



RESEARCH ARTICLE

REVISED **10-year longitudinal study of malaria in children: Insights into acquisition and maintenance of naturally acquired immunity [version 3; peer review: 2 approved]**

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V3 **First published:** 09 Apr 2021, **6:79**
<https://doi.org/10.12688/wellcomeopenres.16562.1>
Second version: 03 Dec 2021, **6:79**
<https://doi.org/10.12688/wellcomeopenres.16562.2>
Latest published: 04 Feb 2022, **6:79**
<https://doi.org/10.12688/wellcomeopenres.16562.3>

Abstract

Background: Studies of long-term malaria cohorts have provided essential insights into how *Plasmodium falciparum* interacts with humans, and influences the development of antimalarial immunity. Immunity to malaria is acquired gradually after multiple infections, some of which present with clinical symptoms. However, there is considerable variation in the number of clinical episodes experienced by children of the same age within the same cohort. Understanding this variation in clinical symptoms and how it relates to the development of naturally acquired immunity is crucial in identifying how and when some children stop experiencing further malaria episodes. Where variability in clinical episodes may result from different rates of acquisition of immunity, or from variable exposure to the parasite.

Methods: Using data from a longitudinal cohort of children residing in an area of moderate *P. falciparum* transmission in Kilifi district, Kenya, we fitted cumulative episode curves as monotonic-increasing splines, to 56 children under surveillance for malaria from the age of 5 to 15.

Results: There was large variability in the accumulation of numbers of

Open Peer Review

Reviewer Status

	Invited Reviewers	
	1	2
version 3		
(revision)		
04 Feb 2022		report
version 2		
(revision)		
03 Dec 2021	report	report
version 1		
09 Apr 2021		
	report	report

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clinical malaria episodes experienced by the children, despite being of similar age and living in the same general location. One group of children from a particular sub-region of the cohort stopped accumulating clinical malaria episodes earlier than other children in the study. Despite lack of further clinical episodes of malaria, these children had higher asymptomatic parasite densities and higher antibody titres to a panel of *P. falciparum* blood-stage antigens.

Conclusions: This suggests development of clinical immunity rather than lack of exposure to the parasite, and supports the view that this immunity to malaria disease is maintained by a greater exposure to *P. falciparum*, and thus higher parasite burdens. Our study illustrates the complexity of anti-malaria immunity and underscores the need for analyses which can sufficiently reflect the heterogeneity within endemic populations.

Keywords

Plasmodium falciparum, Clinical Malaria, Protective Immunity, Longitudinal Cohorts, Growth Curves



This article is included in the [The Francis Crick Institute gateway](#).

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Competing interests: No competing interests were disclosed.

Grant information: This study was funded by the UK Medical Research Council (MR/M003906/1). The samples were collected within the Kilifi malaria immunology cohort studies supported by the Wellcome Trust (B9RTIRO). JWGA, YB, and JL are supported by the Francis Crick Institute, which receives its funding from the UK Medical Research Council, Cancer Research UK, and the Wellcome Trust, UK (FC001101). JL is a Wellcome Senior Investigator (104777). YB is a Crick Africa Network (CAN) Fellow (FC10902). FMN is supported by an MRC/DFID African Research Leadership Award, MB and AR are supported by the Wellcome Trust (206194). For the purpose of Open Access, the authors have applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission. This manuscript is published with permission from the Director, KEMRI.

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How to cite this article: Addy JWG, Bediako Y, Ndungu FM *et al.* **10-year longitudinal study of malaria in children: Insights into acquisition and maintenance of naturally acquired immunity [version 3; peer review: 2 approved]** Wellcome Open Research 2022, 6:79 <https://doi.org/10.12688/wellcomeopenres.16562.3>

First published: 09 Apr 2021, 6:79 <https://doi.org/10.12688/wellcomeopenres.16562.1>

REVISED Amendments from Version 2

This version has a sentence omitted from the methods which was included in error.

Any further responses from the reviewers can be found at the end of the article

Introduction

Malaria is a major global health problem responsible for millions of clinical cases each year with the highest burden of mortality in children under 5 years of age¹. A malaria infection is caused by the protozoan parasite *Plasmodium*, with the most virulent human parasite, *Plasmodium falciparum* (*Pf*), responsible for over 90% of malaria-related morbidity and mortality, mostly in sub-Saharan Africa¹. Subsequent repeated exposure to *Pf* infections eventually leads to the development of partial immunity²⁻⁴. Evidence for such immunity includes the age-associated decrease in frequency and severity of clinical malaria episodes among children living in endemic areas where *Pf* infections in older children present with lower parasite densities, infrequent malaria symptoms and may produce more *Pf*-specific antibodies⁵.

Although repeated clinical episodes of malaria have been shown to lead to substantial and diverse host immune responses⁶ the precise mechanism(s) by which partial immunity to malaria develops and is maintained, remains unclear. Development of partial immunity to malaria likely involves a complex interplay between an antigenically diverse parasite and a dynamic host immune response. Investigating this process within human populations is challenging given the many factors that influence the development and maintenance of immunity to *Pf* including age², genetics, the number of previous clinical episodes⁶ as well as past and current exposure⁷ to the parasite. While some of these factors are relatively easily quantified, accurately estimating total exposure is extremely difficult as not all exposure results in clinical manifestations. Exposure to *Pf* has been demonstrated to be extremely heterogeneous, exhibiting both temporal (seasonal) and micro-geographic variation⁷⁻⁹.

Longitudinal study cohorts, often considered the “goldstandard” in observational studies of natural infection, can provide very useful insights into the development of antimalarial immunity¹⁰. Individuals typically under active surveillance are followed for several years, during which time all clinical cases of malaria are recorded. Given the impracticality of large, continuous entomological surveys, such studies typically estimate parasite exposure based on the incidence of clinical malaria within a specified geographic area¹¹. The aggregate number of episodes an individual experiences is dependent on both the extent of their exposure to the *Pf* parasites and their level of immunity. As such, in areas with reasonably high transmission intensity, the number of episodes an individual experiences

would be expected to decline over time, not necessarily because transmission intensity in that geographic area is reducing, but rather because of the development of partial immunity.

After following 56 individuals over ten-years from a longitudinal study cohort, we are able to compare the rate at which each individual acquires episodes over time, an approach only possible with long-term surveillance datasets. In such an approach, the development of immunity against malaria may be illustrated as a cumulative malaria episode curve (previously used to study the rate of growth in young children¹²), where a plateau in accumulated episodes from children in an endemic region may be considered as evidence of the development of immunity. By visualizing the rate of accumulation of clinical episodes for each child individually, we are better able to capture the heterogeneity of clinical episodes within the population. For a subset of individuals who stop accumulating more episodes within this age-span, we compared the levels of antibodies to selected *Pf*-antigens to help determine if the decline in the rate of accumulating episodes is related to acquisition of immunity or rather reduced exposure to the parasite.

Methods

Ethics and consent

The study protocol and its subsequent amendments received ethical and scientific approval from the Kenyan Medical Research Institute National Ethics Committee (KEMRI SSC 1131 & KEMRI SERU 3149). Written informed consent in the local languages (Swahili or Giriama) was required from parents/guardians for participation.

Study population

The study took place at the KEMRI-Wellcome Trust Research Programme (KWTRP) situated next to the Kilifi County Hospital, Kilifi, Kenya. The hospital serves approximately 500,000 people living in Kilifi County. The children investigated were residents of Junju a community on the southern side of an Indian Ocean creek and inhabited by predominantly Mijikenda people. Over the last 15 years, there has been a gradual, heterogeneous decline in malaria transmission in Kilifi County^{13,14} whereby transmission in Junju village has remained stable with a parasite prevalence of 30%^{15,16} during the dry season. However, there are two high malaria transmission seasons, May to August and October to December, during which parasite prevalence rises beyond 70%. Children are recruited into the cohort at or shortly after birth and actively monitored on a weekly basis for detection of malaria episodes until 15 years of age. Extensive and detailed records of the number and dates of malaria episodes for each child over the period they are enrolled in the cohort are maintained.

The Junju cohort was started in 2005 with children of various ages but has since continuously recruited newly born children, who subsequently drop out of the surveillance at the age of 15 years. The size of the cohort at any one point is 300–400 children. For these analyses, 56 children who were born

between 2001 and 2003 and had completed 10 years of malaria surveillance within the cohort were selected to determine whether there is heterogeneity in the rate of accumulation of clinical episodes with age.

A clinical malaria episode was defined as a body temperature greater than 37.5°C and 2500 parasites per microlitre of blood¹⁷. A year was defined from 1st of April to the 31st of March, capturing the total number of episodes before the wet season, which normally starts in April after a relative dry period of at least four months with minimal *Pf* transmission. For example, 2015 corresponds to the 1st of April 2014 to the 31st of March 2015. Parasite load (determined by microscopy and PCR) and serum antibody levels were measured from blood samples collected at the end of the dry season each year.

Sample collection

Pf episodes are normally diagnosed during weekly active surveillance carried out by a field worker based in the same village as the child. During these visits auxiliary body temperature, and or recent history of fever is taken, and if a child is febrile a blood sample is taken for a *Pf* specific rapid diagnostic test (RDT) and for blood smears. The blood smears are read later to determine the *Pf* parasite densities used in this paper, whilst immediate antimalarial treatments are administered based on the RDT testing.

Additionally, an annual cross-sectional survey is conducted in March, just before the beginning of the rains that marks the beginning of the main malaria transmission season in Kilifi. During these surveys, 5ml of venous blood (for immunological studies) and blood smears for detection and subsequent calculation of the associated cross-sectional *Pf* densities and prevalence. Furthermore, q-rPCR has been applied to all the samples collected since 2007 to complement the microscopy data.

Determination of parasite density

Thick and thin blood films were stained with Giemsa and *Pf*-infected red cells counted against 500 leukocytes and 1,000 red blood cells, respectively. To detect lower parasite densities, a highly sensitive *Pf* -specific PCR assay based on 18 was performed.

A sensitive high qPCR assay was used for detection where 500 µl of whole venous blood was used to extract DNA using an automated DNA extraction and purification method (QIASymphony platform, Qiagen, Germany) according to the manufacturer's instructions. DNA was eluted in 100 µl of DNase free water/elution buffer from which 13.5 µl was used to amplify the 18S ribosomal RNA gene by qPCR (we used Applied Biosystems' TaqMan™ Universal PCR Master Mix (cat no 4318157) which already contains the DNA polymerase (AmpliQ Gold™ DNA Polymerase)) in triplicates in a hydrolysis probe assay using primers and probes previously described. The PCR cycling conditions were carried as described using Applied Biosystems 7500 real-time PCR system. Non-template

control was used as a negative control (in triplicate wells) with parasite quantification against known cultured parasite standards comprising of six serial dilutions of extracted DNA also run-in triplicate.

Antigens for ELISA

Pf -specific plasma IgG plasma antibody responses were quantified against recombinant *Pf* AMA1 (FVO, 3D7 and L32 alleles), MSP1-42 kDa (3D7 and FUP alleles) and MSP3, to which circulating IgG antibodies were associated with clinical protection in previous studies¹⁹⁻²². Recombinant *Pf* antigens were kindly provided by L.H. Miller and colleagues from the Laboratory of Malaria and Vector Research (National Institute of Allergy and Infectious Disease, NIH, Rockville, MD, USA).

Eleven serial dilutions of a purified immunoglobulin reagent (malaria immune globulin [MIG]) prepared from a pool of immune Malawian adults²³ were included in every ELISA plate to provide a standard dilution curve that allowed conversion of optical density (OD) readings to antibody concentrations relative to levels present in MIG²⁴.

ELISA

Plasma samples from the cross-sectional surveys of 2015, 2016 and 2017 were tested for human IgG antibodies specific for AMA1, MSP1-42 and MSP3 antigens using a standard ELISA protocol. Recombinant *Pf* antigens were provided by L. H. Miller (National Institutes of Health, Rockville, MD). Plates were coated overnight at 4 °C, with recombinant proteins at 1 µg/mL in bicarbonate buffer (100 µL/well). One-hundred microliters per well of 1 in 1,000 dilution of test plasma in 0.3% (vol/vol) PBST + EDTA was added after plates had been washed three times with 0.05% (vol/vol) Tween in phosphate buffered saline (PBST), and thereafter blocked with 10% (vol/vol) foetal calf serum (FCS)/PBS (200 µL/well). Plates with test plasma were then incubated for 1.5 h at room temperature in a humidified chamber. Plates were then washed five times before the addition of alkaline phosphatase (AP)-labelled goat anti-human IgG Abs (Sigma) conjugate at 1:2,000 dilution 0.05% PBST at 100 µL/well. After 1h incubation with the conjugate, the plates were washed five times and the human IgG complexed with the AP-labelled conjugate revealed with and P-nitrophenyl phosphate (Sigma). The substrate reaction was stopped with 50 µL/well of 3 M NaOH, after which the plates were left for 5 min in the dark before being read at 405/570 nm. Antibody levels were quantified against respective standard curves on each plate of a purified hyperimmune IgG from immune adults and expressed in arbitrary units²⁵.

Monotonic increasing functions

Spline functions²⁵ were fitted to the 56 children who completed the cohort study from Junju, from the age of 5 to 15. The functional relationship of accumulated malaria episodes over time t , $g(t)$, may be represented as a smoothed function through linear combinations of model coefficients c_k and basis functions $\phi_k(t)$, where

$$g(t) = \sum_{k=1}^k (c_k \phi_k(t)).$$

Shape constrained additive models were used to ensure the accumulated malaria episode function never decreased and followed a monotonic functional relationship with time²⁶. These functions were fitted in R using the SCAM package²⁷. A log-link function was used to model the malaria count data. The smoothing parameter of each SCAM was fixed at 0.01 at 7 basis functions to make lines across all children comparable. The first derivative of the fitted accumulation of malaria episodes ($g'(t)$) represents the estimated number of episodes for that time point, t . Children who stop experiencing episodes in their last three years in the study were considered plateauers and their parasite density and antibody levels were investigated to see if this was due to a drop in exposure.

Statistical analysis

To understand why those children experiencing no more clinical malaria episodes, measurements of the levels of AMA1, MSP1 and MSP3-specific antibodies were compared between plateauers and children who experienced episodes up to the last three years of the cohort study. Antibody measurements were measured from samples taken in 2015, 2016 and 2017 and followed a crossed design structure fitted through a mixed model framework in the R package lme4²⁸.

$$Y = X\beta + Zv + \varepsilon$$

Where, $X\beta$ are the models fixed effects of Group (whether they plateaued in clinical episodes by the age of 12 or did not) and Year (2015, 2016 and 2017) and Zv is the random effect of Participant. An F-test was used to determine the significant differences of the fixed effects based on the Kenward-Roger method²⁹ from the lmerTest R package³⁰. Standard error of the difference was derived from the predictmeans package in R³¹ for each linear mixed effect model. For comparisons between antibodies AMA1 (3D7, L32) and MSP1 (FVO) the

sample sizes for each group and year were: Continuous 11, Plateau 6 (2015); Continuous 10, Plateau 8 (2016) and; Continuous 10, Plateau 8 (2017). The comparison group sizes for antibodies AMA1 (FVO), MSP1 (3D7, FUP) and MSP3 (FVO) were: Continuous 10, Plateau 8 (2015); Continuous 10, Plateau 8 (2016) and Continuous 10, Plateau 8 (2017). Please note that these sample sizes do not add to 56. The samples for antibody comparison comprise children from the 56 who had antibody measurements from 2015 to 2017.

Results

Large between-child variation in accumulation of clinical episodes over time

Figure 1a shows the fitted accumulated number of clinical malaria episodes of all 56 children born between 2001 and 2003 who completed the cohort study period. The inter-quartile range of clinical episodes experienced by the age of 15 was 4–11.25, with a median of 7. The range in accumulated malaria episodes was large, with one child who experienced 32 episodes by the age of 15 compared to another child, who experienced only 1 clinical episode before the age of 15. The fitted year-to-year variation in episodes experienced by each child is given in Figure 1.

By the age of 8, 2 out of 56 children do not go on to experience any further clinical malaria episode over the entire study period. This value increases to 22 out of 56 by age 12. Generally, there does not seem to be any discernible trend in terms of cumulative number of episodes for the 38 children who experienced an episode within the last three years of the study (Figure 2a, c). Of the 22 children who stop experiencing episodes before the last three years, the rate at which they accumulated episodes slowed after an initial peak, but this peak varied for each child (Figure 2b, d). There does not seem to be a specific age where children as a whole suddenly acquire episodes. However, children who stopped experiencing episodes before the last three years of the study did not experience more than 9 episodes.

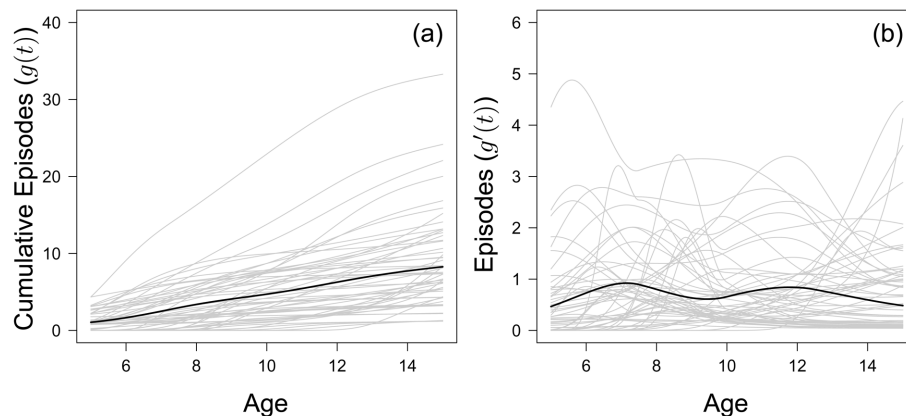


Figure 1. (a) The fitted monotonic increasing functions to cumulative malaria episodes against age for all children (grey) who left the study at 15 years of age, with the mean fitted line (black) to all children. (b) The first derivative of the fitted monotonic functions in (a), considered as the number of episodes each child experiences in a year.

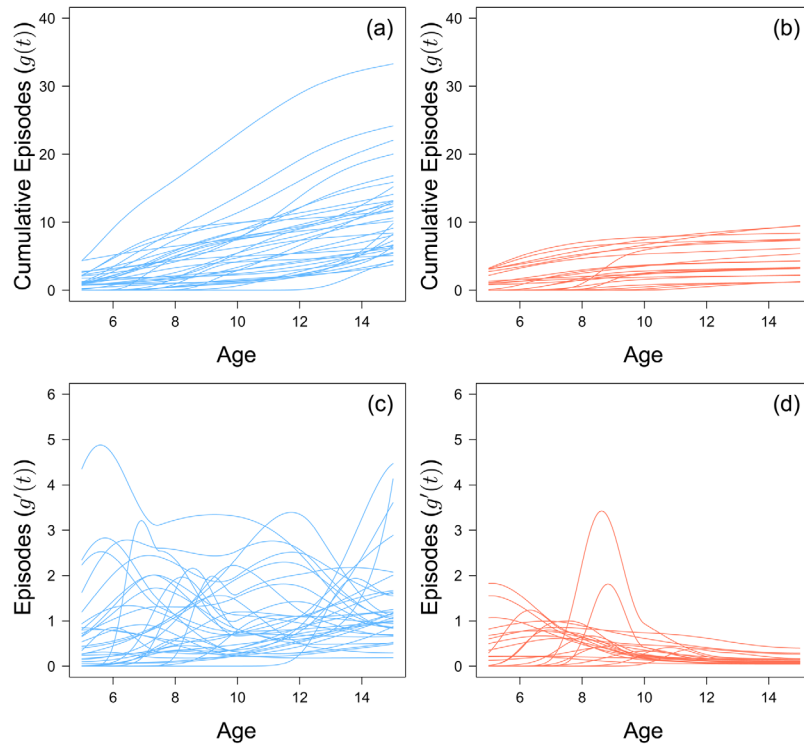


Figure 2. The fitted monotonic increasing functions to cumulative malaria episodes against age for children who experienced a malaria episode between the age of 13 and 15 in blue (a) and plateaued in their episodes at the age of 12 in red (b). The first derivative of the fitted monotonic functions of children who experienced a malaria episode between the age of 13 and 15 in blue (c) and plateaued in their episodes at the age of 12 in red (d).

Children who stop experiencing clinical episodes experienced a higher parasite density

The 22 children who stopped experiencing episodes in the last few years of the study tended to be in the South-West region of the region, whereas the rest of the children were mostly located in the North-East region (Figure 3a–b). When considering their annual asymptomatic (cross-sectional) parasite densities (parasite/mL) of children who were parasite positive, there was little difference until 2010. Of the children who were found to be parasite positive at the time of asymptomatic infection, from 2010, the children who then stopped experiencing episodes had, on average, higher asymptomatic parasite densities than other children (Figure 3c). Children who stopped experiencing episodes in the last three years of the study were also more likely to have positive results from the annual cross-sectional survey (Table 1). This finding agrees with the assumption that ability to carry higher parasitemia and remain asymptomatic is in fact a product of immunity. 2015 was the year with the largest difference and incidentally marked the period when most children within this group experienced their final clinical episode.

Children who stop experiencing clinical episodes are characterized by higher levels of circulating malaria-specific antibodies

Children who plateaued in their accumulation of clinical episodes had higher levels of antibodies, specific for a number of key

Pf antigens compared to children who continuously experienced clinical malaria episodes (AMA1 (3D7 ($F_{1,16} = 6.81$, $p = 0.019$), FVO ($F_{1,16} = 7.77$, $p = 0.013$), L32 ($F_{1,16,01} = 7.11$, $p = 0.017$) and MSP3 (FVO ($F_{1,16} = 22.65$, $p < 0.001$); Table 2). MSP1 was the only antigen for which there were no significant differences between the two groups of children (Figure 5). Although there were small differences for MSP1 3D7 and FUP, small sample sizes were a limitation and larger sample sizes may be needed to detect a small difference.

Further, there were large yearly differences across all groups in the levels of circulating antibodies (AMA1 (3D7 ($F_{2,31,11} = 15.26$, $p < 0.001$), FVO ($F_{2,32} = 12.80$, $p < 0.001$), L32 ($F_{2,31,15} = 12.51$, $p < 0.001$), MSP1 (3D7 ($F_{2,32} = 5.34$, $p = 0.010$), FUP ($F_{2,32} = 19.04$, $p < 0.001$), FVO ($F_{2,32,53} = 16.75$, $p < 0.001$) and MSP3 FVO ($F_{2,32} = 14.22$, $p < 0.001$), Table 2). However, the year effect of antibody production was consistent across all antibody specificities (Figure 4 and Figure 5).

Discussion

From this 10-year observational study, our results demonstrate that small changes in geographic location can impact the accumulation of clinical manifestations of malaria. Children who continued to have episodes throughout the study were generally located in the North-East part of the study area and tended to be characterised by lower asymptomatic parasite densities and lower levels of circulating *Pf*-specific antibodies.

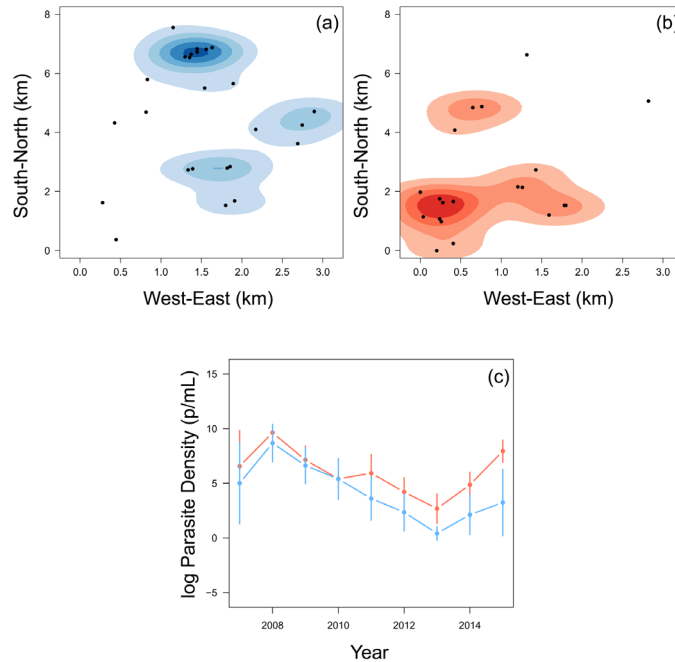


Figure 3. A smoothed histogram of the location of children who experienced a malaria episode between the age of 13 and 15 in blue (a) and plateaued in their episodes at the age of 12 in red (b). The mean (+95% confidence intervals) of the log parasite density of the annual cross-sectional survey for children who experienced a malaria episode between the age of 13 and 15 in blue and plateaued in their episodes by the age of 12 in red (c).

Table 1. The distribution of the 56 children (plateauers and non-plateauers) who had PCR positive and negative annual cross-sectional survey results from 2007 to 2015.

	Year									
	2007	2008	2009	2010	2011	2012	2013	2014	2015	
All										
Positive	14	27	23	29	23	24	19	26	23	
Negative	0	0	32	36	33	32	35	29	33	
% Positive	100.00	100.00	41.82	44.62	41.07	42.86	35.19	47.27	41.07	
Plateauer										
Positive	8	15	11	17	9	15	14	16	16	
Negative	0	0	10	5	13	7	8	6	6	
% Positive	100.00	100.00	52.38	77.27	40.91	68.18	63.64	72.73	72.73	
Non-Plateauer										
Positive	6	12	12	12	14	9	5	10	7	
Negative	0	0	22	21	20	25	27	23	27	
% Positive	100.00	100.00	35.29	36.36	41.18	26.47	15.63	30.30	20.59	

Where increases in parasitaemia were shown to be associated with higher antibody levels²⁰. These results indicate that micro-geographic regions of high parasite exposure³² have an impact on the acquisition of immunity, where children from

the same sub-region develop immunity at different rates based on their exposure to the parasite. Methods of estimating exposure such as molecular “force of infection”, which defines the number of new *Plasmodium* infections over time^{33,34},

Table 2. The Analysis of Variance tables for each antibody response at the Group, Year and Group:Year level, with corresponding F-tests and p-values.

AMA1: 3D7	Sum Square	Mean Square Error	Numerator DF	Denominator DF	F value	p-value
Group	1.43	1.43	1	16	6.81	0.019
Year	6.39	3.20	2	31.11	15.26	<0.001
Group:Year	0.25	0.13	2	31.12	0.61	0.552
AMA1: FVO	Sum Square	Mean Square Error	Numerator DF	Denominator DF	F value	p-value
Group	1.82	1.82	1	16	7.77	0.013
Year	5.99	2.99	2	32	12.80	<0.001
Group:Year	1.40	0.70	2	32	3.00	0.064
AMA1: L32	Sum Square	Mean Square Error	Numerator DF	Denominator DF	F value	p-value
Group	1.93	1.93	1	16.01	7.11	0.017
Year	6.79	3.39	2	31.15	12.51	<0.001
Group:Year	0.14	0.07	2	31.17	0.26	0.776
MSP1: 3D7	Sum Square	Mean Square Error	Numerator DF	Denominator DF	F value	p-value
Group	2.09	2.09	1	16	3.03	0.101
Year	7.35	3.68	2	32	5.34	0.010
Group:Year	0.94	0.47	2	32	0.68	0.513
MSP1: FUP	Sum Square	Mean Square Error	Numerator DF	Denominator DF	F value	p-value
Group	0.92	0.92	1	16	2.58	0.128
Year	13.59	6.80	2	32	19.04	<0.001
Group:Year	0.38	0.19	2	32	0.53	0.596
MSP1: FVO	Sum Square	Mean Square Error	Numerator DF	Denominator DF	F value	p-value
Group	0.03	0.03	1	15.92	0.15	0.702
Year	6.44	3.22	2	32.53	16.75	<0.001
Group:Year	0.06	0.03	2	32.61	0.15	0.858
MSP3: FVO	Sum Square	Mean Square Error	Numerator DF	Denominator DF	F value	p-value
Group	4.68	4.68	1	16	22.65	<0.001
Year	5.88	2.94	2	32	14.22	<0.001
Group:Year	0.50	0.25	2	32	1.21	0.312

and measurement of IgG antibodies to Anopheles salivary gland extracts^{35,36} and a spatially derived prevalence index based upon clinical symptoms³⁷, may provide a more precise picture of exposure in this small study area, and should be considered for future studies. Molecular studies would require intensive sampling, whereas the measurement of antibodies may reflect cumulative exposure more readily than current exposure, and were beyond the scope of the present manuscript.

Human cohort studies provide a unique opportunity to investigate the development of immunity to malaria. However, interpreting such studies is often a challenge as using number of clinical episodes as a measure of immunity makes it difficult to

distinguish between immune individuals and those who are simply less exposed to the parasite. In this study, we analyzed data from a ten-year longitudinal cohort of children using growth curves to capture the heterogeneity of accumulated clinical episodes, allowing for a better interpretation into more immune and less immune individuals. From these curves, large variations in the rate of accumulation of clinical episodes were observed, illustrating the challenges associated with extrapolating from such data to investigate the development of immunity to malaria.

Two sub-populations of children were identified; children who plateaued in the accumulation of clinical episodes at or before

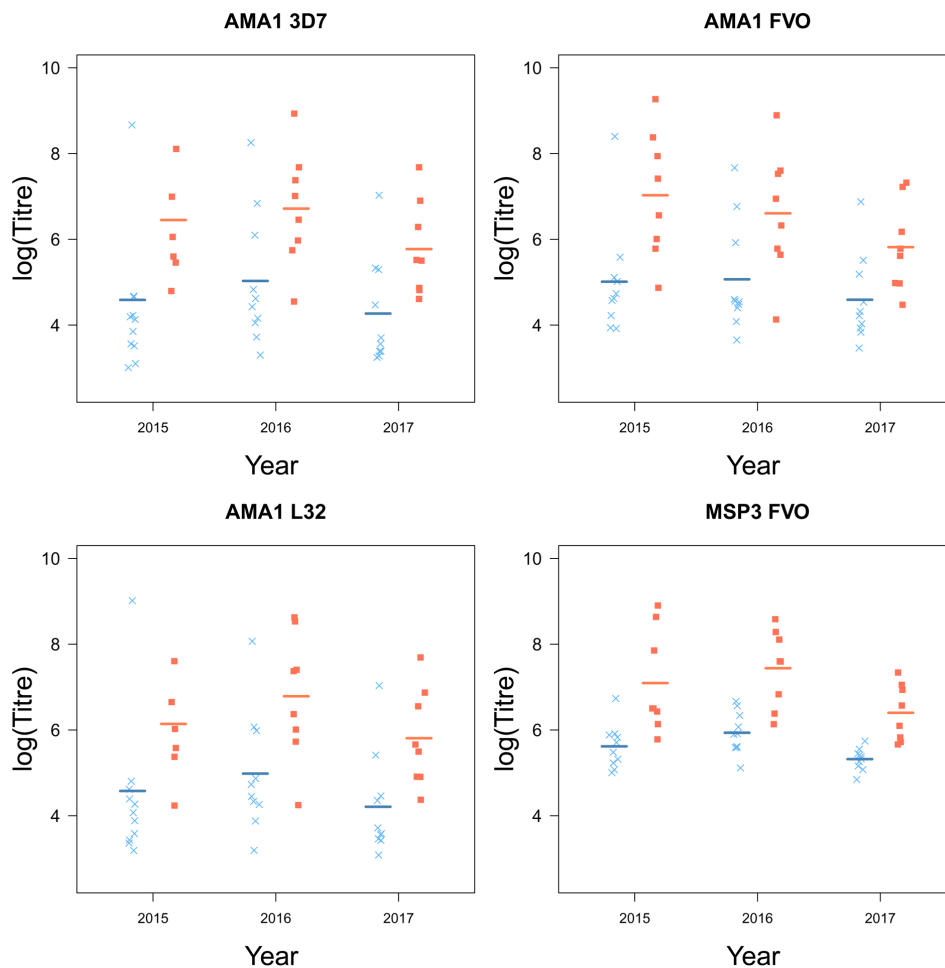


Figure 4. The antigens AMA1 and MSP3 and their strains from children who experienced a malaria episode between the age of 13 and 15 (blue x) and plateaued in their episodes at the age of 12 (red ■) from 2015 to 2017, with fitted values (line) from the linear mixed model.

the age of 12, and those children who continued to experience clinical episodes between the age of 13 and 15. Those children who plateaued in their accumulation of malaria episodes and who were infected at the time of asymptomatic sampling had, on average, higher asymptomatic parasite densities of *Pf* and were generally located in the South-West region of Junju (Figure 3c). Furthermore, children who plateaued had higher levels of circulating malaria-specific antibodies AMA1 and MSP3 (Figure 4). The regional differences in accumulated episodes appear to agree with our findings of spatial differences in the prevalence of clinical malaria among this cohort, with children in the South-West experiencing fewer clinical episodes³⁷. Our results show these regional differences seem to be reflected in the development of protective immunity.

Our findings agree with previous data, which suggest that protection from clinical malaria is associated with higher titres of *Pf*-specific antibodies³⁸ as well as an ability to remain asymptomatic whilst carrying higher parasite densities³⁹. The reducing rate of accumulation of clinical episodes with age is indicative of developing anti-disease immunity, i.e., the ability

to tolerate higher parasite densities without clinical malaria. This could be the result of higher exposure to *Pf* in the micro-environment of South-West Junju. It is intriguing that these higher parasite densities are maintained despite the higher levels of anti-AMA1 and MSP3 antibodies in the plateauing group. This suggests that these antibodies are not contributing significantly to anti-parasite immunity but are rather a reflection of the level of *Pf* infection.

Longitudinal surveillance cohorts are a very powerful tools to study anti-malarial immunity and a growing number of studies are adopting such a design in exploring the immune mechanisms responsible for mediating such immunity^{6,40,41}. These studies often classify individuals within their cohorts as immune or non-immune based on the total accumulated numbers of episodes that an individual has experienced over a period. Given the heterogeneous spatial and temporal distribution of the malaria parasite within a geographic area and study period respectively, such an approach is likely to be confounded by variations in exposure. By assessing each study participant's malaria history over ten years, we can provide a more

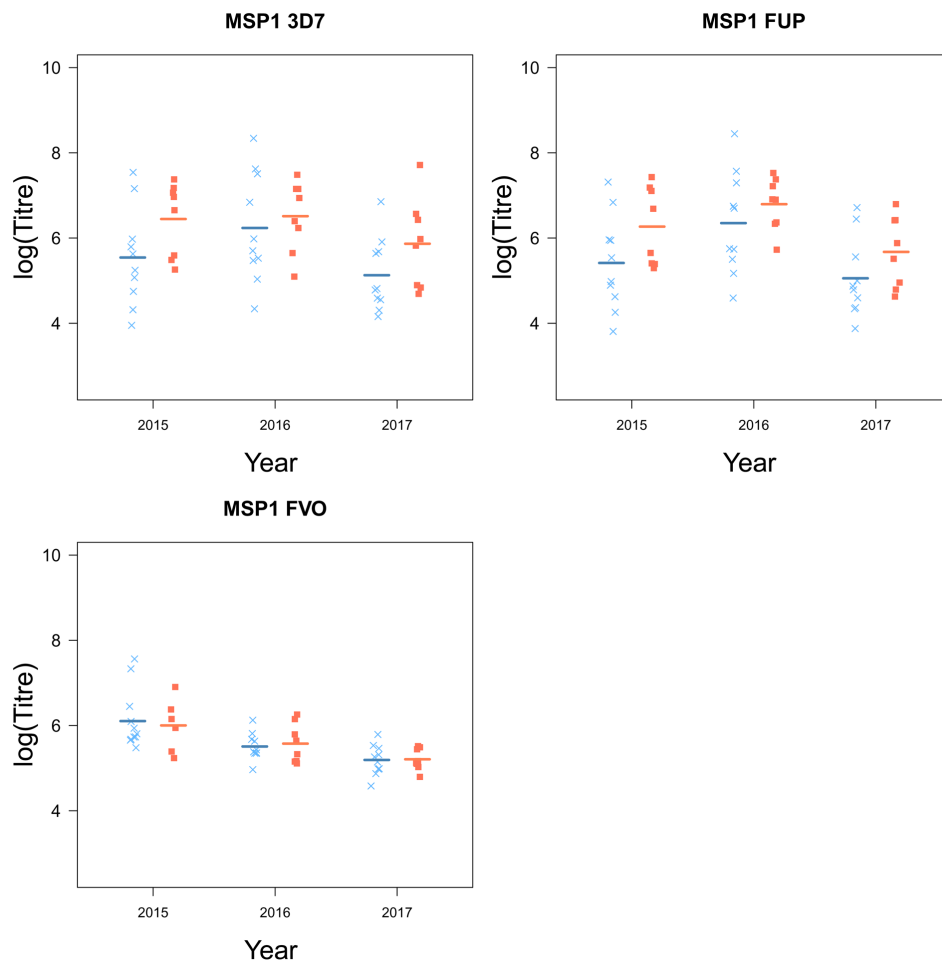


Figure 5. The antigen MSP1 and their strain from children who experienced a malaria episode between the age of 13 and 15 (blue ×) and plateaued in their episodes at the age of 12 (red ■) from 2015 to 2018).

comprehensive analysis of the diversity of malaria history within a cohort, facilitating more accurate identification of individual immune status. This type of cohort analysis, used together with measurements of antibody breadth²⁰, and functional capacity^{42–45} will extend our understanding of targets and mechanisms of protective immunity.

Data availability

Underlying data

Harvard Dataverse: Replication Data for: 10-year longitudinal study of malaria in children: Insights into acquisition and maintenance of naturally acquired immunity. <https://doi.org/10.7910/DVN/X4L47D>⁴⁶.

This project includes the following underlying data:

- Epi_Data_Pub.tab (underlying data file)
- JAddy_Epi_Data_Codebook.pdf (data code book)
- SummaryofAnalysis.html (R analysis script)

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/) (CC-BY 4.0).

Author contributions

JWGA and YB conceived the idea for the manuscript. JWGA performed the analysis. JWGA and YB wrote the manuscript. FMN contributed to acquisition and access to data, contributed to the idea of the manuscript and manuscript preparation. JJV, AJR, FMN, MR, MB, KM and PB contributed to study design and manuscript preparation. JMwa, JMwo, JW, EO, JMu, and KS contributed to malaria surveillance for malaria and laboratory data generation. EO contributed to surveillance for malaria and data management.

Acknowledgments

We would like to thank Dr. L.H. Miller and colleagues at NIH for the recombinant proteins, and Professor Chris Newbold, Oxford, for his critical comments on the manuscript.

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Open Peer Review

Current Peer Review Status:  

Version 3

Reviewer Report 07 February 2022

<https://doi.org/10.21956/wellcomeopenres.19559.r48528>

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Rhea J Longley 

Population Health and Immunity Division, Walter and Eliza Hall Institute of Medical Research, University of Melbourne, Melbourne, Victoria, Australia

My concern has been addressed.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Immunology of infectious diseases

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

Version 2

Reviewer Report 17 December 2021

<https://doi.org/10.21956/wellcomeopenres.19279.r47420>

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Rhea J Longley 

Population Health and Immunity Division, Walter and Eliza Hall Institute of Medical Research, University of Melbourne, Melbourne, Victoria, Australia

Thank you to the authors for addressing my comments. The updated manuscript communicates the research very nicely. I particularly appreciate the updated description of the study design and

inclusion of Table 1.

I am still somewhat confused by the response to my question on the ELISA and use of mixed alleles. I understand the concept behind wanting to mix alleles, but the results (in the text, table and figures) are presented per allele i.e. 3D7 and FVO separately. So were the antigen alleles mixed 1:1 and also assessed individually in the ELISA? Thank you for the clarification.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Immunology of infectious diseases

I confirm that I have read this submission and 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 30 Jan 2022

John Addy, Francis Crick Institute, London, UK

We thank the reviewer for their response.

This part of the methods has been corrected. The sentence "*For AMA1, ELISA plates were coated with a 1:1 mixture of FVO and 3D7 alleles*" was included in error.

Competing Interests: No competing interests were disclosed.

Reviewer Report 13 December 2021

<https://doi.org/10.21956/wellcomeopenres.19279.r47421>

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Rodrigo M Corder 

University of California, Berkeley School of Public Health, Berkeley, CA, USA

I thank the authors for their consideration of my comments. The additional information they provide in the publication satisfies my earlier concerns.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Mathematical modelling of infectious diseases

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

Version 1

Reviewer Report 06 September 2021

<https://doi.org/10.21956/wellcomeopenres.18251.r45152>

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**Rhea J Longley**

Population Health and Immunity Division, Walter and Eliza Hall Institute of Medical Research, University of Melbourne, Melbourne, Victoria, Australia

This manuscript studies the acquisition of clinical episodes of malaria over 10 years and identifies two patterns; one where children stop acquiring further clinical episodes after age 12 and one where they continue to acquire them. They relate this to both asymptomatic parasite densities and anti-malarial antibody responses. The data availability is fantastic and very clear with the additional files outlining the variable information and the script used for analyses. The manuscript is generally well-written but could use further clarification on the points below (in addition to points made by the first reviewer).

Major points:

1. Data from the yearly cross-sectional surveys: the authors present asymptomatic parasite densities in Fig3c but as far as I can tell, they have not presented a summary of this data i.e. # of children with asymptomatic infections at each yearly survey and thus # of children that make up the data presented in Fig3c. I think this is important for their conclusion "children from the same sub-region develop immunity at different rates based on their exposure to the parasite". Are the authors relating exposure to the parasite density or the total # infections (inc asymptomatic) experienced? Are all 56 children infected at each survey (I think no from the dataset)? Are the data in Fig3c from the blood-smears or qPCR (qPCR I believe, but methods states blood smear densities are used in the paper)?
2. ELISA results: in the methods it said the two alleles for AMA1 were mixed, so I am unclear how there are different results presented for both alleles? As the authors state in the introduction, current infections can have a big impact on antibody levels – can they comment on whether individuals from either group had current infections at the time the sampling was made for antibody measurements? In those that have current infections have the authors directly correlated/associated the parasite density with antibody level i.e. to give strength to their statement that antibodies are "a reflection of the level of Pf infection"?

Minor points:

1. Study population: This section in the methods is a little difficult to follow given it's a rolling enrolment and then a sub-group has been used. The 2nd paragraph in particular could be revised so it covers the whole cohort first then describes the 56 kids in this study. Would a schematic help? This might also help clarify the sampling for the yearly surveys, clinical episodes, antibodies etc.

2. Antigen/ELISA technical details: antigens need more info i.e. the expression system and construct – this could be referenced to a prior publication (it seems the references listed more relate to the statement about associations with protection, but the authors could clarify). What hyperimmune sample/s were used for the standard curve? Are they available for others to use i.e. is this a reference pool? How was the standard curve conversion performed? If this has all been done before a reference can be cited.
3. Fig3C: the parasite densities between the two groups are still fairly similar and do vary year on year - can the authors comment on whether that difference in parasite density is meaningful/statistically significant?
4. Discussion on characterising exposure in longitudinal cohorts: how about work that uses genotyping to understand the force of infection (PMID: 22665809¹)/molecular force of blood-stage infection (PMID: 24040428²)? Or antibodies against mosquito salivary gland antigens (PMID: 21175067³, PMID: 22195000⁴)?
5. Final sentence in discussion on mechanisms responsible for partial immunity “*By assessing each study participant’s malaria history over ten years, we were able to provide a more comprehensive analysis of the diversity of malaria history within a cohort, facilitating more accurate identification of individual immune status and ultimately a less confounded investigation of the mechanisms responsible for development of partial immunity to malaria*”. It might be worth also reflecting on work by others suggesting functional antibody assays are needed to identify targets of immunity rather than magnitude alone (PMID: 30723225⁵), as it could be argued the current manuscript does not identify the targets or mechanisms of the acquired immunity in these children – scope for future work.

I cannot comment on technical details of the mathematical models.

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

Partly

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?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Immunology of infectious diseases

I confirm that I have read this submission and 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 19 Nov 2021

John Addy, Francis Crick Institute, London, UK

We thank both reviewers for the positive review of our work. Their comments have added to the manuscript and have improved the quality and readability of the manuscript. We have addressed each point with an answer. In some cases, we have provided more information and clarity, in other cases we have accepted the issue and added to our manuscript accordingly. Responses from all reviewers have been included here.

This manuscript studies the acquisition of clinical episodes of malaria over 10 years and identifies two patterns; one where children stop acquiring further clinical episodes after age 12 and one where they continue to acquire them. They relate this to both asymptomatic parasite densities and anti-malarial antibody responses. The data availability is fantastic and very clear with the additional files outlining the variable information and the script used for analyses. The manuscript is generally well-written but could use further clarification on the points below (in addition to points made by the first reviewer).

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authors relating exposure to the parasite density or the total # infections (inc asymptomatic) experienced? Are all 56 children infected at each survey (I think no from the dataset)? Are the data in Fig3c from the blood-smears or qPCR (qPCR I believe, but methods states blood smear densities are used in the paper)?

Added new Table 1 about yearly summaries cross-sectional surveys which answers this point and addressed in text (see Section Children who stop experiencing clinical episodes experienced a higher parasite density). Exposure to the parasite is assessed using both asymptomatic measurements and clinical episodes. All 56 children live in a malaria region. qPCR data were used for Figure 3c.

ELISA results: in the methods it said the two alleles for AMA1 were mixed, so I am unclear how there are different results presented for both alleles? As the authors state in the introduction, current infections can have a big impact on antibody levels – can they comment on whether individuals from either group had current infections at the time the sampling was made for antibody measurements? In those that have current infections have the authors directly correlated/associated the parasite density with antibody level i.e. to give strength to their statement that antibodies are “a reflection of the level of Pf infection”?

The mixing of the two alleles means that we are only interested in the antibody response to AMA1 – irrespective to whatever alleles. Mixing ensures we minimise missing the response owing to the polymorphism. There are too few samples to do an adequate correlation between parasite density for a few of the antibodies. Added reference of evidence of relationship between parasite and antibodies in text (See the Discussion).

Minor points:

Study population: This section in the methods is a little difficult to follow given it's a rolling enrolment and then a sub-group has been used. The 2nd paragraph in particular could be revised so it covers the whole cohort first then describes the 56 kids in this study. Would a schematic help? This might also help clarify the sampling for the yearly surveys, clinical episodes, antibodies etc.

This question is similar to the other reviewers question, see the response to the other reviewer.

Antigen/ELISA technical details: antigens need more info i.e. the expression system and construct – this could be referenced to a prior publication (it seems the references listed more relate to the statement about associations with protection, but the authors could clarify). What hyperimmune sample/s were used for the standard curve? Are they available for others to use i.e. is this a reference pool? How was the standard curve conversion performed? If this has all been done before a reference can be cited.

This has been addressed in text (see Methods section “Antigens for ELISA”).

Fig3C: the parasite densities between the two groups are still fairly similar and do vary year on year - can the authors comment on whether that difference in parasite density is meaningful/statistically significant?

This difference is on the log-scale and shows that the parasite density is similar in both groups until 2010. This difference illustrates that although the temporal trends look similar, there is obvious spatial effects, plateauers seem to have higher parasite densities since 2010, but tend to live in the South-West. We cannot say which parasite density cut-off is responsible for the development of immunity here. A formal statistical comparison here is would not provide additional information as 95% confidence intervals have been produced.

Discussion on characterising exposure in longitudinal cohorts: how about work that uses genotyping to understand the force of infection (PMID: 226658091)/molecular force of blood-stage infection (PMID: 240404282)? Or antibodies against mosquito salivary gland antigens (PMID: 211750673, PMID: 221950004)?

Changed in text (see Discussion).

Final sentence in discussion on mechanisms responsible for partial immunity "By assessing each study participant's malaria history over ten years, we were able to provide a more comprehensive analysis of the diversity of malaria history within a cohort, facilitating more accurate identification of individual immune status and ultimately a less confounded investigation of the mechanisms responsible for development of partial immunity to malaria". It might be worth also reflecting on work by others suggesting functional antibody assays are needed to identify targets of immunity rather than magnitude alone (PMID: 307232255), as it could be argued the current manuscript does not identify the targets or mechanisms of the acquired immunity in these children – scope for future work.

I cannot comment on technical details of the mathematical models.

Changed in text (see Discussion).

Competing Interests: No competing interests were disclosed.

Reviewer Report 22 July 2021

<https://doi.org/10.21956/wellcomeopenres.18251.r45029>

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Rodrigo M Corder

University of California, Berkeley School of Public Health, Berkeley, CA, USA

This manuscript studies the variation in the number of clinical episodes experienced by children of the same age within the same cohort and relates it to the development of naturally acquired immunity. The analysis presented is particularly important as it highlights the need to account for heterogeneity within endemic populations in order to better define control and elimination measures. This is a well written manuscript and I have a few comments:

1. If antibody levels were compared between plateauers and children who experienced episodes up to the last three years of the cohort study, it is not clear to me why the sample sizes are different between comparisons AMA1(3D7, L32)/MSP1(FVO) and AMA1(FVO)/MSP1(3D7,FUP)/MSP3(FVO) over the years. For example, considering 2015, “continuous” are 11 when the comparison is between antibodies AMA1 (3D7, L32) and MSP1 (FVO), and 10 when comparison is between antibodies AMA1 (FVO), MSP1 (3D7, FUP) and MSP3 (FVO). Also, individuals do not add up to 56 (sample size). Please, clarify it.
2. Sentences like “cumulative episodes by the age of 15”, “with one child accumulating 32 episodes by the age of 15”, “another child, who experienced only 1 clinical episode before the age of 15” and “children who plateaued in their accumulation of clinical malaria episodes did not experience more than 9 episodes” are technically inconsistent if the analysis comprised children aged from 5 to 15. Please, rephrase them. The authors should also consider starting Figure x-tick labels (Age) with 5 and ending it with 15.
3. If my understanding is correct, by selecting 56 children who were born between 2001 and 2003, the age of them should not be equal at a given time. Therefore, why do the authors choose to do antibody analysis over “Year” (Figures 3c, 4 and 5 and Table 1) and not over “Age” (like in Figures 1 and 2)? It seems to me that the analysis over “Age” is one that best assesses each study participant's history of malaria. Please, clarify it.
4. The sentence “MSP1 was the only antigen for which there were no distinct differences between the two groups of children” may lead to wrong inferences. The authors should consider mentioning the sample size of the analysis as one of its limitations and improve the discussion about MSP1 antigen results.

I cannot comment on technical details of antibody methods and techniques.

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

Partly

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?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Mathematical modelling of infectious diseases

I confirm that I have read this submission and 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 19 Nov 2021

John Addy, Francis Crick Institute, London, UK

This manuscript studies the variation in the number of clinical episodes experienced by children of the same age within the same cohort and relates it to the development of naturally acquired immunity. The analysis presented is particularly important as it highlights the need to account for heterogeneity within endemic populations in order to better define control and elimination measures. This is a well written manuscript and I have a few comments:

If antibody levels were compared between plateauers and children who experienced episodes up to the last three years of the cohort study, it is not clear to me why the sample sizes are different between comparisons AMA1(3D7, L32)/MSP1(FVO) and AMA1(FVO)/MSP1(3D7,FUP)/MSP3(FVO) over the years. For example, considering 2015, “continuous” are 11 when the comparison is between antibodies AMA1 (3D7, L32) and MSP1 (FVO), and 10 when comparison is between antibodies AMA1 (FVO), MSP1 (3D7, FUP) and MSP3 (FVO). Also, individuals do not add up to 56 (sample size). Please, clarify it.

The initial part of this study focuses on children who have completed the cohort study and left when they were 15 by 2018 (so 10 years' worth of data for each individual, 56 in total, some children completed the cohort earlier, 2016 or 2015 for example). When comparing antibody data we want to remove all possible confounders, such as age. Also, the antibody data follow a repeated measures design, the same children sampled each year. Further, there is strong year-to-year variation in clinical malaria episodes and parasite densities across the study. So, in order to obtain an unbiased antibody comparison between plateauers and non-plateauers children who have antibody measurements across all three years were considered, and these were all children of the same age. A linear-mixed-model (LMM) was used, to identify the individual effect of each child and this was modelled as a random effect. The reason why 11 children were included for some antibodies and 10 for other, is because these are the same individuals, but there was a missing value for AMA1 (FVO), MSP1 (3D7, FUP) and MSP3 (FVO) antibodies. We reduced the influence this missing value has on our result by using a linear-mixed-model (LMM) and having child as a random effect. This has been clarified in the text (see Statistical Analysis section).

Sentences like “cumulative episodes by the age of 15”, “with one child accumulating 32 episodes by the age of 15”, “another child, who experienced only 1 clinical episode before the age of 15” and “children who plateaued in their accumulation of clinical malaria episodes did not experience more than 9 episodes” are technically inconsistent if the analysis comprised children aged from 5 to 15. Please, rephrase them. The authors should also consider starting Figure x-tick labels (Age) with 5 and ending it with 15.

Changed in text (see “Large between-child variation in accumulation of clinical episodes over time” section).

If my understanding is correct, by selecting 56 children who were born between 2001 and 2003, the age of them should not be equal at a given time. Therefore, why do the authors choose to do antibody analysis over “Year” (Figures 3c, 4 and 5 and Table 1) and not over “Age” (like in Figures 1 and 2)? It seems to me that the analysis over “Age” is one that best assesses each study participant’s history of malaria. Please, clarify it.

Following from above, to ensure Age was not confounding our plateauers vs non-plateauers comparison, only children of the same age and over three years were considered. We could not adequately do an Age comparison due to the oldest children leaving the study after one antibody sample. Therefore, any Age comparison (only data from 2015) would be confounded by 2015 being a high transmission year. Age is not a variable of interest in the manuscript but rather whether children plateau or not in their clinical symptoms. As this illustrates the complexity of longitudinal malaria cohort studies.

The sentence “MSP1 was the only antigen for which there were no distinct differences between the two groups of children” may lead to wrong inferences. The authors should consider mentioning the sample size of the analysis as one of its limitations and improve the discussion about MSP1 antigen results.

Changed in text (see “Children who stop experiencing clinical episodes are characterized by higher levels of circulating malaria-specific antibodies” section).

Competing Interests: No competing interests were disclosed.