Identifying immune correlates of protection against *Plasmodium falciparum* through a novel approach to account for heterogeneity in malaria exposure.

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Key points: Associations of antibodies targeting *P. falciparum* with reduced risk of clinical malaria were reported in 24-months-old children, but not before, only when considering malaria exposure. This highlights the importance of age and exposure levels when studying correlates of protective immunity.

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ABSTRACT

Background

A main criterion to identify malaria vaccine candidates is the proof that acquired immunity against them is associated with protection from disease. The age of the studied individuals, heterogeneous malaria exposure and assumption of the maintenance of a baseline immune response can confound these associations.

Methods

IgG/IgM levels were measured by Luminex® in Mozambican children monitored for clinical malaria from birth until 3 years of age, together with functional antibodies. Studied candidates were pre-erythrocytic and erythrocytic antigens, including EBAs/PfRhs, MSPs, DBLs, and novel antigens merely or not previously studied in malaria-exposed populations. Cox regression models were estimated at 9 and 24 months of age, accounting for heterogeneous malaria exposure or limiting follow-up according to the antibody's decay.

Results

Associations of antibody responses with higher clinical malaria risk were avoided when accounting for heterogeneous malaria exposure or when limiting the follow-up time in the analyses. Associations with reduced risk of clinical malaria were found only at 24 months old, but not younger children, for IgG breadth and levels of IgG targeting $EBA140_{III-V}$, CyRPA, DBL5ε and DBL3x, together with C1q-fixation activity by antibodies targeting $MSP1_{19}$.

Conclusions

Malaria protection correlates were identified, only in children aged 24 months old when accounting for heterogeneous malaria exposure. These results highlight the relevance of

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considering age and malaria exposure, as well as the importance of not assuming the maintenance of a baseline immune response throughout the follow-up. Results may be misleading if these factors are not considered.

INTRODUCTION

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Since 2007, many efforts are focused on malaria elimination, with many settings reporting a dramatic decline in malaria cases. A highly efficacious vaccine could supplement the waning naturally acquired immunity (NAI) against malaria in these areas. Given the limited protection conferred by RTS,S vaccination[1], second-generation vaccines are an urgent requirement. A crucial criterion to identify malaria vaccine candidates is not only the validation of a specific antigen as target of NAI but also the confirmation that this immune response is associated with reduced risk of disease in the target population.

Importantly, malaria cases are not equally distributed in endemic countries. This geographical disparity is observed at all transmission levels[2-4] and can be as pronounced as between households in the same village[4]. Variations in local transmission intensity and differential mosquito attraction seem to be major reasons for this heterogeneity in malaria exposure[5]. This phenomenon has major implications when studying correlates of protection, since unexposed individuals can be erroneously considered as protected[5,6]. In fact, inconsistent results are commonly found in the literature, where antibodies against the same antigen in similar populations are differentially associated with protection[7-13]. These discrepancies might be partially explained by the confounding effect of heterogeneous malaria exposure, together with the age of the studied population, as shown by several studies highlighting the role of age in protective immunity[14].

Previous approaches to account for heterogeneous malaria exposure are mainly focused on excluding malaria-unexposed individuals from the analyses[15-17]. Blood-stage infection at any time during the follow-up is the most common indicator of exposure. However, this methodology is logistically costly and is largely used in short-term follow-ups, given that it requires active case detection (ACD)[15]. Besides, it frequently implies the case treatment, limiting the identification of correlates of protection from symptomatic disease. For long-term follow-ups, in addition to blood-stage infection, clinical episodes have also been used as an

exposure indicator[17]. However, even focusing the analyses in those individuals reporting an infection and/or clinical episode during the follow-up, the microheterogeneity in the exposure level can affect the rate of immunity acquisition and consequently the identification of protective correlates. Therefore, it is not only important to focus the analyses on malaria-exposed individuals, but also to consider the exposure level, indicated by serology, clinical or demographic data. Similarly, results from longitudinal studies can also be confounded by the long follow-ups during which the outcome can occur, according to the assumption that the immune responses at baseline represent a stable measure of immune competence[6]. Hence, the follow-up of a longitudinal study can be higher than the actual time that the specific antibodies remain in blood, at least, at protective levels[6,18]. Approaches to control these phenomena are thought to contribute to a deeper understanding of NAI against vaccine candidates.

We hypothesized that correlates of protection are better identified when studying only malaria-exposed individuals, when limiting the follow-up time according to the antibodies' decay and in older compared to younger children. To address this, we analyzed antibody responses and their functionality to *Plasmodium falciparum* antigens and assessed the association with clinical malaria risk in Mozambican children. The antigens studied are expressed during pre-erythrocytic and erythrocytic stages, including Erythrocyte-Binding Antigens (EBAs), *P. falciparum* Reticulocyte-binding Homologs (PfRhs), Merozoite Surface Proteins (MSPs) and novel antigens scarcely or not tested yet in longitudinal studies, namely P41, AARP (Apical Asparagine-Rich Protein) or CyRPA (Cysteine-Rich Protective Antigen).

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METHODS

Study area and population

The study was conducted in the Manhiça District, southern Mozambique[19], where a demographic surveillance system (DSS) provides accurate information on its population (see supplementary methods). Malaria transmission is perennial with some seasonality, coinciding with the rainy season (November-April). *P. falciparum* and *Anopheles funestus* are the predominant species and vector, respectively. Entomological inoculation rate in 2002 was 38 infective bites/person/year[19].

Study population comprised children from three cohorts studied between 2002 and 2007, in placebo-controlled trials (see supplementary methods and[20-22]). Children from the placebo arm of each cohort were selected to exclude the effect of any intervention in the acquisition of immunity. Briefly, cohort A[20] involved newborns followed from birth until 2 years of age (cross-sectional visits at 0, 2.5, 5.5, 10.5, 15 and 24 months). Cohort B[22] included children enrolled at 5 months old who underwent cross-sectional visits at 5, 9, 12 and 24 months. Finally, cohort C[21] involved newborns followed from birth until 12 months of age (cross-sectional visits at 0, 3, 9 and 12 months). For cohorts A and C, information about maternal malaria infections (peripheral and placental -acute, chronic, past-) was available, together with maternally-transferred antibodies (measured in cord blood). Infections were detected by microscopy (OM) and malaria episodes by passive case detection (PCD, except for cohort A, see supplementary methods). The National Mozambican and the Hospital Clínic of Barcelona Ethics Review Committees approved these studies. Written informed consent was signed at enrollment.

Antibody measurement

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AARP, PTRAMP (*Plasmodium* Thrombospondin-Related Apical Merozoite Protein), $MSP1_{19}$, MSP3, PfRh1, $PfRh2_{40}$, $PfRh4_{30}$, $PfRh4_{90}$, PfRh5, CyRPA, P41, VAR2CSA Duffy Binding-Like domains (DBL3X, DBL5ε and DBL6ε), DBLα (CR1-binding minimal domain from the *R29var1* PfEMP1), $PfRh2_{2030\text{-}2528}$, EBA175-PfF2, regions III-V of EBA175 ($EBA175_{III\text{-}V}$) and EBA140 ($EBA140_{III\text{-}V}$), and pre-erythrocytic antigens CSP (Circumsporozoite Protein), LSA1 (Liver Stage Antigen-1) and TRAP (Thrombospondin-Related Anonymous Protein) were produced in *E. coli*; AMA1 (Apical membrane antigen-1) in *Pichia pastoris* and gSG6-P1 (peptide from mosquito saliva) was synthesized by GenScript (see supplementary Table 1 and [23-30]). IgG and IgM responses were determined using Luminex xMAP™ beads (see supplementary methods). Briefly, multiplexed beads[31] were incubated with plasma samples, and antibody levels were measured as described elsewhere[31]. Positive, negative and background controls were added to each plate. Net Median Fluorescence Intensity (MFI) was obtained by subtracting blank values and prior to inter-plate normalization. Negative MFI values were substituted by the half-value of the minimum positive value for log-transformation.

Functional assays

In a subset of children with available samples (n=67), functional features of merozoite-specific antibodies at 24 months were studied. Opsonic phagocytosis (OPA)[32] and complement fixation assays (CFA)[33] are detailed in supplementary methods. Briefly, for OPA, freshly isolated merozoites were ethidium bromide-stained and incubated with plasma samples. THP-1 monocytes were added after opsonization. The number of THP-1 cells with internalized merozoites was determined by flow cytometry and the ratio to a positive control value was reported as the relative phagocytosis index (RPI%). In CFA, plates were coated with $MSP1_{19}$ or MSP3, and plasma samples incubated to allow antibody:antigen recognition. Human C1q was added, detected and its binding was measured by Optic Density.

Definitions and statistical analysis

Cross-sectional samples were grouped as follows: birth (cohort A and B); 2.5 months (cohort A); 5 months (cohort A -5.5 months-, cohort B -3 months-, cohort C -5 months-); 9 months (cohort A -10.5 months-, cohort B -9 months-, cohort C -9 months-); 12 months (cohort A -15 months-, cohort B -12 months, cohort C -12 months-); and 24 months (cohorts A and C). Clinical malaria was defined as axillary temperature ≥37.5ºC or reported fever in the preceding 24 hours with positive parasitemia by OM. Children were not considered at risk of malaria after 28 days of reporting an episode.

Seropositive threshold was defined by the mean plus 3 standard deviation of IgG and IgM antibody levels of 82 and 85 Spanish naïve donors, respectively. Breadth of antibody responses was determined by the sum of seropositive responses for each child. Malaria-exposed individuals were those that met, at least, one of the following criteria during the follow-up: report a previous *P. falciparum* infection or clinical episode; being seropositive for IgM levels against the pre-erythrocytic antigens CSP or LSA1; or a mother with reported infection during pregnancy (placental or peripheral).

Parametric or non-parametric tests were applied according to the variables distribution. Wilcoxon rank-sum (or Kruskal-Wallis) and chi-square tests were used to compare means or prevalence between groups, respectively. Correlation between variables were assessed by Spearman's correlation test.

To study the effect of age and infection on antibody levels, we performed crude and adjusted linear regression models. Cox proportional hazard models were used to calculate hazard ratios (HR) of clinical malaria. To test the hypothesis that protective associations of antibodies differ with age, we compared the HR in children at 9 and 24 months of age during a year follow-up. To compare the importance of accounting for malaria-exposure heterogeneity, two Cox models were estimated: (i) adjusted by infection at sampling, age,

gender, neighborhood of residence and cohort and (ii) limited to malaria-exposed individuals and adjusted by covariates specified in (i) and by exposure variables: IgM levels at sampling against pre-erythrocytic antigens; IgM levels against a mosquito saliva antigen (gSG6-P1) at sampling; and the number of previous malaria episodes. IgM adjustments were not done when analyzing IgM or functional immune responses' association with clinical malaria risk. Hazards proportionality was evaluated by studying the Schoenfeld residuals. The time to 25% of baseline (birth) antibody levels were estimated using linear mixed-effect regression models with data from birth to 5 months from children without infection or clinical episode during that time. These estimated times were used to limit the follow-up in Cox regression models when indicated.

P-values were corrected by the Benjamini-Hochberg procedure[34]. Statistical analyses were performed using Stata version 14 (StataCorp. College Station, TX, USA) and graphs with Prism7 (GraphPad) and R Core Team (2016, Vienna, Austria).

RESULTS

Antibody dynamics during the first two years of life

A total of 1142 samples were tested for IgG and IgM against 23 *P. falciparum* antigens. Characteristics of children studied in each cross-sectional visit are specified in Table 1. Maternal IgG antibodies (measured in the newborns) were, in general, lost during the first months up to 5 months of age when the antibody responses increased again. As expected, IgM antibody responses increased since birth (Figure 1). Main determinants of antibody levels in children from 5 months old were infection at sampling and age (Supplementary Table 2).

Association of antibody responses with risk of clinical malaria

Malaria-exposed children represented 84.2% of the studied population at 9 months old (208 out of 247) and 83.1% at 24 months of age (148 out of 178). Malaria-exposed children had higher breadth of IgG and IgM antibody responses at 9 and 24 months old compared to children considered non-exposed (Wilcoxon rank-sum test, $p<0.001$).

In order to identify correlates of protection while studying the importance of accounting for heterogeneous malaria exposure, Cox regression models adjusted by *P. falciparum* infection, gender, age, neighborhood of residence and cohort were compared to models estimated after accounting for malaria exposure. At 9 and 24 months of age, initial models showed significant associations of IgG and IgM levels with higher risk of clinical malaria (HR>1). However, when considering malaria exposure, these associations with increased risk disappeared, and protective associations emerged, only at 24 months of age. Breadth of IgG antibody responses was associated with a slightly reduced risk of clinical malaria (HR 0.93 [95% Confidence Interval 0.89, 0.97]; p=0.015). There were stronger protective associations for IgGs against CyRPA (HR 0.57 [95% CI 0.39, 0.83]; p=0.017), $EBA140_{III-V}$ (HR 0.55 [95% CI 0.39, 0.79]; p=0.009), DBL3x (HR 0.60 [95% CI 0.44, 0.81]; p=0.009) and DBL5ε (HR 0.55

239 [95% CI 0.40, 0.77]; p=0.009) (Figure 2). The specific antibodies differentially associated with
240 reduced risk at 24 versus 9 months of age were not necessarily higher in the older group,
241 being this the case for IgGs against CyRPA, DBL3X and DBL5ε (Wilcoxon rank-sum test,
242 p>0.050). In contrast, no association of specific IgMs with reduced risk was observed once
243 malaria exposure was considered. Some associations with higher clinical malaria risk were
244 still found (although lost after multiple comparisons correction, Supplementary Figure 1).
245
246 We also hypothesized that limiting the follow-up time according to the antibody's decay
247 would allow to better identify correlates of protection. The time to 25% of baseline levels was
248 calculated for all the specific IgGs (Figure 3). This time ranged from 78 (95% CI [92, 64];
249 p<0.001) for PTRAMP to 550 days (95%CI [397, 703]; p<0.001) for P41. These times were
250 used to limit the follow-up time in Cox regression models. As shown in Supplementary Figure
251 2, this approach allowed to decrease the number of significant associations of specific
252 antibodies with higher risk of clinical disease in standard analyses, but not when
253 heterogeneity in malaria exposure was considered.
254
255 ***Functional immune responses***
256 For a subset of children at 24 months of age (n=67), we analyzed the capacity of (i)
257 phagocytosis induction by antibodies targeting merozoites and (ii) C1q fixation by MSP_{19} and
258 MSP3-specific antibodies. Association of C1q fixation with reduced malaria risk was found
259 only for MSP_{19} when considering malaria exposure (HR 0.11 [95% CI 0.02, 0.57]; p=0.009;
260 n=45), but not for MSP3 specific antibodies (HR 0.5 [95% CI 0.2, 1.3); p=0.143). $MSP1_{19}$ nor
261 MSP3-specific antibodies were associated with protection in this subset (p=0.994 and 0.922,
262 respectively). Their C1q-fixation activity did not correlate for $MSP1_{19}$-specific antibodies, in
263 contrast to antibodies targeting MSP3 (Spearman's rho=0.37, p<0.001 for IgG and
264 rho=0.212, p=0.03 for IgM). RPI increased with increasing breadth of IgG responses
265 (Spearman's rho=0.47, p-value<0.001), and was significantly higher in those children
266 considered exposed compared to those considered non-exposed (Wilcoxon rank-sum test,

267 p<0.001). However, RPI was not associated with reduced risk of clinical malaria even when
268 considering malaria exposure.

DISCUSSION

In this study, we describe the dynamics of antibody responses against *P. falciparum* antigens in the first two years of life, and report correlates of protective immunity in children from Mozambique. This is the first study to point the association of antibodies targeting CyRPA and AARP with reduced malaria risk and to assess functionality of antibody responses in such young children.

Studies restricted to infants are considered less adequate to analyze correlates of protection given the confounding effect of *in utero* exposure and maternal transferred antibodies. However, this study is focused in children old enough to have lost maternal antibodies and built their own immunity, but young enough to still be the target population of routine vaccinations. It is known that antibodies need a mature immune system to perform certain functions[35], reason that can explain the lack of protective associations at 9 months compared to 24 months of age, as well as discrepancies with other studies with older children[13,32,33]. It is possible that specific antibody levels at 9 months are below protective thresholds[18], compared to 24 months. The protective threshold can be defined by intrinsic immune system features changing with age, as previously suggested[14]. This observation is in agreement with studies describing higher RTS,S vaccine efficacy in older children[1].

IgGs targeting CyRPA, DBL3x, DBL5ε, $EBA140_{III-V}$ were associated with reduced clinical malaria risk at 24 months of age, in addition to AARP, DBLα, LSA1, $PfRh_{2030}$ and P41, that lost significance after multiple comparisons correction. Some of them have already been shown as correlates of protection[13], but CyRPA and AARP are shown as correlates of protection for the first time. Unexpectedly, association of antibodies against DBL5ε, a VAR2CSA domain which binds to Chondroitin Sulphate A (CSA), with lower malaria risk was consistently found. *P. falciparum*-infected erythrocytes binding to CSA was first reported in pediatric isolates[36], suggesting the possible contribution of CSA-binding to malaria

infection and disease in children[37]. However, we cannot discard DBL5ε-specific antibodies as markers of other immune effectors responsible for such observation. We did not identify PfRh5-specific antibodies as correlates of protection, as previously reported[13]. These antibodies can act through the disruption of the PfRh5/CyRPA/Ripr complex[25], which can be also mediated by CyRPA-specific antibodies. IgM association with higher risk was reduced after accounting for malaria exposure. However, IgM targeting EBA175-PfF2 at 24 months of age was associated with higher clinical malaria risk, which can be explained by mechanisms such as IgM masking[38]. Finally, we report that complement fixation by antibodies targeting $MSP1_{19}$ is associated with protection[33], compared to IgG or IgM levels against $MSP1_{19}$. This confirms previous results where $MSP1_{19}$, but not MSP3, are targets of antibodies fixing complement[33], and highlights the importance of studying not only specific antibody levels, but also functionality of immune responses to not miss any potential correlate.

Importantly, correlates of protection were identified only when accounting for heterogeneity in malaria exposure by (a) including only malaria-exposed individuals; and (b) considering different exposure levels by clinical, serological and demographic data. By doing so, the effect of heterogeneous malaria exposure was reduced, and therefore correlates of protection could be identified. Limiting the follow-up time did not have an impact on our conclusions when considering malaria exposure, probably by the longer maintenance of protective immunity in malaria-exposed population, although this approach was shown useful when exposure variables were not available. This will happen in low transmission contexts where malaria exposure will be more difficult to define due to the expected drop in clinical episodes.

This study has some limitations. First, truly exposed individuals could be excluded from the analysis, given the restrictive definition of exposure used and the lack of serological data after 24 months. In this line, the PCD and OM methodologies can lead to underestimate the

exposed population. However, the study area has an established DSS, that allowed a rigorous and complete documentation of clinical malaria cases over the study period. Furthermore, our definition of malaria-exposure included clinical, parasitological and serological markers (IgMs). Another potential limitation is the methodology to control the follow-up time. Given that protective thresholds are unknown, the time to 25% of baseline maternal antibody levels was arbitrarily selected to allow antibodies to decrease while remaining higher than the seropositivity cutoff. Other limitations include a lacking evaluation of combination of antibody responses associated with malaria risk and of functional immunity, that needs to be further studied in bigger populations, with a larger panel of antigens and including subclasses characterization[32,33,39,40]. This will provide enough statistical power to identify, if any, more correlates of protection, since unfortunately, availability of samples was limited.

Altogether, we report new correlates of clinical malaria protection, and the importance of age in these studies. We also confirm the crucial requirement to account for heterogeneity in malaria exposure during vaccine development: from the identification of candidates to the evaluation of its efficacy. This need will become more apparent as malaria transmission drops, since the probability of including malaria-unexposed population in clinical trials will increase.

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DECLARATION OF INTERESTS

The authors have declared that no conflict of interest exists.

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TABLES

Table 1. Characteristics of children in the cross-sectional visits.

		Birth	2,5 months	5 monhts	9.5 months	12 months	24 months	p-value
Age (months) [1]		0.00 (0.00) [143]	2.60 (0.09) [71]	4.73 (1.12) [252]	9.60 (0.63) [247]	13.06 (1.31) [256]	24.16 (0.42) [178]	-
Males [2]		61 (43%)	32 (45%)	116 (46%)	110 (45%)	118 (46%)	85 (48%)	0.9631 [3]
Malaria infection at sampling [2]		14 (10%)	12 (17%)	25 (10%)	35 (14%)	16 (6%)	66 (37%)	< 0.0001 [3]
Cohort [2,4]	A	70 (49%)	71 (100%)	70 (28%)	69 (28%)	72 (28%)	71 (40%)	< 0.0001 [3]
	B	0 (0%)	0 (0%)	110 (44%)	108 (44%)	113 (44%)	107 (60%)	
	C	73 (51%)	0 (0%)	72 (29%)	70 (28%)	71 (28%)	0 (0%)	
	Total	143 (100%)	71 (100%)	252 (100%)	247 (100%)	256 (100%)	178 (100%)	
Number of previous malaria episodes [2]	0	143 (100%)	72 (99%)	239 (92%)	202 (77%)	192 (74%)	110 (59%)	-
	1	0 (0%)	1 (1%)	19 (7%)	37 (14%)	40 (15%)	32 (17%)	
	2	0 (0%)	0 (0%)	2 (1%)	13 (5%)	14 (5%)	19 (10%)	
	3	0 (0%)	0 (0%)	1 (0%)	8 (3%)	9 (3%)	7 (4%)	
	4	0 (0%)	0 (0%)	0 (0%)	1 (0%)	3 (1%)	4 (2%)	
	>4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (1%)	15 (8%)	
Malaria-exposed Children by Cohort [2,4]	A	41 (56%)	61 (86%)	63 (90%)	59 (86%)	59 (82%)	64 (90%)	0.0002 [5]
	B	-	-	90 (82%)	89 (82%)	95 (84%)	84 (79%)	
	C	57 (78%)	-	64 (89%)	60 (86%)	65 (92%)	-	
	Total	98 (69%)	61 (86%)	217 (86%)	208 (84%)	219 (86%)	148 (83%)	

1: Arithmetic Mean (SD) [n]
2: n (column percentage)
3: Chi-squared test
4: See supplementary methods for description
5: Chi-squared test of "Total" row

Con formato

Con formato

493 **FIGURE LEGENDS**

494 **Figure 1. Boxplots of antibody levels for each cross-sectional visit.** For each age
495 category in ascending order, n = 143, 71, 252, 247, 256, and 178, respectively. MFI: Mean
496 Fluorescence Intensity.

497

498 **Figure 2. Hazard ratios and the 95% confidence interval of clinical malaria for IgG**
499 **levels against *P. falciparum* antigens for children at 9 (a, b) and 24 months (c, d).**
500 Panels (a) and (b) represent the hazards obtained from Cox-regression models adjusted by
501 infection status, gender, age, neighborhood of residence and cohort. Panels (b) and (d)
502 represent the hazards obtained from Cox-regression models adjusted by infection status,
503 gender, age, neighborhood of residence and cohort and that accounted for heterogeneity in
504 malaria exposure (by excluding non-exposed individuals and adjusting for exposure
505 variables: number of previous malaria episodes, IgM levels against pre-erythrocytic and
506 gSG6-P1 antigens at sampling).

507

508 **Figure 3. Time to the drop of antibody levels to their 25% levels.** The time, in days, to
509 the drop to a 25% of the antibody levels at birth was calculated with a mixed-effect models
510 including children from birth to 5 months of age who did not report any infection nor clinical
511 episode.