appearance of subpatent infections) as no direct measurement of liver parasitemia can be done in humans.

Some changes are required to clarify some aspects and make the work more scientifically sound:

ABSTRACT

In the Abstract background, specify if the statement "it is unclear whether immunity that affects the establishment of infections develops following continuous natural exposure" refers to children or in general, and if it refers to naturally acquired immunity as typically perceived (mostly against blood stages) or here if specific for pre-erythrocytic immunity. This also applies to the main Introduction. Although sterilizing immunity does not develop, parasite densities tend to decrease with age.

In the Abstract Methods it would be relevant to specify the intensity of malaria transmission and the specific area of study (location) within Burkina Faso, to know how repeatedly are they exposed to Pf. Also clarify if it is qPCR or nested PCR (also in results, it is a bit confusing when each is used). Liver stage antigen: which one? Specify also that the functional sporozoite assays are *in vitro*.

In the results, mention if CSP antibody titres correlated with hepatocyte invasion and if the latter correlated to delayed time to qPCR +ve.

INTRODUCTION

See comments in the Abstract section.

METHODS

- Can it be clarified if children did not receive antimalarial treatment, if during the weekly monitoring visits the nested PCR (done 48h after bleeding) was Pf positive but they had no symptoms (at least

for 35 days)? If so, this was accepted by ethics committees easily? Were the families communicated of a positive PCR reaction in absence of symptoms?
- Indicate where the sporozoites were generated.
- Indicate if and how the patent parasitemias were also monitored and taken into account in the analysis.

RESULTS
- When presenting data on median invasion inhibition, please comment on whether these % are high, moderate or low, and give some references.
- The following statement does not look convincing by looking at the Fig S3A scatter plot despite the P=0.02 and Spearman's ρ=0.60 that appears driven by exteme values: "There was a positive correlation between gliding and invasion inhibition suggesting that *in vitro* gliding inhibition by naturally acquired antibodies might serve as a good surrogate for *in vitro* hepatocyte invasion inhibition." The correlation in Fig S3B is clearer.
- Fig 3A: interesting to see that there is not always overlap of the three metrics, expected to be discussed. Why was hepatocyte invasion inhibition not included?
- Explain the rationale for categorizing in high and low responders. Was the analysis also done with continuous values but did not yield significant insights?
- The joint analysis of the antibody levels to CSP and blood stage lysate and the functional pre-erythrocytic responses is helpful, but shouldn't there be a model with the 3-4 responses together? If not, explain why.

DISCUSSION
- It is not convincing to say that children 5-10 years in a highly endemic area of Burkina Faso have limited blood stage immunity. They will have quite high antibody responses to blood stage antigens already. Better to reformulate.
- It is surprising the lack of difference in antibody levels for CSP between these children and naive volunteers. Even if CSP is not as highly immunogenic as many blood stage antigens, the responses are usually significant and detectable, even in children. Could there be a problem with the capture antigen? Seasonality by itself may not totally explain this, or it would imply they are very short lived. The potential inhibition by blood stage has been proposed but it would also affect LSA1.
- It is not well discussed how the affection of gliding and invasion before liver stage is detected as delay in positive qPCR in the blood, how do authors think this works

As mentioned, the study is valuable due to the lack of data on functional pre-erythrocytic antibody responses in exposed African children.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: malaria immunology and vaccines

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 22 Apr 2019

Bronner Goncalves, London School of Hygiene and Tropical Medicine, London, UK

Comment

"The study by Barry and colleagues is important to shed more light into the efficiency of naturally-acquired sporozoite immunity to interfere with the parasite development at the pre-erythrocytic stages. Functional antibodies are assessed in vitro in plasma from P. falciparum exposed children for their capacity to interfere with sporozoite gliding and hepatocyte invasion which are required for progress with their life cycle to liver and blood stages, and these are related to titers. The outcome measured is blood stage parasitemia (delay of appearance of subpatent infections) as no direct measurement of liver parasitemia can be done in humans.

Some changes are required to clarify some aspects and make the work more scientifically sound:

ABSTRACT

In the Abstract background, specify if the statement "it is unclear whether immunity that affects the establishment of infections develops following continuous natural exposure" refers to children or in general, and if it refers to naturally acquired immunity as typically perceived (mostly against blood stages) or here if specific for pre-erythrocytic immunity. This also applies to the main Introduction. Although sterilizing immunity does not develop, parasite densities tend to decrease with age."

Answer
In the sentence mentioned by the reviewer, we refer to the fact that there is little or no evidence that naturally acquired immunity against liver stage parasites blocks initiation of blood-stage infection after parasite inoculation by *Anopheles* mosquitoes, including in adults. The term "establishment of infections" in that sentence is equivalent to "release of merozoites, from hepatocytes, in the blood".

We now modified two similar sentences in the *Abstract* and *Introduction* section:

"Individuals living in malaria-endemic regions develop naturally acquired immunity against severe malarial disease, but it is unclear whether immunity against pre-erythrocytic stages that blocks initiation of blood-stage infection after parasite inoculation develops following continuous natural exposure." (Abstract)

"Individuals living in malaria-endemic regions can develop naturally acquired immunity against severe malaria disease and death, but it is unclear whether immunity that reduces, entirely or partially, the probability of blood-stage infection after parasite inoculation develops following natural exposure." (Introduction section)

Comment

"In the Abstract Methods it would be relevant to specify the intensity of malaria transmission and the specific area of study (location) within Burkina Faso, to know how repeatedly are they exposed to Pf. Also clarify if it is qPCR or nested PCR (also in results, it is a bit confusing when each is used). Liver stage antigen: which one? Specify also that the functional sporozoite assays are in vitro.

In the results, mention if CSP antibody titres correlated with hepatocyte invasion and if the latter correlated to delayed time to qPCR +ve."

Answer

We now mention in the *Abstract* the study area and clarify which liver stage antigen was studied:

"We cleared schoolchildren living in an area (health district of Saponé, Burkina Faso) with highly endemic seasonal malaria of possible sub-patent infections and examined them weekly for incident infections by nested PCR. Plasma samples collected at enrolment were used to quantify antibodies to the pre-erythrocytic-stage antigens circumsporozoite protein (CSP) and Liver stage antigen 1 (LSA-1). In vitro sporozoite gliding inhibition by naturally acquired antibodies was assessed using Plasmodium falciparum NF54 sporozoites; hepatocyte invasion was assessed using the human HC-04 hepatoma cell line and NF54 sporozoites. The associations between antibody responses, functional pre-erythrocytic humoral immunity phenotypes and time to infection detected by 18S quantitative PCR were studied."

We have also included information on the correlation between *in vitro* hepatocyte invasion inhibition and anti-CSP antibody levels:

"*In vitro hepatocyte invasion was inhibited by naturally acquired antibodies (median invasion inhibition, 19.4% [IQR 15.2-40.9%]), and there were positive correlations between invasion inhibition and gliding inhibition (P=0.005, Spearman's ρ=0.67) and between invasion inhibition and CSP-specific antibodies (P=0.002, Spearman's ρ=0.76)*."

The hepatocyte invasion inhibition phenotype was not included in the survival analysis as only 16 participants had samples tested with this assay.

Comment

"INTRODUCTION
See comments in the Abstract section."

Answer

We addressed these comments in our answer to the first comment of Reviewer 3.

Comment

"METHODS
Can it be clarified if children did not receive antimalarial treatment, if during the weekly monitoring visits the nested PCR (done 48h after bleeding) was Pf positive but they had no symptoms (at least for 35 days)? If so, this was accepted by ethics committees easily? Were the families communicated of a positive PCR reaction in absence of symptoms?"

Answer

In this study, we monitored children with weekly samples to identify new infections during the transmission season. We used nested PCR to detect *early, i.e. low density, infections* and characterize for the first time the evolution of naturally acquired acute infections in partially immune children. Children with parasites detected by nested PCR were closely monitored for development of symptoms and treated with first line therapy when symptoms developed. Burkina Faso's national malaria guidelines did not include treatment of asymptomatic parasite carriage, consequently initial detection of parasites, often at low levels, by nested PCR was not sufficient to prompt administration of antimalarials. It should be noted, and we have clarified this in the *Methods* section, that children received a long-lasting net at the start of the study and were guaranteed access to care 24 hours per day. Moreover, children were clinically examined on a daily basis in the first week of detection of infection. In this manner, the study adhered to the national guidelines but provided improved (access to) care for all study participants.

Parents and guardians were informed that study participation could involve intensive follow-up if parasites were detected in their child's blood samples and were oriented to contact the study staff if their child developed symptoms. The study was approved by the ethics committee of the London School of Hygiene and Tropical Medicine (reference number 9008) and the Ministry of Health in Burkina Faso (reference number 2015-3-033). Of note, previous studies with similar design were performed that described the development of malaria symptoms in children after detection of patent infections (e.g. M. A. Missinou, B. Lell, P. G. Kremsner, *Uncommon asymptomatic Plasmodium falciparum infections in Gabonese children*. Clin Infect Dis 2003). In the study by Missinou and colleagues, children were followed every second week for detection of infection by blood smear, and after malaria infection was diagnosed, children were clinically monitored with daily blood smears. In large cohort studies conducted in the context of the International Centers of Excellence for Malaria Research (ICEMR) program in Uganda, participants of all age groups (including much younger and with less prior malaria exposure compared to the current study) are followed every 4 weeks with asymptomatic infections sometimes remaining untreated for more than a year (Moss et al. *Malaria Epidemiology and Control Within the International Centers of Excellence for Malaria Research.* Am J Trop Med Hyg. 2015). An important difference between some of these studies and ours is that we used molecular methods to be able to detect infections from the early, sub-patent, phases and we also characterized malaria infectiousness over time,

which will be described in a separate manuscript (*in preparation*). In conclusion, we appreciate the discussion about the necessity to treat asymptomatic infections and believe this is an important discussion to have, but the current study followed national guidelines and is by no means unique in following asymptomatic infections without treatment but with excellent care.

Comment

"Indicate where the sporozoites were generated."

Answer

As described in the *Methods* section, *Anopheles stephensi* mosquitoes were infected by standard membrane feeding assays on *P. falciparum* NF54 gametocyte cultures. These experimental mosquito infections were performed in the insectarium of the Department of Medical Microbiology of the Radboud University Nijmegen Medical Centre.

Comment

"Indicate if and how the patent parasitemias were also monitored and taken into account in the analysis."

Answer

The analysis presented in this manuscript focuses on the influence of immune factors on infection establishment, i.e. appearance of falciparum parasites in the blood. Consequently, all different comparisons presented relate to the first visit of each participant when parasites were detected by sensitive molecular methods. Blood smears were also performed for all study visits, before and after infection detection, but results were not immediately available. The relationship between blood smear results and parasite and gametocyte levels quantified by molecular methods after infection detection will be described in detail in a separate manuscript (*in preparation*).

Comment

"RESULTS
When presenting data on median invasion inhibition, please comment on whether these % are high, moderate or low, and give some references."

Answer

The assessment of whether *in vitro* hepatocyte invasion inhibition was high, moderate or low requires comparisons of inhibition percentages between different studies, which might be less valid than within-study comparisons between samples from different participants tested with the same protocol. Furthermore, without an established quantitative relationship between the results of this assay and *in vivo* infection incidence, semi-quantitative statements of this type are difficult to interpret. Some studies that performed the same *in vitro* assay were cited:

Behet et al. *The complement system contributes to functional antibody-mediated responses induced by immunization with Plasmodium falciparum malaria sporozoites*. Infect Immun. 2018

Sattabongkot et al. *Establishment of a human hepatocyte line that supports in vitro development of the exo-erythrocytic stages of the malaria parasites Plasmodium falciparum and P. vivax.* Am J Trop Med Hyg. 2006

Comment

"The following statement does not look convincing by looking at the Fig S3A scatter plot despite the P=0.02 and Spearman's ρ=0.60 that appears driven by extreme values: "There was a positive correlation between gliding and invasion inhibition suggesting that in vitro gliding inhibition by naturally acquired antibodies might serve as a good surrogate for in vitro hepatocyte invasion inhibition." The correlation in Fig S3B is clearer."

Answer

In our analysis of the correlation between *in vitro* hepatocyte invasion inhibition and *in vitro* sporozoite gliding inhibition, we used the Spearman's rank correlation method, which assessed the correlation between the ranking of the values in these two phenotypes, rather than the values themselves. As additional descriptive information, we added the following sentence:

"*7/8 plasma samples from strong gliding inhibitors reduced hepatocyte invasion by 20% or more, whereas only 2/8 poor gliding inhibitors inhibited at least 20% of hepatocyte invasion. There was a positive correlation between gliding and invasion inhibition (Figure S3A in Extended data14, P=0.005, Spearman's ρ=0.67), suggesting that in vitro gliding inhibition by naturally acquired antibodies might serve as a good surrogate for in vitro hepatocyte invasion inhibition.*"

Comment

"Fig 3A: interesting to see that there is not always overlap of the three metrics, expected to be discussed. Why was hepatocyte invasion inhibition not included?"

Answer

The *in vitro* hepatocyte invasion assay was only performed in a subset of the study population (16 children) and for this reason was not included in this figure and in the survival analysis. The invasion experiments are highly laborious and resource intensive. We used these as supportive experiments in our study, to confirm the relevance of gliding assays, but invasion assays did not form the core of our analysis. We therefore feel that our approach to perform these experiments on a selective number of samples is justified.

Comment

"Explain the rationale for categorizing in high and low responders. Was the analysis also done with continuous values but did not yield significant insights?"

Answer

The survival analysis was only performed with binary variables. The rationale for categorizing the study population in high and low responders, based on the median, was to facilitate interpretation of the results and avoid assumptions about linearity of effect.

Comment

"The joint analysis of the antibody levels to CSP and blood stage lysate and the functional pre-erythrocytic responses is helpful, but shouldn't there be a model with the 3-4 responses together? If not, explain why."

Answer

Since CSP-specific antibody levels are highly correlated with sporozoite gliding inhibition (see *Results* section), we decided not to include these two variables in the same model. Additionally, the limited sample size also influences the number of predictors that can be reasonably included in our multivariate analyses.

Comment

"DISCUSSION
It is not convincing to say that children 5-10 years in a highly endemic area of Burkina Faso have limited blood stage immunity. They will have quite high antibody responses to blood stage antigens already. Better to reformulate."

Answer

We agree that children in highly endemic areas will have considerable immunity by the age of 5 years. However, our statement compared immunity in children relative to adults: "*We selected children aged 5–10, who have limited blood stage immunity (a potential confounder when studying pre-erythrocytic immunity) compared to semi-immune adults, ….*". We have now modified the sentence to avoid misinterpretation:

"We selected children aged 5–10, who have lower blood stage immune responses (a potential confounder when studying pre-erythrocytic immunity) compared to semi-immune adults, and who allow repeated blood sampling compared to toddlers."

Comment

"It is surprising the lack of difference in antibody levels for CSP between these children and naive volunteers. Even if CSP is not as highly immunogenic as many blood stage antigens, the responses are usually significant and detectable, even in children. Could there be a problem with the capture antigen? Seasonality by itself may not totally explain this, or it would imply they are very short lived. The potential inhibition by blood stage has been proposed but it would also affect LSA1."

Answer

Thank you for these suggestions. The reviewer suggested that suppression of immune responses by blood-stage parasites would, theoretically, affect responses to both antigens, LSA-1 and CSP. We agree and have now modified the *Discussion* section accordingly:

"These low titres could be related to the timing of the study recruitment (prior to the transmission season after ~7 months of very low malaria exposure), which would imply that responses to CSP are short lived. The antibody levels could also have been influenced by repeated blood-stage infections in our cohort, which may have suppressed immune responses against the pre-erythrocytic stages, although, in theory, responses to both LSA-1 and CSP would be affected."

The reviewer also suggested that there might have been a problem with the antigen used in the CSP assay. The significant correlations between anti-CSP levels and functional phenotypes (i.e. sporozoite gliding inhibition or hepatocyte invasion inhibition) suggest that this is not the case (or that even if there was an issue, the interpretation of the relative values is still valid), and this is supported by the fact that a subset of children had particularly high antibody responses to CSP (Figure 2A).

Comment

"It is not well discussed how the affection of gliding and invasion before liver stage is detected as delay in positive qPCR in the blood, how do authors think this works"

Answer

We observed an association between sporozoite gliding inhibition and time to parasite positivity by qPCR. This could either reflect an ability of these immune responses to completely neutralize some sporozoite inoculums, leading to a delay in patency because this population would have no merozoites being released into the bloodstream from a first sporozoite inoculum. Alternatively, the number of sporozoites that reach hepatocytes can be reduced by immune responses targeting sporozoites and thus, the number of merozoites released upon completion of the hepatic phase of parasite development. This would result in a lower starting point of blood stage infection and plausibly a longer time to reach detectability of infections by PCR. Lastly, and we believe this is well explained in the revised manuscript, it is possible that the number of released merozoites is unaltered in the population with high sporozoite immunity, but the concurrent higher blood stage immunity dampens parasite growth and results in a longer time to reach detectability of infections by PCR.

Competing Interests: No competing interests were disclosed.

Reviewer Report 01 February 2019

https://doi.org/10.21956/wellcomeopenres.16280.r34445

Peter F. Billingsley
Sanaria Inc., Rockville, MD, USA

This ms measures antibody responses in Burkinabe children after drug clearance and subsequent *Plasmodium falciparum* infections, then attempts to explain delays in time to infection in terms of antibodies against circumsporozoite protein (CSP) and whole parasites. The authors demonstrate that, in individuals who show greatest delays in time to parasitemia, there were stronger functional antibody responses against sporozoites which reduced gliding motility and liver cell invasion *in vitro*. The major question addressed in this ms is whether these anti-sporozoite responses are affecting time to parasitemia in these children. From the data presented, the answer is “maybe”. The problem the authors face is that for almost every infection that stimulates anti-sporozoite antibodies, that infection also stimulates blood stage antibodies as the infection progresses. It is these latter that show the best negative correlation in reduction in time to parasitemia, and while separating out the functional anti-sporozoite effects is informative it is not definitive. This is illustrated in Figure 3A, where of the 29 participants with anti-sporozoite functional activities in one or both of the assays, 17 also have higher than median anti-blood stage responses. I do not see a way around this at the participant level and the authors clearly acknowledge this difficulty in their discussion; one is trying to see subtle effects on infection (in this case time to infection no children we uninfected during the study) using markers that autocorrelated and cannot therefore be separated from one another in terms of cause and effect.
One additional complication is not considered as far as I can tell. In order to have become infected, these children must have been bitten by mosquitoes. The mosquito bites induce antibody responses. The sporozoites used in the assays for gliding motility and hepatocyte invasion were from crude homogenates that contain salivary gland material. It is possible (though I admit not too likely) that there was some functional interaction between these two. Could antibodies against (*Anopheles stephensi*) salivary glands be present in these sera (of children bitten by African anophelines), and if so, could they have affected the *in vitro* functional antibody assays?
Nevertheless, the ms provides important information concerning anti-sporozoite functional antibodies, even if a clinical outcome (delayed time to parasitemia) cannot be ascribed to them.
Other points:

1. Given the comments above and the stronger correlation of blood stage antibodies with delay to parasitemia, the authors should consider changing the abstract (and perhaps the title) to include this information. It is buried in the paper but should be more explicit.
2. At the start of the introduction, the authors highlight the protective efficacies of RTS,S and PfSPZ Vaccine. However, they should also note that the outcomes, efficacies are different, RTS,S being time to clinical malaria, PfSPZ Vaccine being time to parasitemia. This has direct relevance to the ms.
3. Also in the introduction, the authors suggest that there is a discrepancy between protection with sporozoites and naturally induced protection not preventing infection. However, they do not consider the obvious reason for this – dose. Mosquitoes deliver a few (median 50 sporozoites per bite) now and again over a transmission season, while the sporozoite vaccines are delivering thousands of sporozoites per dose and defined intervals.
4. The authors say that there are sporozoite-specific IgG and IgM antibodies in the malaria exposed children, but such specificity has not been demonstrated, only reactivity. The term should be changed.
5. What do the authors mean by the title “Evidence of natural risk-modifying pre-erythrocytic immunity”?
6. The authors note an important variable that could not be accounted for in their studies, namely variability in exposure. One of the authors has worked on antibody responses to mosquito saliva as a marker for exposure risk: would it not be possible to measure antibody responses against mosquito bites here?

Minor points:

- Introduction, paragraph 1. The sentence ending " but it is unclear whether immunity that prevents or reduces infection incidence develops following natural exposure". This is a misuse of the term incidence – prevention of infection in one person will reduce incidence in the population. I think the authors mean "that prevents infection or reduces infection intensity in an individual….". In the same paragraph, change the word 'suggests' (…suggests that sterilizing immunity…" to demonstrates; I think the evidence is pretty clear).
- Results, paragraph 2. Should read Parasitological and immunological data from the remaining 51 children followed intensively were…" What does "data from this child were censored after the onset of symptoms" mean? Could have any one of many interpretations.
- In the results section "Naturally acquired antibodies in children neutralize in vitro sporozoite gliding motility" the authors should change the term "considerably larger' for something more scientific (significantly greater"?).
- Authors should give sample sizes (n values) used in the curves for figure 3B,C.
- In the discussion, the authors say "To our knowledge, this is one of the first studies to show that there are functional antibodies against pre-erythrocytic malaria stages in malaria-exposed children." Shouldn't the other studies be referenced here?
- The authors should check the axis labeling of figures: for example (% infected) ought to be "Percent children infected with P. falciparum", in Figure S1 the abbreviations/units are not explained, and so on.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: Sanaria has studies running in both Burkina Faso and in Nijmegen testing our sporozoite vaccines. Sanaria's interest is in the infectivity sporozoites and protection against sporozoite infections. This ms is directly relevant to our vaccines in this regard, but complimentary rather than conflicting. Like the authors, we are trying to both protect against sporozoite infections and understand the background factor affecting infectivity.

Reviewer Expertise: Malaria vaccinology, malaria sporozoites, malaria sporozoite vaccines, medical entomology, mosquito biology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 22 Apr 2019

Bronner Goncalves, London School of Hygiene and Tropical Medicine, London, UK

Comment

"This ms measures antibody responses in Burkinabe children after drug clearance and subsequent Plasmodium falciparum infections, then attempts to explain delays in time to infection in terms of antibodies against circumsporozoite protein (CSP) and whole parasites. The authors demonstrate that, in individuals who show greatest delays in time to parasitemia, there were stronger functional antibody responses against sporozoites which reduced gliding motility and liver cell invasion in vitro. The major question addressed in this ms is whether these anti-sporozoite responses are affecting time to parasitemia in these children. From the data presented, the answer is "maybe". The problem the authors face is that for almost every infection that stimulates anti-sporozoite antibodies, that infection also stimulates blood stage antibodies as the infection progresses. It is these latter that show the best negative correlation in reduction in time to parasitemia, and while separating out the functional anti-sporozoite effects is informative it is not definitive. This is illustrated in Figure 3A, where of the 29 participants with anti-sporozoite functional activities in one or both of the assays, 17 also have higher than median anti-blood stage responses. I do not see a way around this at the participant level and the authors clearly acknowledge this difficulty in their discussion; one is trying to see subtle effects on infection (in this case time to infection no children we uninfected during the study) using markers that autocorrelated and cannot therefore be separated from one another in terms of cause and effect.

One additional complication is not considered as far as I can tell. In order to have become infected, these children must have been bitten by mosquitoes. The mosquito bites induce antibody responses. The sporozoites used in the assays for gliding motility and hepatocyte invasion were from crude homogenates that contain salivary gland material. It is possible (though I admit not too likely) that there was some functional interaction between these two. Could antibodies against (Anopheles stephensi) salivary glands be present in these sera (of children bitten by African anophelines), and if so, could they have affected the in vitro functional antibody assays?

Nevertheless, the ms provides important information concerning anti-sporozoite functional antibodies, even if a clinical outcome (delayed time to parasitemia) cannot be ascribed to them."

Answer

Thank you. We agree with this assessment of the difficulty in separating pre-erythrocytic-stage- and blood-stage-specific immune responses. We have now included in the *Discussion* section a statement that emphasizes this point:

"We observed that high responses in the asexual stage lysate assay with unknown functionality were also associated with longer time to infection. We thus cannot rule out a supportive role for

asexual antibody responses in the observed associations. Indeed, as pointed out by one of the reviewers of the first version of this manuscript, 17/29 (58.6%) children with high anti-sporozoite responses in either CSP-specific or sporozoite gliding assays had above-median asexual lysate responses versus 8/22 (36.4%) children with below-median anti-sporozoite activities."

The reviewer also suggested that the functional activity of the plasma samples from study participants could be partially related to targeting antigens of salivary gland material rather than targeting sporozoites antigens. This is a very novel idea and an area of research that is both exciting and relatively new. There are indeed some indications that saliva responses may influence both the viability of sporozoites (Dragovic et al. *Immunization with AgTRIO, a Protein in Anopheles Saliva, Contributes to Protection against Plasmodium Infection in Mice.* Cell, Host & Microbe 2018) and (not the topic of this paper) the transmission of parasites from man to mosquito (Yamamoto et al. *Inhibition of Malaria Infection in Transgenic Anopheline Mosquitoes Lacking Salivary Gland Cells*. PLoS Pathogens 2016). How important such responses are compared to parasite-specific responses is unclear. Our assumption, based on the available literature, is that responses against salivary proteins would have a considerably smaller impact on sporozoite invasion compared to anti-sporozoite responses.

Moreover, the *in vitro* work that we have done is based on sporozoites harvested from *Anopheles stephensi*, that is not present in West Africa (see Sinka et al. *A global map of dominant malaria vectors.* Parasites & Vectors. 2012). Whilst many salivary proteins appear to be conserved among *Anophelines*, others are distinct between *Stephensi* and *Gambiae s.l.* (Arca *et al.* BMC Genomics 2017). Responses to conserved salivary proteins would thus have to be functionally important in invasion assays and differentially produced by individuals with high sporozoite responses/low sporozoite responses. Our previous work on the salivary protein gSG6 suggested an early saturation of responses to this protein (Stone *et al.* PLoS ONE 2012) and correlations between asexual, sporozoite and salivary gland responses (Proietti *et al.* Am J Trop Med Hyg 2013), making it difficult to disentangle the different responses. In conclusion, there is no convincing evidence that salivary gland responses could explain our current findings but it is a theoretical possibility that we cannot rule out completely. We have thus added the following underlined sentence to the paragraph where we describe some of the limitations of this study:

"Another limitation of our study is that we were only able to determine humoral responses. Cellular responses to pre-erythrocytic stages have been implicated in malaria protection in multiple studies. It is conceivable that by quantifying both antibody and cellular responses we would be able to better define natural immunological phenotypes associated with differential malaria risk. Immunity to mosquito saliva might have also influenced sporozoite invasion although the magnitude of such an effect compared to the established impact of anti-sporozoite responses is currently unknown and it is currently unknown whether this would require responses to antigens that are conserved between An. stephensi mosquitoes used for sporozoite production and vectors that study participants were naturally exposed to."

Comment

"Other points:
Given the comments above and the stronger correlation of blood stage antibodies with delay to parasitemia, the authors should consider changing the abstract (and perhaps the title) to include this information. It is buried in the paper but should be more explicit."

Answer

To address this comment and comments from Reviewer 1, we have now modified the *Abstract*:

"Survival analysis indicated longer time to infection in individuals displaying higher-than-median sporozoite gliding inhibition activity (P=0.01), although this association became non-significant after adjustment for blood-stage immunity (P = 0.06).

"In summary, functional antibodies against the pre-erythrocytic stages of malaria infection are acquired in children who are repeatedly exposed to Plasmodium parasites. This immune response does not prevent them from becoming infected during a malaria transmission season, but might delay the appearance of blood stage parasitaemia. Our approach could not fully separate the effects of pre-erythrocytic-specific and blood-stage-specific antibody-mediated immune responses in vivo; epidemiological studies powered and designed to address this important question should become a research priority."

Comment

"At the start of the introduction, the authors highlight the protective efficacies of RTS,S and PfSPZ Vaccine. However, they should also note that the outcomes, efficacies are different, RTS,S being time to clinical malaria, PfSPZ Vaccine being time to parasitemia. This has direct relevance to the ms."

Answer

The efficacy metric used in the PfSPZ vaccine trial is mentioned in the *Introduction* section. We now included information on the outcome used to assess efficacy of the RTS,S vaccine:

"The most advanced malaria vaccine, RTS,S (trade name, Mosquirix), induces immune responses that target P. falciparum circumsporozoite protein (CSP), and thereby the pre-erythrocytic stages of malaria, and has been shown to be partially effective in delaying the time to clinical malaria episodes."

Comment

"Also in the introduction, the authors suggest that there is a discrepancy between protection with sporozoites and naturally induced protection not preventing infection. However, they do not consider the obvious reason for this – dose. Mosquitoes deliver a few (median 50 sporozoites per bite) now and again over a transmission season, while the sporozoite vaccines are delivering thousands of sporozoites per dose and defined intervals."

Answer

We have now modified the following sentence in the *Introduction* section:

"The results of this and other vaccine trials and the efficient immunisation of malaria-naive individuals with multiple infected mosquito bites while receiving chloroquine contrast with the limited epidemiological evidence of naturally acquired functional immunity to Plasmodium

pre-erythrocytic stages, which could be possibly linked to the lower number of sporozoites in natural parasite inoculations or to the frequency of host-vector contacts. Individuals living in malaria-endemic regions can develop naturally acquired immunity against severe malaria disease and death, but it is unclear whether immunity that reduces, entirely or partially, the probability of blood-stage infection after parasite inoculation develops following natural exposure."

Comment

"The authors say that there are sporozoite-specific IgG and IgM antibodies in the malaria exposed children, but such specificity has not been demonstrated, only reactivity. T44he term should be changed."

Answer

We agree with the reviewer that the specificity of these antibodies has not been demonstrated, and have now modified the title of the sub-section in the manuscript:

"*Sporozoite-specific IgG and IgM antibodies in malaria-exposed children*"
to:
"*Naturally acquired IgG and IgM antibodies targeting sporozoites*"

We also modified the following sentence:

"In addition to antigen-specific assays, IgG and IgM antibodies recognizing Plasmodium sporozoites were quantified using fluorescently labelled anti-IgG and anti-IgM antibodies by flow cytometric analysis. Levels of antibodies targeting whole sporozoites were significantly higher in the study participants compared to malaria-naive donors (Figure 2D, E, P=0.01 and 0.04 for IgG and IgM, respectively). Strong correlations between IgG and IgM antibodies targeting whole sporozoites (Figure S1A in Extended data 14 , P=0.0005, Spearman's ρ=0.75), and between CSP-IgG antibody levels and whole sporozoite IgG antibodies (Figure S1B in Extended data 14 , Spearman's ρ=0.83, P<0.0001) were also observed."

We also replaced the expression "*sporozoite specific*" with more accurate expressions in other paragraphs of the manuscript.

Comment

"What do the authors mean by the title "Evidence of natural risk-modifying pre-erythrocytic immunity"?"

Answer

This sub-section title refers to the observation that functional immune responses against pre-erythrocytic stages were associated with delay in infection appearance. As discussed and addressed in answers to other comments, this evidence is based on the univariate survival analysis. "Risk" here is being used loosely, and not in the epidemiological sense of probability of an event during a specified time period.

Comment

"The authors note an important variable that could not be accounted for in their studies, namely variability in exposure. One of the authors has worked on antibody responses to mosquito saliva as a marker for exposure risk: would it not be possible to measure antibody responses against mosquito bites here?"

Answer

Although quantification of antibody responses to mosquito saliva antigens would provide some information on exposure to *Anopheles* mosquitoes generally, as shown in a previous study in the same area (*Guelbéogo et al, Variation in natural exposure to anopheles mosquitoes and its effects on malaria transmission. eLife 2018*), only a small proportion of blood-fed mosquitoes are parasite carriers, and the prevalence of infection in malaria vectors varies from household to household. For these reasons, we believe quantification of immunity against mosquito saliva antigens alone would not adequately adjust for exposure. Moreover, saliva responses are complicated to use in areas of high mosquito exposure. There is considerable evidence for a level of immune tolerance that reduces responses to mosquito saliva proteins in older individuals with high mosquito exposure. This would complicate the interpretation of mosquito salivary gland responses considerably in our cohort experiencing high but heterogeneous mosquito exposure.

Comment

"Minor points:
Introduction, paragraph 1. The sentence ending " but it is unclear whether immunity that prevents or reduces infection incidence develops following natural exposure". This is a misuse of the term incidence – prevention of infection in one person will reduce incidence in the population. I think the authors mean "that prevents infection or reduces infection intensity in an individual….". In the same paragraph, change the word 'suggests' (…suggests that sterilizing immunity…" to demonstrates; I think the evidence is pretty clear)."

Answer

We modified the sentence below for clarification:

"Individuals living in malaria-endemic regions can develop naturally acquired immunity against severe malaria disease and death, but it is unclear whether immunity that reduces, entirely or partially, the probability of blood-stage infection after parasite inoculation develops following natural exposure."

We prefer to keep the word 'suggests' in that same paragraph as "high incidence of blood-stage re-infection after effective anti-malarial treatment in adults" is a strong but not conclusive evidence that sterilizing immunity does not develop.

Comment

"Results, paragraph 2. Should read Parasitological and immunological data from the remaining 51 children followed intensively were…" What does "data from this child were censored after the onset of symptoms" mean? Could have any one of many

interpretations."

Answer

In that sentence, we meant that any visits and samples collected after the development of symptoms were disregarded and the child was classified as uninfected during the entire valid follow-up period.

We replaced the first sentence of that paragraph with the following sentence:

"Parasitological and immunological data from the remaining 51 children followed intensively were analysed."

Comment

"In the results section "Naturally acquired antibodies in children neutralize in vitro sporozoite gliding motility" the authors should change the term "considerably larger' for something more scientific (significantly greater"?)."

Answer

The modified sentence is:

"To characterize the effect of human plasma on sporozoite gliding motility that is independent of naturally acquired immunity, sporozoites were incubated in the presence of plasma from malaria-naive individuals (n=5) and showed an average gliding trail surface of 1,427(95% CI 646.6-2,207) pixels, significantly greater than the gliding trail surface of sporozoites incubated with 30 µg/ml of a monoclonal anti-CSP antibody, our positive control,141 (95% CI 71.9-225.4) pixels."

Comment

"Authors should give sample sizes (n values) used in the curves for figure 3B,C."

Answer

We have now included this information in the figure legend.

Comment

"In the discussion, the authors say "To our knowledge, this is one of the first studies to show that there are functional antibodies against pre-erythrocytic malaria stages in malaria-exposed children." Shouldn't the other studies be referenced here?"

Answer

We modified the sentence:

"To our knowledge, this is the first study to show that there are functional antibodies against pre-erythrocytic malaria stages in malaria-exposed children."

Comment
"The authors should check the axis labeling of figures: for example (% infected) ought to be "Percent children infected with P. falciparum", in Figure S1 the abbreviations/units are not explained, and so on."

Answer

We have now modified the legends, rather than axes' labels, of the following figures:

Figure 1

"… The black line represents the percentage of the study population that remained uninfected at different time-points (y-axis)."

Figure 3

"…. In (B), Kaplan-Meier curves for children with high and low circumsporozoite protein (CSP) responses and gliding inhibition (GI) phenotypes are presented (N = 51); in (C), curves for participants categorized based on their responses to asexual stage antigens (AL) are shown (N = 51). In both panels (B) and (C), the y-axis corresponds to the percentage of the population uninfected at different time points."

Competing Interests: No competing interests were disclosed.

Reviewer Report 03 January 2019

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?

Silvia Portugal
Malaria Infection Biology and Immunity Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD, USA

In this manuscript Barry, Behet and colleagues address an important question aiming to possibly appoint an effective role in protection from clinical malaria to liver-stage immunity acquired naturally in malaria endemic areas. I believe the work presented is a significant contribution to our understanding of naturally acquired immunity to pre-erythrocytic stages of *P. falciparum* and I make a few comments and suggestions that may improve the current version of the manuscript.
The data shows that ABs developed during past malaria cases can reduce sporozoite motility and hepatocyte invasion *in vitro* suggesting that ABs acquired during natural infections can reduce new liver-stage infections. However, the contribution of a possible similar effect *in vivo* is, in my view, less clear from the data presented, and more caution may be needed to discuss the results. All the children followed throughout the study became parasite positive by qPCR, indicating that even children with strong

inhibiting ABs were unable to block liver-stage infection efficiently. Furthermore, *high response to asexual stage lysate* might be confounding the analysis. I would add to the manuscript (or supplemental) figures the association of CSP ABs with asexual stage lysate ABs, and also the association of whole SPZ ABs with asexual stage lysate ABs, to give the reader an idea of how close these parameters are.

I also suggest including, if that has not been done already, the *Reported bed net use* in the multivariate analyses, as this could also be a factor increasing time to PCR positive and clinical malaria.

The authors cite the study by Tran et al.[1] where it was shown that, in Mali, time to PCR positive was independent of age, while time to clinical malaria increased with age, and where as stated *it was concluded that there no or very limited evidence for an age dependent acquisition of immunity protecting from infection.* Similarly, in the present manuscript, *in vitro* functional data of higher humoral response against pre-erythrocytic stages does not (independently of blood-stage immunity) protect from infection. So, I would rephrase the last sentence in first paragraph of the discussion to add a bit more caution in interpreting what may be causing partial protection.

At the end of section *Evidence of natural risk-modifying pre-erythrocytic immunity*the authors should, in my view, clearly state that *High gliding inhibition activity*does not independently associate in a statistically significant way, with *protection against falciparum infection*on the multivariate analyses where blood stage immunity was included; the P value is above 0.05 (0.055) and the CI includes 1, making the relative risk not statistically significant.

I believe the manuscript could be improved by presenting the quantitative analysis of the *18s* qPCR upon first parasite detection and determine if there is a negative association with the inhibitory capacity of the individuals' ABs. It would also be very interesting to question if time from first PCR positive to time of presentation of symptoms is different between poor and strong *in vitro* inhibitors. If the *in vitro* data showing gliding inhibition and reduced hepatocyte invasion are significant *in vivo*, one would expect a lower inoculum in the liver and thus a lower parasitaemia on the first PCR positive time-point. And then potentially a slower progression to clinical malaria. I believe with the data generated in this manuscript these analyses could be done, and would enrich the story.

It is not totally clear to me how individuals were selected for the flow cytometry assays. Survival, gliding inhibition, CSP, LSA1 and asexual lysate ELISAs were performed for the 51 participants, but flow cytometry data presented in fig2 D and E was obtained from 17 Burkinabes only; how were those selected and what is their time to PCR+ in the survival analysis. If they are the 8 poor and 8 strong inhibitors as defined by their gliding inhibition it should be stated in the methods (seems to be so, given supFig3, but there is one extra?).
I also suggest to pinpoint these 8 poor and 8 strong inhibitors in fig1 so that the reader would be informed of their time to PCR+ and time to malaria symptoms.

I would be more cautious when citing ref 31, I believe the study by Michael Stewart et al.[2] shows that non-motile SPZ are unable to invade, but is not clearly showing a direct association between % of human AB affecting motility and those levels correlating directly with invasion either.

Minor points:
In table 1, I would not refer to the 6 children who were PCR positive at the 3 weeks after treatment time-point as *Persisting parasites post-treatment*as I do not think that it can be excluded that the children were re-infected after clearance of PQ.
I recommend adding a brief description of the method in ref 45 in the section *In vitro sporozoite infectivity*

assay of a human hepatoma cell line inmaterial and methods.
On page 3 below table 1 there is mention to *field PCR* which may be a mistake.
The data from the *in vitro* gliding inhibition by LSA IgG seems to be not shown. I think it should be clarified in the text that that is indeed the case. Likewise, if the LSA-1-specific IgG antibodies correlation with sporozoite invasion inhibition is data not shown I would clearly state it in the text.
In figS2A I would specify that is IgG in the figure x axis and use the label CSP IgG titer instead of CSP antibody titer.
Figure S3D is called before Figure S3C, I would call figures in ascending and alphabetical order instead.

References

1. Tran TM, Li S, Doumbo S, Doumtabe D, Huang CY, Dia S, Bathily A, Sangala J, Kone Y, Traore A, Niangaly M, Dara C, Kayentao K, Ongoiba A, Doumbo OK, Traore B, Crompton PD: An intensive longitudinal cohort study of Malian children and adults reveals no evidence of acquired immunity to Plasmodium falciparum infection.*Clin Infect Dis*. 2013; **57** (1): 40-7 PubMed Abstract | Publisher Full Text
2. Stewart MJ, Nawrot RJ, Schulman S, Vanderberg JP: Plasmodium berghei sporozoite invasion is blocked in vitro by sporozoite-immobilizing antibodies.*Infect Immun*. 1986; **51** (3): 859-64 PubMed Abstract

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: malaria, immunoparasitology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 22 Apr 2019

Bronner Goncalves, London School of Hygiene and Tropical Medicine, London, UK

We would like to thank the reviewers for their insightful comments and suggestions. The modifications we made to address their comments improved the quality of our manuscript and

clarified the interpretation of our results.

Reviewer 1

Comment

"In this manuscript Barry, Behet and colleagues address an important question aiming to possibly appoint an effective role in protection from clinical malaria to liver-stage immunity acquired naturally in malaria endemic areas. I believe the work presented is a significant contribution to our understanding of naturally acquired immunity to pre-erythrocytic stages of P. falciparum and I make a few comments and suggestions that may improve the current version of the manuscript.

The data shows that ABs developed during past malaria cases can reduce sporozoite motility and hepatocyte invasion in vitro suggesting that ABs acquired during natural infections can reduce new liver-stage infections. However, the contribution of a possible similar effect in vivo is, in my view, less clear from the data presented, and more caution may be needed to discuss the results. All the children followed throughout the study became parasite positive by qPCR, indicating that even children with strong inhibiting ABs were unable to block liver-stage infection efficiently. Furthermore, high response to asexual stage lysate might be confounding the analysis. I would add to the manuscript (or supplemental) figures the association of CSP ABs with asexual stage lysate ABs, and also the association of whole SPZ ABs with asexual stage lysate ABs, to give the reader an idea of how close these parameters are."

Answer

In response to this comment and to a comment from Reviewer 2, we modified the *Abstract* to mention that our study did not fully address the correlation between liver-stage- and blood-stage-specific immunities and that additional studies are necessary.

"Survival analysis indicated longer time to infection in individuals displaying higher-than-median sporozoite gliding inhibition activity (P=0.01), although this association became non-significant after adjustment for blood-stage immunity (P = 0.06)."

"In summary, functional antibodies against the pre-erythrocytic stages of malaria infection are acquired in children who are repeatedly exposed to Plasmodium parasites. This immune response does not prevent them from becoming infected during a malaria transmission season, but might delay the appearance of blood stage parasitaemia. Our approach could not fully separate the effects of pre-erythrocytic-specific and blood-stage-specific antibody-mediated immune responses in vivo; epidemiological studies powered and designed to address this important question should become a research priority."

We have also modified the first paragraph of the *Discussion* section:

"Our epidemiological data together with the in vitro data provide evidence that there is partially effective pre-erythrocytic immunity that influences individual level infection incidence, although we cannot exclude the contribution of immunity against asexual blood-stage antigens to this observation."

Additionally, we agree that the inclusion of a figure showing the correlation between liver-stage immune responses and asexual stage lysate responses would be informative for the readers. We included a scatter plot of CSP-specific immune responses versus asexual stage lysate responses (Figure S4).

"***Figure S4.*** *CSP antibody levels versus immune responses against asexual stage antigens. In this figure, the x-axis shows CSP antibody levels; the y-axis represents antibody responses against asexual stage parasites lysate. Both axes are in log-scale; one child with undetectable CSP response is not included in this graph.*"

We also modified the legend of Figure 3 to refer to this supplementary figure:

"*To assess the effect of immune responses on infection risk, children were classified based on whether the results of their assays were higher or lower than the study population median (see Results): in (A), vertically aligned cells represent the same participant, and orange cells indicate that assay results are higher than the median (see also Figure S4, that shows a scatter plot of CSP antibody levels and immunity against asexual lysate).*"

Comment

"I also suggest including, if that has not been done already, the Reported bed net use in the multivariate analyses, as this could also be a factor increasing time to PCR positive and clinical malaria."

Answer

The analysis in this manuscript concerns time to infection detection, regardless of whether symptoms are present or not. In this cohort, reported bed net use at enrollment had no effect on time to infection (hazard ratio 1.16, 95% CI [0.66 – 2.03], P = 0.61). In analyses that included reported bed net use at enrollment and either CSP immune responses or sporozoite gliding inhibition phenotype as binary variables, neither the hazard ratio point estimates nor the confidence intervals changed significantly (data not shown). Of note, only data on reported bed net use were collected and it is evident that this is an imprecise indicator of personal protection.

Comment

"The authors cite the study by Tran et al. where it was shown that, in Mali, time to PCR positive was independent of age, while time to clinical malaria increased with age, and where as stated it was concluded that there no or very limited evidence for an age dependent acquisition of immunity protecting from infection. Similarly, in the present manuscript, in vitro functional data of higher humoral response against pre-erythrocytic stages does not (independently of blood-stage immunity) protect from infection. So, I would rephrase the last sentence in first paragraph of the discussion to add a bit more caution in interpreting what may be causing partial protection."

Answer

Our multivariate analyses showed that the effects of functional phenotypes became non-significant

after adjustment for blood stage immunity. However, we disagree with the reviewer that we can conclude the opposite, i.e. that “higher humoral response against pre-erythrocytic stages does not (independently of blood-stage immunity) protect from infection”. The study was not designed, and might not have been powered, to assess the separate effects of the correlated pre-erythrocytic-stage and blood-stage immunities on malaria infection establishment. A study with a larger sample size, where a sufficient number of individuals would have relatively high liver-stage immunity and relatively low asexual responses, and vice-versa, would be more suitable to address this question.

We have modified the *Discussion* section and the *Abstract* to mention that our approach did not fully separate the effects of these two types of immunity (see response to the first comment).

Comment

At the end of section Evidence of natural risk-modifying pre-erythrocytic immunity the authors should, in my view, clearly state that High gliding inhibition activity does not independently associate in a statistically significant way, with protection against falciparum infection on the multivariate analyses where blood stage immunity was included; the P value is above 0.05 (0.055) and the CI includes 1, making the relative risk not statistically significant.

Answer

With the small sample size, we believe a very strong focus on the P-value threshold of 0.05 is potentially problematic. In general, the P-value and strict thresholds for interpreting significance are criticized by many (e.g. Greenland et al. Eur J Epidemiol 2016; 31: 337-50; Lang *et al.* Epidemiology 1998; 9:7-8), illustrated also by a recent comment in *Nature* (Amrhein V, Greenland S, McShane B. *Scientists rise up against statistical significance.* Nature 2019). With our small sample size, we agree that all results should be interpreted with caution (also related to the limited sample size, but mostly due to the correlations between blood stage and pre-erythrocytic humoral immunity). The sentence in the *Results* section has now been modified to:

“In a model that also included the results of the asexual stage lysate assay, the relationship between anti-CSP responses and time to malaria infection was not statistically significant. Despite a clear trend for a protective effect, the association between inhibition of in vitro sporozoite gliding motility and time to falciparum infection also did not reach statistical significance after adjustment for blood-stage immunity (hazard ratio, 0.55; 95% CI, 0.29–1.01).”

Comment

“I believe the manuscript could be improved by presenting the quantitative analysis of the 18s qPCR upon first parasite detection and determine if there is a negative association with the inhibitory capacity of the individuals’ ABs. It would also be very interesting to question if time from first PCR positive to time of presentation of symptoms is different between poor and strong in vitro inhibitors. If the in vitro data showing gliding inhibition and reduced hepatocyte invasion are significant in vivo, one would expect a lower inoculum in the liver and thus a lower parasitaemia on the first PCR positive time-point. And then potentially a slower progression to clinical malaria. I believe with the data generated in this manuscript these analyses could be done, and would enrich the story.”

Answer

Thank you, we agree that presenting data on the *18S* qPCR-based parasite levels at the time of infection detection would be informative and have now included the sentence below in the *Methods* section:

"The first scheduled weekly visit or intensive follow-up visit when parasitaemia of at least 0.1 parasites per µl was detected by 18S qPCR was considered the time of infection incidence. Using this criterion, the median 18S qPCR-based parasitaemia at infection detection was 2.2 parasites per µl and the interquartile range was 0.4 – 80.4. This threshold of parasitaemia was chosen to minimise false-positive results."

The median *18S* qPCR parasite density during the first parasite-positive visits of children with higher-than-median sporozoite gliding inhibition activity was 1.0 (interquartile range 0.3 – 28.2) parasites per µl; for those children whose plasma inhibited sporozoite gliding with efficiency below the population median, the parasite density at first detection was 4.7 (median; interquartile range 0.5 – 136.2). The interpretation of associations between these parasite levels and immune phenotypes, however, is not straightforward. Immune responses that reduce liver parasite burden and influence the size of the parasite inoculum in the blood would most likely influence the time required for parasitaemia to reach the detection threshold, but it is not obvious that the parasite levels at which we first detect infection have a direct relation with liver stage immunity. For example, even if after a week (the time between consecutive scheduled visits), parasite levels in blood-stage infections starting on the same day would be higher in children with higher liver-to-blood parasite inocula, if "lower liver-to-blood inoculum" infections remain undetectable, they might be detected at higher levels afterwards.

Regarding the evolution of parasite levels during these natural infections, and the eventual development of symptoms, another manuscript, with a more comprehensive analysis of immune responses against blood stage antigens, is being prepared.

Comment

"It is not totally clear to me how individuals were selected for the flow cytometry assays. Survival, gliding inhibition, CSP, LSA1 and asexual lysate ELISAs were performed for the 51 participants, but flow cytometry data presented in fig2 D and E was obtained from 17 Burkinabes only; how were those selected and what is their time to PCR+ in the survival analysis. If they are the 8 poor and 8 strong inhibitors as defined by their gliding inhibition it should be stated in the methods (seems to be so, given supFig3, but there is one extra?).

I also suggest to pinpoint these 8 poor and 8 strong inhibitors in fig1 so that the reader would be informed of their time to PCR+ and time to malaria symptoms."

Answer

We have rectified this. In the original submission 17 individuals were included in the flow assays (the 16 selected and one additional individual with no hepatocyte invasion data). For consistency, we now presented data for the 8 poor and 8 strong gliding inhibitors with available invasion data in

all figures where a selection of the total study population of 51 individuals was used. These 16 individuals were selected based on highest gliding inhibition (84-99%) and lowest gliding inhibition (-34 – 20%), as illustrated in supplementary figure 2B. To ensure consistency, we updated Figure 2, Figure S2 and Figure S3. This had no impact on any of the effects, significance levels changed marginally and have been updated. We have now included this information in the *Methods* section:

"Recognition of whole sporozoites by naturally acquired IgG and IgM antibodies was determined by an in vitro flow-cytometry-based antibody opsonization assay that was presented in detail elsewhere. Due to limited plasma availability and available sporozoite numbers, 16 naturally exposed children with highest (8) and lowest (8) gliding activity were selected for sporozoite opsonization assays and invasion assays, allowing us to investigate potential correlations."

The median time to infection detection in the group of children classified as strong inhibitors of sporozoite gliding motility was 41 days (range 5 – 64 [N = 7]; 1/8 child did not have parasites detected); poor gliding inhibitors (N = 8) had a median time to infection of 34 days (range 13 – 48).

Comment

"I would be more cautious when citing ref 31, I believe the study by Michael Stewart et al. shows that non-motile SPZ are unable to invade, but is not clearly showing a direct association between % of human AB affecting motility and those levels correlating directly with invasion either."

Answer

We cite the study by Stewart et al in the following sentence:

"It has been demonstrated that Plasmodium parasites use the system of adhesion-based motility, gliding, to actively penetrate host cells[30]; and that the invasive ability of sporozoites is directly associated with their motility[31]."

This is an accurate statement about that study, where the authors concluded that "sporozoite invasiveness is associated with sporozoite motility".

Comment

"Minor points:
In table 1, I would not refer to the 6 children who were PCR positive at the 3 weeks after treatment time-point as Persisting parasites post-treatment as I do not think that it can be excluded that the children were re-infected after clearance of PQ."

Answer

We modified Table 1 to 'Presence of parasites post-treatment'.

Comment

"I recommend adding a brief description of the method in ref 45 in the section In vitro sporozoite infectivity assay of a human hepatoma cell line in material and methods."

Answer

"Neutralization of P. falciparum sporozoite hepatocyte invasion by naturally acquired antibodies was assessed in a flow-cytometry–based in vitro invasion assay as previously described with small adaptations. Briefly, freshly dissected P. falciparum NF54 sporozoites were added to heat-inactivated plasma samples (10% final concentration) from malaria-naive or malaria-exposed individuals and pre-incubated for 30 minutes at 4 ˚C. Subsequently, the sporozoite-plasma mixtures (5.10^4 sporozoites in the presence of 10% plasma) were added to HC-04 hepatocytes in 96-well plates. Following 3 hours of incubation at 37 ˚C in 5% CO_2, invaded and intracellular sporozoites were stained with an Alexa Fluor 488-conjugated anti-CSP antibody. Flow cytometric analysis…."

Comment

"On page 3 below table 1 there is mention to field PCR which may be a mistake."

Answer

One child in the study did not develop symptoms nor had parasites detected by nested PCR performed in Ouagadougou. We have now modified the sentence to:

"The median time from confirmation of the absence of parasites (i.e. 3 weeks after anti-malarial treatment) to infection detection by nested PCR or onset of symptoms was 28 days; one child who did not have parasites detected by nested PCR was not included in this calculation."

Comment

"The data from the in vitro gliding inhibition by LSA IgG seems to be not shown. I think it should be clarified in the text that that is indeed the case. Likewise, if the LSA-1-specific IgG antibodies correlation with sporozoite invasion inhibition is data not shown I would clearly state it in the text."

Answer

Although no correlation plot is shown for LSA-1 titers and gliding inhibition, the lack of association with gliding inhibition is included in the *Results* section:

"In vitro gliding inhibition did not correlate with LSA-1 IgG antibody levels (P=0.11, Spearman's ρ=0.23)…"

"Sporozoite invasion inhibition correlated with IgG targeting whole sporozoites (Figure S3B in Extended data14, P=0.004, Spearman's ρ=0.67) but not with whole sporozoite IgM antibody levels (Figure S3C in Extended data 14 , P=0.13, Spearman's ρ=0.38). There was a correlation of hepatocyte invasion inhibition with CSP-specific IgG antibodies (Figure S3D in Extended data14, P=0.002, Spearman's ρ=0.76), but not with LSA-1 specific IgG antibodies (P=0.08, Spearman's ρ=0.48)."

In response to the next comment and to comments by another reviewer, we have changed the

order of the sentences and removed the word ‘specific’ from the section above and replaced it with “targeting whole sporozoites”.

Comment

“In figS2A I would specify that is IgG in the figure x axis and use the label CSP IgG titer instead of CSP antibody titer.
Figure S3D is called before Figure S3C, I would call figures in ascending and alphabetical order instead.”

Answer

We have now modified Figure S2 and rephrased the text so Figure S3C is mentioned before Figure S3D (see answer to previous comment).

Competing Interests: No competing interests were disclosed.