



## RESEARCH ARTICLE

# Functional antibodies against *Plasmodium falciparum* sporozoites are associated with a longer time to qPCR-detected infection among schoolchildren in Burkina Faso [version 1; referees: 1 approved, 2 approved with reservations]

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## Abstract

**Background:** Individuals living in malaria-endemic regions develop naturally acquired immunity against severe malarial disease, but it is unclear whether immunity that affects the establishment of infections develops following continuous natural exposure.

**Methods:** We cleared schoolchildren in Burkina Faso of possible sub-patent infections and examined them weekly for incident infections by PCR. Plasma samples collected at enrolment were used to quantify antibodies to the pre-erythrocytic-stage antigens circumsporozoite protein (CSP) and liver stage antigen. 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 these functional pre-erythrocytic immunity phenotypes and time to PCR-detected infection were studied.

**Results:** A total of 51 children were monitored; the median time to first detection of infection by PCR or development of clinical symptoms was 28 days. Anti-CSP antibody titres showed a strong positive association with sporozoite gliding motility inhibition ( $P < 0.0001$ , Spearman's  $\rho = 0.76$ ). *In vitro* hepatocyte invasion was inhibited by naturally acquired antibodies (median invasion inhibition, 19.4% [IQR 15.2-40.9%]), and there was a positive correlation between gliding and invasion inhibition ( $P = 0.02$ , Spearman's  $\rho = 0.60$ ). Survival analysis indicated longer time to infection in individuals displaying higher-than-median sporozoite gliding inhibition activity ( $P = 0.01$ ).

## Open Peer Review

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|  | 1   | 2   | 3   |
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**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 and consequently needs to be considered in the evaluation of malaria vaccines.

### Keywords

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

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## 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<sup>1</sup>. Alongside the RTS,S subunit vaccine, several other pre-erythrocytic stage vaccines are under development, based on subunit or whole-parasite vaccination<sup>2-5</sup>. 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<sup>6</sup>. The results of this and other vaccine trials and the efficient immunisation of malaria-naïve individuals with multiple infected mosquito bites while receiving chloroquine<sup>7</sup> contrast with the limited epidemiological evidence of naturally acquired functional immunity to *Plasmodium* pre-erythrocytic stages. Individuals living in malaria-endemic regions can develop naturally acquired immunity against severe malaria disease and death<sup>8,9</sup>, but it is unclear whether immunity that prevents or reduces infection incidence develops following natural exposure<sup>9</sup>. 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<sup>10,11</sup>. 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<sup>12,13</sup>. 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<sup>13</sup>.

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-piperazine (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 intensively followed children 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

**Table 1. Study population.**

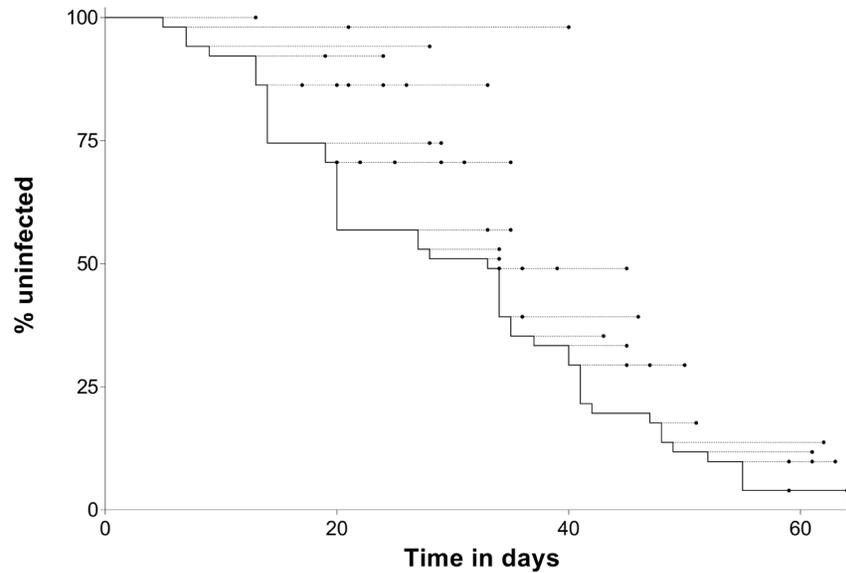
| Variable   | Value            |
|--|------------------|
| <b>Number of individuals</b>   |                  |
| Screened and parasite-free by microscopy                               | 58               |
| Persisting parasites post-treatment                                    | 6                |
|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

\*First weekly visit. IQR, interquartile range.

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 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 field 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.

### Sporozoite-specific IgG and IgM antibodies in malaria-exposed children

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-naïve 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-naïve 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-naïve donors (Figure 2B,  $P=0.006$ ). As expected, asexual blood stage-specific antibody titers in naturally exposed children were higher compared to malaria-naïve donors (Figure 2C,  $P<0.0001$ ). In addition to antigen-specific assays, IgG and IgM antibodies



**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. 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.

recognizing *Plasmodium* sporozoites were quantified using fluorescently labelled anti-IgG and anti-IgM antibodies by flow cytometric analysis. Whole sporozoite-specific IgG and IgM antibody levels in the study participants were significantly higher than in malaria-naïve donors (Figure 2D, E,  $P=0.02$  and  $0.004$  for IgG and IgM, respectively). Strong correlations between sporozoite-specific IgG and IgM antibodies (Figure S1A in Extended data<sup>14</sup>,  $P=0.0005$ , Spearman's  $\rho=0.75$ ), and between CSP-IgG antibody levels and whole sporozoite-specific IgG antibodies (Figure S1B in Extended data<sup>14</sup>, Spearman's  $\rho=0.83$ ,  $P<0.0001$ ) were also observed.

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

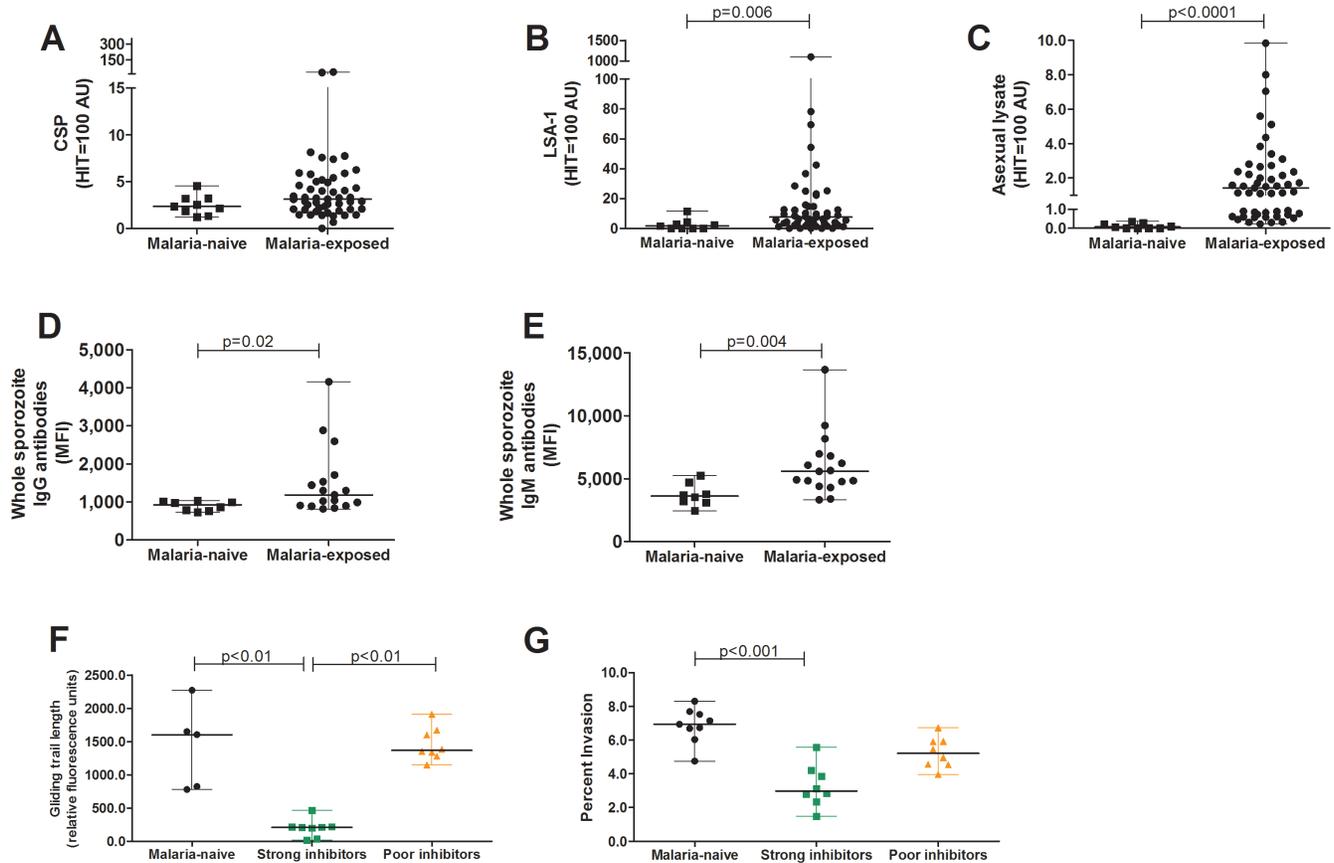
The neutralizing activity of naturally acquired antibodies against sporozoite motility was determined in an *in vitro* assay. Sporozoites pre-treated with PBS showed an average gliding trail surface of 7,392 (95% confidence interval [CI] 4,151-9,388) pixels. To characterize the effect of human plasma on sporozoite gliding motility that is independent of naturally acquired immunity, sporozoites were incubated in the presence of plasma from malaria-naïve individuals ( $n=5$ ) and showed an average gliding trail surface of 1,427 (95% CI 646.6-2,207) pixels, which is considerably larger than the gliding trail surface of sporozoites incubated with 30  $\mu\text{g}/\text{ml}$  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-naïve donors (median 1605.7, 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-naïve donors (Figure 2F,  $P<0.01$ ). *In vitro* gliding inhibition did not correlate with LSA-1 IgG antibody levels ( $P=0.11$ , Spearman's  $\rho=0.23$ ), but correlated with CSP-specific IgG antibodies (Figure S2A in Extended data<sup>14</sup>,  $P<0.0001$ , Spearman's  $\rho=0.76$ ), whole sporozoite-specific IgG (Figure S2B in Extended data<sup>14</sup>,  $P<0.0001$ , Spearman's  $\rho=0.81$ ) and IgM antibodies (Figure S2C in Extended data<sup>14</sup>,  $P=0.01$ , Spearman's  $\rho=0.61$ ). While sporozoite-specific IgG and IgM antibody levels of poor gliding inhibitors did not significantly differ from malaria-naïve adults, sporozoite-specific antibody levels of strong gliding inhibitors were significantly higher compared to poor gliding inhibitors (Figure S2D in Extended data<sup>14</sup>,  $P<0.0001$  and  $P=0.004$  for IgG and IgM antibodies, respectively) and malaria-naïve adults (Figure S2D, E in Extended data<sup>14</sup>,  $P<0.0001$  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



**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 ( $n=17$ ) 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.

gliding inhibitors (see previous section) also prevented hepatocyte invasion more effectively compared to malaria-naive donors (Figure 2G,  $P<0.001$ ). There was a positive correlation between gliding and invasion inhibition (Figure S3A in Extended data<sup>14</sup>,  $P=0.02$ , Spearman's  $\rho=0.60$ ), suggesting that *in vitro* gliding inhibition by naturally acquired antibodies might serve as a good surrogate for *in vitro* hepatocyte invasion inhibition. As with gliding inhibition, sporozoite invasion inhibition correlated positively with sporozoite-specific IgG (Figure S3B in Extended data<sup>14</sup>,  $P=0.004$ , Spearman's  $\rho=0.71$ ) and CSP-specific IgG antibodies (Figure S3D in Extended data<sup>14</sup>,  $P=0.002$ , Spearman's  $\rho=0.76$ ), but not with LSA-1-specific IgG antibodies ( $P=0.08$ , Spearman's  $\rho=0.48$ ). In contrast, sporozoite-specific IgM antibody levels did not correlate with invasion inhibition (Figure S3C in Extended data<sup>14</sup>,  $P=0.14$ , Spearman's  $\rho=0.42$ ).

### 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  $\geq 0.1$  parasites/ $\mu$ l) 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<sup>15</sup> and parasite carriage<sup>16,17</sup>. 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

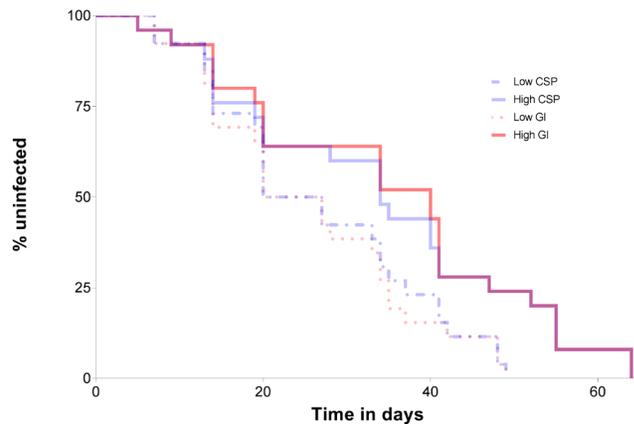
assay). Additional analyses (Extended data<sup>14</sup>) show that functional responses against pre-erythrocytic stages were also associated with infection risk when different parasite density thresholds are used to define infection.

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 models were fit to assess the mutually adjusted effects of these immune responses on time to infection detection (Table 2). While the relationship between

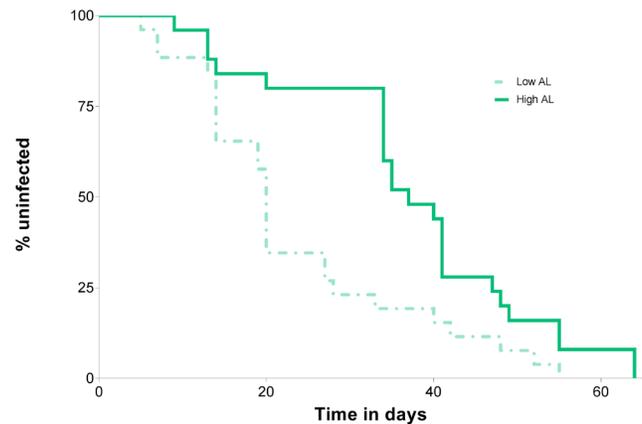
**A**



**B**



**C**



**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. In (B), Kaplan-Meier curves for children with high and low circumsporozoite protein (CSP) responses and gliding inhibition (GI) phenotypes are presented; in (C), curves for participants categorized based on their responses to asexual stage antigens (AL) are shown.

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

| Variable                              | Hazard ratio | 95% CI    | P-value |
|---------------------------------------|--------------|-----------|---------|
| <b>Model I</b>                        |              |           |         |
| 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    |
| <b>Model II</b>                       |              |           |         |
| 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.

anti-CSP responses and time to malaria infection was not statistically significant in a model that also included results of the asexual stage lysate assay, higher inhibition of *in vitro* sporozoite gliding motility remained associated with protection against falciparum infection 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 immunity that influences individual level infection incidence.

Sterile protection against malaria can be readily demonstrated in human and animal experimental models<sup>7,18,19</sup>, but not following natural malaria exposure<sup>13</sup>. 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<sup>11,13</sup>. 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, and who allow repeated blood sampling compared to toddlers. Since submicroscopic infections are prevalent in the area<sup>20</sup>, 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<sup>21–24</sup>, 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-naïve 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) and may also be influenced by repeated blood-stage infection in our cohort, which may have suppressed immune responses against the pre-erythrocytic stages<sup>25</sup>. 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<sup>12</sup>. 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<sup>26,27</sup>. 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<sup>28</sup> and hepatocyte

invasion<sup>29</sup> 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<sup>30</sup>; and that the invasive ability of sporozoites is directly associated with their motility<sup>31</sup>. 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 study undertaken in Indonesia, hepatocyte invasion inhibition was associated with higher anti-CSP antibody titres<sup>32</sup>. 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 one of the first studies 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<sup>33</sup>. 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<sup>34</sup>. 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<sup>35</sup>. 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<sup>36,37</sup>. 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<sup>38</sup>.

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<sup>20</sup>. 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<sup>39</sup> 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 RNeasy Protect 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<sup>40</sup>. Genomic DNA from the same extraction was used to test for human haemoglobinopathies haemoglobin S and C<sup>41</sup>.

### 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<sup>42</sup>. 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<sup>18</sup>.

### Human hepatoma HC-04 cell line

The HC-04 human hepatoma cell line<sup>29</sup> 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<sub>2</sub>.

### 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)<sup>43,44</sup> in naturally exposed children (n=51) and malaria-naïve 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)<sup>19</sup>.

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<sup>45</sup>. Flow cytometric analysis was performed with a LSRII flow cytometer (BD BioSciences); data analysis by FlowJo software (version 10.0.8, Tree Star).

Malaria-naïve donors are healthy malaria-naïve European volunteers who participated in CPS-immunization trial (immunization of malaria-naïve human volunteers under chloroquine prophylaxis with sporozoites delivered by mosquito bites) at the Radboud University Medical Center (Nijmegen, The Netherlands)<sup>46</sup>. 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<sup>47</sup>) 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<sub>2</sub>, 4% CO<sub>2</sub> and 3% O<sub>2</sub>. 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<sup>47</sup>), 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<sup>28</sup>. 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<sup>28</sup>.

#### *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<sup>45</sup>. 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 S4](#) in Extended data<sup>14</sup>). 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. 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](mailto: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><sup>14</sup>.

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

##### Extended data

The results of sensitivity analyses (see Results section) and Figures S1-4 are available from the Dryad repository.

**Figure S1. Correlation analyses of naturally acquired sporozoite-specific IgG and IgM antibodies.** (A) Scatter plot of sporozoite-specific IgG and IgM antibodies targeting *P. falciparum* NF54 sporozoites is shown (n=17). (B) Correlation analysis for *P. falciparum* NF54 sporozoite-specific 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 sporozoite-specific (B) IgG or (C) IgM antibodies and *in vitro* gliding inhibition by naturally acquired antibodies was conducted with samples from 17 children. Recognition of *P. falciparum* NF54 sporozoites by sporozoite-specific (D) IgG and (E) IgM antibodies from naturally exposed children (n= 17) 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 Wallist 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-naïve 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-specific (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. 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://dx.doi.org/10.5061/dryad.n1m33qq14>.

Data are available under the terms of the [Creative Commons Zero “No rights reserved” data waiver](#) (CC0 1.0 Public domain dedication).

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## Version 1

Referee Report 19 February 2019

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**Carlota Dobaño** 

Barcelona Institute for Global Health (ISGlobal), Hospital Clinic – Universitat de 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  $\rho=0.60$  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.
- 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.**

Referee 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.**

Referee Report 03 January 2019

<https://doi.org/10.21956/wellcomeopenres.16280.r34444>



**Silvia Portugal**

Center for Infectious Diseases, Parasitology, Heidelberg University Hospital, Heidelberg, Germany

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.<sup>1</sup> 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.<sup>2</sup> 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* in material 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.**

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