MAJOR ARTICLE

TITLE

Declining malaria transmission differentially impacts on the maintenance of humoral immunity to *Plasmodium falciparum* in children

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Declining malaria transmission results in reductions in total antibodies to merozoite antigens in children. However, some important functional antibodies and antibodies to infected-erythrocytes are sustained for many years. This has implications for vaccine development and understanding malaria risk in populations.

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ABSTRACT

BACKGROUND: We investigated the poorly understood impact of declining malaria transmission on maintenance of antibodies to *P. falciparum* merozoite antigens and infected erythrocytes (IEs), including functional immunity.

METHODS: In a 3-year longitudinal cohort of 300 Kenyan children, antibodies to different merozoite AMA1 and MSP2 alleles, IE surface antigens, and antibody functional activities were quantified.

RESULTS: Over a period in which malaria transmission declined markedly, AMA1 and MSP2 antibodies decreased substantially; estimated half-lives of antibody duration were 0.8 and 1-3 years, respectively. However, 69-74% of children maintained their sero-positivity to AMA1 alleles and 42-52% to MSP2 alleles. Levels and prevalence of anti-merozoite antibodies were consistently associated with increasing age and concurrent parasitaemia. Antibodies promoting opsonic phagocytosis of merozoites declined rapidly (half-life 0.15 years). In contrast, complement-fixing antibodies to merozoites did not decline and antibodies to IE surface antigens expressing virulent phenotypes were much better maintained (half-life 4-10 years).

CONCLUSIONS: A decline in malaria transmission is associated with reduction in naturally-acquired immunity. However, loss of immunity is not universal; some key functional responses and antibodies to IEs were better maintained and these may continue to provide some protection. Findings have implications for malaria surveillance and control measures and informing vaccine development.

Key words: malaria, *P. falciparum*, immunity, children, Africa, antibodies, complement, phagocytosis

INTRODUCTION

In areas of moderate to high malaria endemicity, naturally-acquired immunity to malaria is characterized by protection against clinical disease and control of high density parasitaemia [1, 2]. Antibodies play a major role in naturally-acquired immunity to *Plasmodium falciparum* malaria [1, 2] and predominantly target the blood-stages, including merozoites and infected erythrocytes (IEs). Anti-malarial antibodies typically increase with age, exposure and transmission intensity and the link between antibody acquisition and the level of malaria exposure has been largely established (reviewed in [3], [4]). As a result of intensified control efforts, and other factors (e.g. change in malaria policies and practices, changes in users' and health providers' behaviours), *P.falciparum* transmission has declined in many regions in recent years, and these declines have been associated with higher rates and severity of clinical malaria [5-7], which may be attributed to declining naturally-acquired immunity in populations. While there is evidence that declines in malaria transmission are associated with reductions in antibodies to blood-stage antigens [8-10], what is less clear is how rapidly antibody levels to different targets decline in the context of declining transmission, whether significant humoral immune responses are maintained after reductions in transmission, or the impact of changing transmission on functional antibody responses.

Reported estimated half-lives of antibodies to blood-stage malaria antigens range from weeks to years (reviewed in [3]). These data are mainly for merozoite antigens, whereas there are limited data on the maintenance of functional antibody responses associated with protection (e.g. opsonisation of merozoites for phagocytic clearance and complement fixation on merozoites) [11, 12]. The maintenance and function of antibodies to IE surface antigens or merozoite antigens might be impacted by their different presentation to the immune system, but there are

limited data comparing the decay of antibodies to different blood-stage antigens. Also, different kinetics of antibody responses specific for different alleles might give some indication of the relative prevalence and dynamics of circulating parasite strains, and antibody decay rates for different alleles may vary [13, 14]. Greater knowledge on how declining malaria transmission affects maintenance of immunity, especially functional immune responses, is required in order to identify biomarkers of exposure, evaluate the impact of interventions, and help identify populations at risk (reviewed in [4]), as well as inform the development of long-lasting vaccines.

Here, we examined the impact of declining *P. falciparum* transmission on the maintenance of antibodies to *P. falciparum* antigens in a 3-year longitudinal cohort of Kenyan children. We measured antibody responses to two representative merozoite antigens, apical membrane antigen 1 (AMA1) and merozoite surface protein 2 (MSP2). The two antigens are important targets of naturally-acquired antibodies, including functional antibodies, that have been associated with protection against clinical disease in our study population [11, 15, 16], and are established vaccine candidates [17, 18]. We included different alleles of AMA1 and MSP2 to assess patterns of allele-specific antibodies over time. We examined the acquisition of antibody responses in relation to age and parasitaemia over time, and calculated antibody decay rates. Furthermore, we determined the maintenance of functional antibodies to merozoites and compared maintenance of antibodies between merozoite and IE surface antigens to determine whether different response-types are maintained differently.

METHODS

Study design and population

This cohort study was conducted in Ngerenya (Kilifi district, Kenya) [19] where biannual malaria transmission occurs (May-July and November-December), and comprised ~300 children aged 0.5-10 years who were followed for 3 years from May 2002 to October 2004. This was an aging cohort; median age 3.7 years in May 2003 and 5.3 years in October 2004. Venous blood was collected in May (high transmission) and October (low transmission) in 2002-2004 (6 time-points). The same children were seen at most time points and 186 children were present at all 6 sampling points. At each time-point, presence of *P. falciparum* parasitaemia was assessed among all children by light microscopy. Active malaria case detection was performed weekly; children who were febrile (temperature ≥ 37.5°C) or had a recent history of fever or illness had a blood smear performed. Malaria was defined as any parasitaemia with fever in children <1 year and a parasitaemia ≥2,500/µl of blood with fever in children ≥1 year [20]. Ethics approval was obtained from the Ethics Committee of the Kenya Medical Research Institute, and the Alfred Health Human Research and Ethics Committee. Parents/guardians of each participant provided written informed consent.

For cross-sectional analyses of antibody prevalence at each time-point, all available children were included (n=270 to 298). For longitudinal analysis of changes in antibody levels over time, only children who were sampled at all time points were included (n=186). For estimating antibody half-life during a period of minimal malaria transmission, only children who were sampled at all three time-points that corresponded to the decline in malaria prevalence (October 2003, May 2004, October 2004), and were aparasitaemic at the time of sampling and during the interval periods, were included. From those, a subset of 71 children (who were positive for antibodies to merozoite antigens at October 2003) were selected for analysis of maintenance of functional antibodies and antibodies to IEs.

Antibody measurements

IgG among serum samples was measured by standard ELISA, as described [21] using recombinant AMA1 (W2mef, HB3, and 3D7 alleles) and MSP2 (3D7 and FC27 alleles), which were expressed in *E. coli* [15, 22]. For functional antibodies to merozoites we used intact purified merozoites of the D10 isolate [23]. Merozoite opsonic phagocytosis was performed as described elsewhere [24]. Antibody-mediated fixation of C1q to the surface of merozoites, a biomarker of classical complement activation that leads to inhibition of merozoite invasion and merozoite lysis, was measured as described [25]. IgG reactivity to surface antigens of IEs (3D7 and IT4*var19* isolates) was evaluated using an established flow-cytometry-based approach [26]. Further details are provided in Supplementary Methods.

Data Analysis

Analyses were performed using STATA software. Antibody sero-positivity threshold was defined as the mean reactivity of negative controls plus 3 standard deviations [27]. Prevalence of antibodies to AMA1 and MSP2 between different age groups and different time-points were compared using the Chi-square test. Antibody levels across groups at a single time point were compared using Kruskal-Wallis and Mann-Whitney tests. MANOVA for repeated measures using the Wilks Lambda criteria was used to test for differences in antibody levels over time on the $T - 1$ absolute differences between subsequent measurements. Estimated mean antibodies half-lives were determined from linear mixed effect models as previously described [21].

RESULTS

Declining malaria transmission associated with decreased levels of antibodies to merozoite antigens

Antibodies to merozoite antigens AMA1 and MSP2, including different alleles, were measured at each of the six cross-sectional surveys from May 2002 to October 2004. In the later part of the study, October 2003 to October 2004, malaria transmission significantly decreased. Parasite prevalence at cross-sectional bleeds dropped from 14.0% (May-2002) to 3.7% (October-2004) (**Figure 1A**). The incidence of any detectable parasitaemia (of any density) was <3% by active surveillance between May and October 2004 (**Table S2**).

This decline in transmission was accompanied by significant reductions in the prevalence of antibodies to all AMA1 (**Table 1**) and MSP2 alleles (**Table 2**) within most age groups over that period (2-8-year olds for AMA1 and MSP2-FC27; 3 and 5-7-year olds for MSP2-3D7). Furthermore, from October 2003 levels of antibodies to all AMA1 and MSP2 alleles significantly decreased between cross-sectional bleeds (**Figure 1B; Tables 3, 4**) ($P < 0.001$).

Despite declining transmission and immune responses, AMA1 and MSP2 antibody prevalence and levels were consistently higher among older children at every time point (**Table 1 and 2;** $P < 0.001$ to $P = 0.033$; $P < 0.001$, respectively), such that median antibody levels were significantly higher amongst 7-10-year olds compared with 1-3-year olds ($P < 0.001$; **Tables 2, 3**, **S3**, **S4** and **S5**). Similar associations with age were observed for antibody responses to all alleles of AMA1 and MSP2 and to schizont protein extract used as a proxy for *P. falciparum* blood-stage exposure **(Table S6)**. The prevalence of antibodies to MSP2-3D7 was higher than to MSP2-FC27 allele, reflecting the moderately higher prevalence of MSP2-3D7 genotype infections in the study population [28].

At each time point, children with parasitaemia (including any density) at time of sampling had higher levels of antibodies to all alleles of AMA1 (**Tables 3, 4, S3**) and MSP2 (**Tables 4, S5**) compared to aparasitaemic children ($P < 0.01$). This suggests that on-going exposure to infection helped maintain higher levels in the cohort, and the declining transmission and exposure therefore resulted in declining antibody levels. Associations between antibodies and increasing age were observed in aparasitaemic children, but less consistently amongst parasitaemic children. Similar results were observed for antibody responses to all alleles of MSP2 and AMA1 and to schizont extract (**Table S6**).

Decay rates of antibodies to merozoite antigens with declining malaria transmission

We examined the rate of decline of levels and prevalence of antibodies to AMA1 and MSP2 between October 2003 and October 2004 as malaria transmission declined. While there was inter-individual variation and fluctuation over time, overall cohort antibody levels to all AMA1 and MSP2 alleles significantly declined in all age groups during this interval (**Figure 1B; Tables 1, 2**). We estimated the mean half-life of antibodies to AMA1 or MSP2 between October 2003 and October 2004 [21] in children who were seropositive at the October 2003 contact point and had no recorded parasitaemia at the time of sample collection or during this 12-month period of follow-up (**Figure 1B**). Estimated half-lives of antibodies to the different AMA1 alleles were similar at around 0.8 years (9.6 months; range 0.78-0.83) and comparable to antibodies to MSP2 FC27 allele (1.1 years), but substantially shorter than that of antibodies to MSP2 3D7 allele (3.4 years). No significant differences in decay rates were found between age groups, although confidence intervals were wide.

Despite declining malaria transmission, many children among the whole cohort maintained their seropositive status for antibodies to AMA1 and/or MSP2 (**Table 5**); 69-73% of children who were seropositive at October 2003 maintained sero-positivity to each AMA1 allele at October 2004, but overall antibody reactivity had declined substantially. For MSP2 alleles, 42-52% who were positive at October 2003 remained seropositive at October 2004. Not all children had declining antibodies during this period. For example, for AMA1-W2mef 28.5% of children showed no significant decline between October 2003 to May 2004 (33.8% and 31% for 3D7 and HB3 alleles); for MSP2, proportions were 32.1% and 27.8% for 3D7 and FC27 alleles, respectively.

Maintenance of functional antibodies to merozoites and antibodies to infected erythrocytes surface antigens

We evaluated maintenance of functional antibodies to merozoites as malaria transmission declined. We studied antibodies that promote opsonic phagocytosis of merozoites and antibodies that fix complement on the surface of merozoites as these two functional measures of merozoite antibodies have been associated with protective immunity in longitudinal studies of children [24, 25, 29]. We measured antibody levels over the last 12 months of the study (October 2003 to October 2004) among children who had no recorded episode of parasitaemia during that period.

Antibodies with opsonic phagocytosis activity declined rapidly with a half-life of just 0.15 years (1.8 months; 95% reference range 0.09-0.4 years), significantly shorter than the half-lives of antibodies to individual merozoite antigens (**Figure 2A**). Despite declining levels, the prevalence of phagocytosis-promoting antibodies remained consistently high, from 92.3% in

October 2003 to 89.4% in October 2004 suggesting some level of functional opsonic phagocytosis activity is retained for long periods of time.

In contrast, levels of complement-fixing antibodies did not appreciably decline (0.7-∞) (**Figure 2B**). Accordingly, the proportion of children seropositive for complement-fixing antibodies remained constant from 78% in October 2003 to 81.3% in October 2004. However, there was a wide range of observed responses with some individuals exhibiting a measurable decline and others remaining stable throughout. No significant correlations were found between the measures of functional antibodies to merozoites, consistent with the different rates of decline.

We measured levels of antibodies to surface antigens of IEs given they could be maintained differently to merozoite antigens due to their different presentation to the immune system (**Figures 2C, 2D**). We previously reported that these antibodies are acquired in an age-dependent manner [12, 24] in our study population and predominantly target *Pf*EMP1 [30, 31]. We included two genetically distinct isolates expressing PfEMP1 types associated with virulent phenotypes and disease pathogenesis: i) isolate IT4var19 expresses *var19* (containing a DC8 arrangement) which mediates adhesion to cerebral endothelial cells; ii) a 3D7 isolate we identified that expresses group A *var* genes (including a DC13 arrangement) associated with virulent properties (**Table S1**). Antibodies to IE surface antigens expressed by both isolates were relatively stable as malaria transmission declined (half-lives 10.5 [3.2-∞] and 4 [1.4-∞] years for 3D7 and IT4var19 IEs, respectively).

Collectively these results show that while total levels of antibodies to individual merozoite antigens are sensitive to changes in malaria transmission, specific antibody functional activity is better maintained, especially their capacity to fix complement. Antibodies to IEs

expressing PfEMP1 variants associated with disease pathogenesis also appear to be better maintained.

DISCUSSION

There is concern that recent reductions in malaria transmission in many endemic countries [6, 32-35] may lead to rapid declines in naturally-acquired immunity, leaving many at increased risk of malaria and severe complications. Declining *P. falciparum* prevalence in our cohort was accompanied by decreasing antibodies to two key merozoite antigens, AMA1 and MSP2; this was consistent across different alleles of MSP2 and AMA1. In the context of low parasite prevalence and absence of clinical malaria, most children maintained seropositivity to AMA1, whereas fewer children retained seropositivity for MSP2. The broad similarity in estimated half-lives of antibodies to AMA1 and MSP2 (approximately 9 months and 1-3 years, respectively, with overlapping confidence intervals) suggests that differences in maintenance of sero-positivity could be related to lower starting levels of antibodies to MSP2.

Different kinetics of antibody responses specific for different alleles might give some indication of the relative prevalence and dynamics of circulating parasite strains [13, 14]. Antibody responses to AMA1-W2mef increased from May 2002 before declining and reaching levels comparable to the other two AMA1 alleles by October 2003. This coincided with an increase in the incidence of parasitaemia (of any density) and clinical malaria between May 2002 and May 2003; *P. falciparum* strains carrying a W2mef-like AMA1 allele may have been more prevalent during that time. The higher prevalence of antibodies to MSP2-3D7 is consistent with infections with this genotype being moderately more prevalent.

Rather than determining absolute decay rates of antibodies in the absence of any exposure, we estimated antibody maintenance in a practical setting where malaria transmission had dramatically reduced, an increasingly common situation globally. Maintenance of antibodies was highly variable among individuals, from rapid decay to no decline. Overall estimated half-lives of 1-3 years for antibodies to AMA1 or MSP2 indicate that antibodies to merozoite antigens decline relatively quickly; however, such decay rates and the wide variation in antibody maintenance among individuals imply that detectable levels of these antibodies could be maintained for several years. Our findings are largely consistent with studies in pregnant women [21] and a small study of adults in South-East Asia [36]. In contrast, some studies in African children reported rapid declines in antibodies to merozoite antigens when measured immediately after treatment for acute malaria [37, 38]. Given antibodies initially decay rapidly following an acute infection, estimates of antibody half-lives will be much shorter if measured following an acute episode than measured in uninfected subjects as we have done [3, 39, 40]. Published evidence suggests impairment in the induction of B cell memory to malaria [41], which may partly explain the lack of sustained responses.

We complemented observations on the dynamics of antibody levels by providing important new data on maintenance of functional antibodies to merozoite antigens on which very little is currently known. We measured antibodies that mediate opsonic phagocytosis and complement fixation on merozoites as these have emerged as likely mechanisms of immunity and promising functional correlates of immunity [12, 24, 25, 29]. Functional complement-fixing antibodies to merozoites were better maintained than total antibodies to merozoite antigens. This may suggest that complement fixation requires only low levels of antibodies and antibody levels must decline below a certain threshold before functional activity is lost. Alternatively,

complement fixation might be mediated by specific antibodies that are better maintained over time due to their role in immunity. Functional activity is dependent on multiple antibody properties, such as affinity, subclass, allotype, glycosylation, and epitope specificity, and not just total antibody levels [42]. In contrast, opsonic phagocytosis-promoting antibodies rapidly declined, suggesting this functional response is more sensitive to decrease in antibody levels. However, most children remained positive for opsonic phagocytosis activity over the 12-month period even though overall activity did decline quickly. While antibody decay was estimated among children who had no parasitaemia detected during surveillance using microscopic evaluation of blood smears, it is possible that we missed some parasitaemic events; data on parasitaemia detected using more sensitive PCR methods was not available. Our findings indicate that some forms of immunity may be better maintained, and highlight the need for assessing antibody function in population studies rather than simply antibody levels. The sustained persistence of some key elements of immunity is consistent with epidemiologic evidence suggesting that some level of immunity may be maintained for extended periods after decline or interruption malaria transmission [3].

Our previous studies showed that the prevalence of growth-inhibitory antibodies in this cohort was low [43]; therefore these were not evaluated. However, we previously showed that growth-inhibitory antibodies were higher at times of higher malaria transmission [43]. It was not possible to investigate the relationship between declining immunity and subsequent risk of malaria in this study. Prior studies [15, 44] have suggested that there is a threshold magnitude of antibodies required to mediate immunity. Our results finding a decline in total antibodies to merozoites suggest that susceptibility to malaria is likely to have increased in children as a result of this decline.

Antibody acquisition longevity may also vary according to antigen specificity and parasite life-stage [21, 38, 45]; therefore we compared maintenance of antibodies to merozoite antigens versus IE surface antigens. For our studies we used isolates that expressed PfEMP1 variants associated with disease pathogenesis as we reasoned that antibodies to these variants are likely to have a role in immunity. Levels of antibodies to IE surface antigens were better maintained than levels of antibodies to merozoite surface antigens despite a lower sero-positivity at the beginning of the study. This may be due to their different presentation to the immune system and suggests that some individuals can maintain effective antibody-mediated immunity to IE surface antigens. We have previously reported that the majority (>80%) of antibody reactivity to IEs targets PfEMP1 [30, 31]; however, a minor proportion of antibody reactivity may target other surface antigens. IE surface antibodies correlate with functional opsonic phagocytosis activity [31], a mechanism thought to be important in mediating IE clearance. Our results suggest inherent differences in maintenance of antibodies to antigens of IEs versus merozoites; they also suggest that generating long-lived immune responses to malaria is possible. Similarly, we previously found antibodies to *Pf*EMP1 had much slower decay rates than antibodies to merozoite antigens in pregnant women [21]. Relatively short-lived antibodies suggest an inherent weakness in naturally-acquired immunity to merozoites. Understanding differences in antibody maintenance between antibodies to merozoites and IEs may yield important insights to guide vaccine design. Antibodies may be valuable biomarkers to enhance malaria surveillance [4]. Understanding acquisition and maintenance of antibodies is crucial in assessing their utility in serosurveillance approaches. Our results suggest antibodies to AMA1 and MSP2 are relatively sensitive biomarkers of changes in malaria transmission, consistent with findings in other populations [46, 47].

In conclusion, by evaluating antibodies to merozoite antigens, IE surface antigens, and functional antibodies in a longitudinal cohort of children, we have provided significant new insights into the impact of declining malaria on immunity. The data provide estimates of decay rate of antibodies in a practical context of declining population malaria transmission. Furthermore, the findings indicate that certain elements of immunity may persist for extended periods following reduction in transmission. There was a decline in the prevalence and levels of antibodies to merozoite antigens AMA1 and MSP2 associated with falling prevalence of *P. falciparum* amongst children. Opsonic phagocytosis-promoting antibodies decayed more rapidly following declining transmission, whereas complement-fixing antibodies to merozoites or antibodies to IE surface antigens were much better maintained. Understanding the great variability among individuals in antibody maintenance may be very valuable for informing vaccine and biomarker development and implementation. With upscaling of malaria control and progress towards elimination in some regions, results from this study provide important insights on how immunity may be affected by changes in malaria transmission intensity and provide information to aid selection of biomarkers for monitoring transmission and identifying susceptible populations or groups for targeted interventions.

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FIGURE LEGENDS

Figure 1. Decline in parasite prevalence over the study period.

A. Decline of prevalence of parasite prevalence at each cross sectional bleed. At each time-point, presence of *P. falciparum* parasitaemia was assessed by light microscopy. Data are presented as parasite prevalence (of any density), with the number of parasitaemic children over the total number of children tested indicated for each cross sectional survey time point.

B. Number of episodes of malaria at each cross sectional bleed. Data are presented as number of episodes of malaria (i.e. any parasitaemia accompanied by fever in children <1 year and a parasitaemia ≥2,500 IEs/µl of blood accompanied by fever in children ≥1 year) for each time point. The percentage of children with malaria is indicated for each cross sectional survey time point.

C. Mean antibody levels to merozoite antigens over the study period. Antibody levels against different alleles of AMA1 and MSP2 were measured by standard ELISA. Data are presented as locally weighted scatter plot smoothing (LOWESS) curves (the numbers of subjects included at each time point are: May 2002, 298; Oct 2002, 294; May 2003, 285; Oct 2003, 294; May 2004, 279; Oct 2004, 273). The mean half-life of antibodies is shown for each antibody; this was calculated for the period between October 2013 and October 2014 when malaria transmission declined, and included children who were present at each time-point and had no malaria parasitaemia detected during that time period.

Figure 2. Decline in levels of functional antibodies to merozoites and antibodies to IE surface antigens over the study period.

In a subset of 70 samples, antibody function was measured at three time-points (October 2003, May 2004, and October 2004). The predicted mean antibody levels (and 95% confidence intervals shown in grey) over time are shown. Day 0 represents October 2003 with days of follow-up indicated on the X-axis. Mean functional antibody half-lives were calculated from the fixed effects slope component of a mixed effects model and are represented in years. Antibody half-life for each antibody measure is as follows: (**A**) Opsonic phagocytosis of merozoites, 0.15 (0.09-0.40); (**B**) complement (C1q) fixation on merozoites (0.70-∞); (**C**) antibodies to surface antigens of 3D7 IEs, 10.5 (3.2-∞), and (**D**) antibodies to surface antigens A4var19 IEs 4.0 (1.4-∞).

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Table 1. Seropositivity to AMA1 alleles by age at each cross-sectional survey

Age (years)	Seroprevalence (95% CI)						*P*-value[b]
	May 2002[a]	October 2002[a]	May 2003[a]	October 2003[a]	May 2004[a]	October 2004[a]	
AMA1 (W2mef)							
0	26.7 (10.6 - 42.9)	67.6 (52.3 - 83)	26 (9.1 - 42.9)	43.5 (22.7 - 64.3)	19.1 (1.8 - 36.4)	33.4 (8.6 - 58.2)	0.182
1	14.6 (4.5 - 24.8)	21.9 (7.3 - 36.5)	34.7 (15.9 - 53.4)	11.8 (0.8 - 22.9)	16 (1.3 - 30.8)	8.4 (-3.1 - 19.7)	0.385
2	33.4 (18.3 - 48.4)	48.8 (32.8 - 64.7)	11.4 (1.9 - 20.9)	15.7 (2.8 - 28.5)	6.5 (-2.4 - 15.3)	3.2 (-3.1 - 9.3)	**<0.001**
3	48.8 (33.3 - 64.4)	59 (43.3 - 74.7)	34.4 (17.6 - 51.2)	43.3 (27 - 59.5)	32.5 (17.1 - 47.8)	17.9 (3.4 - 32.4)	**0.002**
4	50 (32.4 - 67.7)	76.7 (61.3 - 92.2)	40 (24.6 - 55.5)	47.7 (32.3 - 63)	17.3 (3.2 - 31.3)	33.4 (16.1 - 50.6)	**0.001**
5	61.8 (47.6 - 75.9)	71.5 (58.6 - 84.3)	48.3 (29.7 - 66.9)	46.2 (26.6 - 65.8)	45.5 (28.2 - 62.8)	42.5 (25.3 - 59.7)	**0.007**
6	65.8 (50.5 - 81.2)	75.9 (60 - 91.8)	52.8 (36.2 - 69.4)	62 (47 - 76.9)	54.2 (33.8 - 74.7)	41.7 (21.5 - 62)	**0.032**
7	74 (55.5 - 92.4)	82.9 (70.2 - 95.6)	71 (54.7 - 87.3)	52 (32 - 72.1)	57.9 (42 - 73.9)	55.3 (39.2 - 71.4)	**0.007**
8[c]	-	100 (0 - 0)	75 (55.5 - 94.6)	81.9 (68.4 - 95.3)	72 (54 - 90.1)	41.7 (21.5 - 62)	**0.005**
9[c]	-	-	-	-	81.3 (61.5 - 101.1)	72 (54 - 90.1)	0.506
***P*-value[d]**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	
AMA1(HB3)							
0	26.7 (10.6 - 42.9)	56.8 (40.6 - 73.1)	37.1 (18.4 - 55.7)	34.8 (14.8 - 54.8)	23.9 (5.1 - 42.6)	33.4 (8.6 - 58.2)	0.361
1	14.6 (4.5 - 24.8)	9.4 (-1 - 19.7)	34.7 (15.9 - 53.4)	14.8 (2.6 - 26.9)	12 (-1.1 - 25.1)	8.4 (-3.1 - 19.7)	0.619
2	33.4 (18.3 - 48.4)	20.6 (7.7 - 33.5)	25 (12.1 - 38)	21.9 (7.3 - 36.5)	9.7 (-1 - 20.4)	3.2 (-3.1 - 9.3)	**0.001**
3	46.4 (30.9 - 61.9)	46.2 (30.3 - 62.1)	31.3 (14.9 - 47.7)	43.3 (27 - 59.5)	32.5 (17.1 - 47.8)	17.9 (3.4 - 32.4)	**0.017**
4	53.2 (35.5 - 70.8)	66.7 (49.5 - 83.9)	55 (39.4 - 70.7)	45.3 (30 - 60.6)	27.6 (11 - 44.3)	30 (13.3 - 46.8)	**0.002**
5	68.1 (54.6 - 81.7)	57.2 (43.1 - 71.3)	62.1 (44.1 - 80.2)	46.2 (26.6 - 65.8)	45.5 (28.2 - 62.8)	39.4 (22.4 - 56.4)	**0.004**
6	79 (65.8 - 92.2)	55.2 (36.7 - 73.7)	61.2 (44.9 - 77.4)	57.2 (42 - 72.4)	54.2 (33.8 - 74.7)	37.5 (17.7 - 57.4)	**0.004**
7	74 (55.5 - 92.4)	77.2 (63 - 91.4)	83.9 (70.7 - 97.1)	52 (32 - 72.1)	52.7 (36.5 - 68.8)	52.7 (36.5 - 68.8)	**0.002**
8[c]	-	100 (0 - 0)	85 (68.9 - 101.2)	84.9 (72.4 - 97.4)	80 (64 - 96.1)	45.9 (25.4 - 66.3)	**0.002**
9[c]	-	-	-	-	75 (53 - 97.1)	76 (58.9 - 93.2)	0.943
***P*-value[d]**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	
AMA1(3D7)							
0	23.4 (7.9 - 38.8)	48.7 (32.3 - 65.1)	29.7 (12.1 - 47.3)	26.1 (7.7 - 44.6)	23.9 (5.1 - 42.6)	26.7 (3.4 - 50)	0.384
1	8.4 (0.4 - 16.3)	3.2 (-3.1 - 9.3)	34.7 (15.9 - 53.4)	20.6 (6.8 - 34.5)	8 (-3 - 19)	12.5 (-1.1 - 26.1)	0.386
2	35.9 (20.6 - 51.3)	20.6 (7.7 - 33.5)	13.7 (3.4 - 24)	18.8 (5 - 32.6)	13 (0.9 - 25)	6.3 (-2.4 - 14.9)	**0.002**
3	46.4 (30.9 - 61.9)	35.9 (20.6 - 51.3)	28.2 (12.3 - 44.1)	37.9 (22 - 53.8)	29.8 (14.8 - 44.8)	17.9 (3.4 - 32.4)	**0.027**
4	40.7 (23.3 - 58)	63.4 (45.8 - 81)	47.5 (31.8 - 63.3)	40.5 (25.4 - 55.6)	17.3 (3.2 - 31.3)	33.4 (16.1 - 50.6)	**0.017**
5	63.9 (49.9 - 77.8)	49 (34.8 - 63.2)	51.8 (33.2 - 70.4)	46.2 (26.6 - 65.8)	39.4 (22.4 - 56.4)	30.4 (14.4 - 46.3)	**0.003**
6	71.1 (56.4 - 85.8)	55.2 (36.7 - 73.7)	50 (33.4 - 66.7)	57.2 (42 - 72.4)	41.7 (21.5 - 62)	33.4 (14 - 52.7)	**0.004**

7	65.3 (45.3 - 85.3)	68.6 (53 - 84.3)	77.5 (62.4 - 92.5)	52 (32 - 72.1)	50 (33.9 - 66.2)	50 (33.9 - 66.2)	**0.022**
8[c]	-	100 (0 - 0)	75 (55.5 - 94.6)	87.9 (76.6 - 99.3)	76 (58.9 - 93.2)	41.7 (21.5 - 62)	**0.003**
9[c]	-	-	-	-	81.3 (61.5 - 101.1)	72 (54 - 90.1)	0.506
***P*-value**[d]	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	

Notes

[a] Cross sectional survey when blood was collected.

[b] *P*-values calculated using a Chi-square test for trend for IgG prevalence in the same age group at different cross sectional bleeds (*P*-values ≤ 0.05 indicated in bold type).

[c] Missing values are due to a lack of children in that age group.

[d] *P*-values calculated using a Chi-square test for trend for IgG prevalence in different age groups at the same cross sectional bleed (*P*-values ≤ 0.05 indicated in bold type).

All samples included in analysis.

Table 2. Seropositivity to MSP2 alleles by age at each cross-sectional survey

Age (years)	Seroprevalence (95% CI)						*P*-value[b]
	May 2002[a]	October 2002[a]	May 2003[a]	October 2003[a]	May 2004[a]	October 2004[a]	
MSP2(3D7)							
0	10 (-1 - 21)	2.8 (-2.7 - 8.1)	3.8 (-3.6 - 11)	0 (0 - 0)	0 (0 - 0)	6.7 (-6.5 - 19.8)	0.246
1	10.5 (1.7 - 19.2)	3.2 (-3.1 - 9.3)	3.9 (-3.8 - 11.5)	0 (0 - 0)	4 (-3.9 - 11.9)	4.2 (-4.1 - 12.4)	0.150
2	15.4 (3.9 - 27)	0 (0 - 0)	6.9 (-0.8 - 14.4)	3.2 (-3.1 - 9.3)	6.5 (-2.4 - 15.3)	3.2 (-3.1 - 9.3)	0.156
3	31.8 (17.3 - 46.2)	10.3 (0.6 - 20)	0 (0 - 0)	16.3 (4.2 - 28.4)	13.6 (2.3 - 24.8)	3.6 (-3.5 - 10.7)	**0.013**
4	18.8 (5 - 32.6)	13.4 (1 - 25.8)	20 (7.4 - 32.7)	19.1 (7 - 31.2)	6.9 (-2.6 - 16.4)	10 (-1 - 21)	0.249
5	49 (34.5 - 63.5)	14.3 (4.4 - 24.3)	13.8 (1 - 26.7)	3.9 (-3.8 - 11.5)	9.1 (-1 - 19.1)	6.1 (-2.3 - 14.4)	**<0.001**
6	47.4 (31.3 - 63.6)	27.6 (11 - 44.3)	27.8 (12.9 - 42.7)	31 (16.8 - 45.2)	8.4 (-3.1 - 19.7)	0 (0 - 0)	**<0.001**
7	52.2 (31.3 - 73.2)	20 (6.5 - 33.6)	22.6 (7.6 - 37.7)	28 (10 - 46.1)	23.7 (10 - 37.5)	10.6 (0.6 - 20.5)	**0.009**
8[c]	-	0 (0 - 0)	30 (9.4 - 50.7)	27.3 (11.8 - 42.8)	28 (10 - 46.1)	25 (7.3 - 42.8)	0.860
9[c]	-	-	-	-	37.5 (12.9 - 62.2)	28 (10 - 46.1)	0.529
***P*-value**[d]	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	
MSP2(FC27)							
0	13.4 (1 - 25.8)	5.5 (-2.1 - 12.9)	7.5 (-2.8 - 17.6)	4.4 (-4.3 - 13)	4.8 (-4.7 - 14.2)	0 (0 - 0)	0.113
1	18.8 (7.6 - 30)	6.3 (-2.4 - 14.9)	7.7 (-2.8 - 18.2)	0 (0 - 0)	0 (0 - 0)	8.4 (-3.1 - 19.7)	**0.012**
2	12.9 (2.2 - 23.5)	10.3 (0.6 - 20)	6.9 (-0.8 - 14.4)	3.2 (-3.1 - 9.3)	6.5 (-2.4 - 15.3)	0 (0 - 0)	**0.026**
3	31.8 (17.3 - 46.2)	15.4 (3.9 - 27)	9.4 (-1 - 19.7)	24.4 (10.3 - 38.4)	16.3 (4.2 - 28.4)	3.6 (-3.5 - 10.7)	**0.026**
4	18.8 (5 - 32.6)	40 (22.1 - 58)	20 (7.4 - 32.7)	21.5 (8.9 - 34.1)	6.9 (-2.6 - 16.4)	10 (-1 - 21)	**0.024**
5	23.5 (11.2 - 35.7)	18.4 (7.4 - 29.4)	20.7 (5.7 - 35.8)	23.1 (6.5 - 39.7)	9.1 (-1 - 19.1)	6.1 (-2.3 - 14.4)	**0.031**
6	31.6 (16.6 - 46.7)	20.7 (5.7 - 35.8)	27.8 (12.9 - 42.7)	26.2 (12.7 - 39.8)	0 (0 - 0)	4.2 (-4.1 - 12.4)	**0.003**
7	39.2 (18.7 - 59.7)	34.3 (18.3 - 50.4)	35.5 (18.3 - 52.7)	20 (4 - 36.1)	10.6 (0.6 - 20.5)	10.6 (0.6 - 20.5)	**0.000**
8[c]	-	0 (0 - 0)	50 (27.5 - 72.6)	45.5 (28.2 - 62.8)	36 (16.8 - 55.3)	12.5 (-1.1 - 26.1)	**0.012**
9[c]	-	-	-	-	50 (24.6 - 75.5)	36 (16.8 - 55.3)	0.381
***P*-value**[d]	**0.033**	**0.004**	**<0.001**	**<0.001**	**<0.001**	**0.001**	

Notes

[a] Cross sectional survey when blood was collected.

[b] *P*-values calculated using a chi^2 test for trend for IgG prevalence in the same age group at different cross sectional bleeds (*P*-values ≤ 0.05 indicated in bold type).

[c] Missing values are due to a lack of children in that age group.

[d] *P*-values calculated using a chi^2 test for trend for IgG prevalence in different age groups at the same cross sectional bleed (*P*-values ≤ 0.05 indicated in bold type).

All samples included in analysis.

Table 3. Levels of antibodies to AMA1 by age group and parasitaemia status

AMA1 (W2mef allele)		All Samples					Aparasitemic					Parasitemic				
		0 years	1-3 years	4-6 years	7-10 years	*P*-value[a]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[a]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[a]
May 2002[b]	N[c]	30	128	117	23		30	109	93	15		0	19	24	8	
	Median	0.04	0.01	0.24	0.24	**<0.001**[d]	0.04	-0.01	0.10	0.44	**<0.001**[d]	0.00	0.65	0.89	1.30	0.710
	IQR	(-0.01 - 0.18)	(-0.02 - 0.17)	(0.03 - 1.18)	(0.1 - 1.9)		(-0.01 - 0.18)	(-0.03 - 0.09)	(0.02 - 0.59)	(0.13 - 1.67)		(0 - 0)	(0.11 - 1.67)	(0.43 - 2.02)	(0.06 - 2.19)	
October 2002[b]	N[c]	37	110	108	36		36	105	99	31		1	5	9	5	
	Median	0.70	0.17	0.66	0.66	**<0.001**[d]	0.73	0.17	0.63	1.60	**<0.001**[d]	-0.04	1.27	1.29	0.86	0.433
	IQR	(0.1 - 1.43)	(0.02 - 0.47)	(0.2 - 1.66)	(0.51 - 2.37)		(0.12 - 1.48)	(0.01 - 0.39)	(0.18 - 1.63)	(0.49 - 2.34)		(-0.04 - -0.04)	(1.13 - 2.2)	(0.27 - 2.19)	(0.66 - 2.47)	
May 2003[b]	N[c]	27	102	105	51		26	93	88	40		1	9	17	11	
	Median	0.01	0.01	0.07	0.07	**<0.001**[d]	0.01	0.00	0.05	0.30	**<0.001**[d]	-0.04	0.04	0.58	2.21	**0.013**[d]
	IQR	(-0.03 - 0.13)	(-0.03 - 0.1)	(0 - 0.73)	(0.08 - 2.06)		(-0.02 - 0.13)	(-0.02 - 0.07)	(0 - 0.46)	(0.07 - 1.51)		(-0.04 - -0.04)	(-0.04 - 0.3)	(0.12 - 1.53)	(0.19 - 2.34)	
October 2003[b]	N[c]	23	103	110	58		23	93	91	45		0	10	18	13	
	Median	0.08	0.02	0.14	0.14	**<0.001**[d]	0.08	0.01	0.07	0.31	**<0.001**[d]	0.00	1.08	1.73	2.19	0.123
	IQR	(0.02 - 0.24)	(-0.01 - 0.1)	(0.02 - 0.78)	(0.07 - 1.92)		(0.02 - 0.24)	(-0.01 - 0.05)	(0.01 - 0.49)	(0.07 - 1.18)		(0 - 0)	(0.21 - 1.71)	(0.44 - 2.13)	(1.61 - 2.26)	
May 2004[b]	N[c]	21	93	86	79		21	88	84	69		0	5	2	10	
	Median	0.00	0.00	0.05	0.05	**<0.001**[d]	0.00	0.00	0.05	0.24	**<0.001**[d]	0.00	1.10	0.22	1.72	0.117
	IQR	(-0.02 - 0.08)	(-0.02 - 0.05)	(0.01 - 0.28)	(0.06 - 1.31)		(-0.02 - 0.08)	(-0.02 - 0.04)	(0.01 - 0.28)	(0.03 - 1.02)		(0 - 0)	(0.58 - 1.55)	(0.1 - 0.34)	(0.98 - 2.03)	

October 2004[b]	N[c]	15	84	87	87		15	80	87	81		0	4	0	6	
	Median	0.02	-0.01	0.03	0.03	**<0.001**[d]	0.02	-0.02	0.03	0.15	**<0.001**[d]	0.00	0.36	0.00	1.12	0.286
	IQR	(-0.01 - 0.21)	(-0.03 - 0.02)	(-0.02 - 0.26)	(0.02 - 0.88)		(-0.01 - 0.21)	(-0.03 - 0.01)	(-0.02 - 0.26)	(0.01 - 0.75)		(0 - 0)	(0.26 - 0.48)	(0 - 0)	(0.2 - 1.5)	

Notes

[a] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[b] Cross sectional survey.

[c] Number of samples tested by ELISA. Data for the 3D7 and HB3 alleles of AMA1 are provided in the Supplementary Material

[d] *P*-values ≤ 0.05 when comparing antibody levels between children 1-3 years and children 7-10 years using a Mann Whitney U test (*P*-values not shown).

Table 4. Levels of antibodies to MSP2 by age group and parasitaemia status

MSP2 (3D7 allele)		All Samples					Aparasitemic					Parasitemic				
		0 years	1-3 years	4-6 years	7-10 years	*P*-value[a]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[a]	0 years	1-3 years	4-6 years	7-10 years	*P*-value[a]
May 2002[b]	N[c]	30	128	117	23		30	109	93	15		0	19	24	8	
	Median	0.04	0.13	0.33	0.33	**<0.001**[d]	0.04	0.10	0.28	0.29	**<0.001**[d]	0.00	0.89	1.14	1.76	0.640
	IQR	(0.02 - 0.13)	(0.03 - 0.4)	(0.12 - 1.09)	(0.11 - 1.29)		(0.02 - 0.13)	(0.03 - 0.3)	(0.12 - 0.82)	(0.11 - 0.87)		(0 - 0)	(0.58 - 1.58)	(0.16 - 2.08)	(0.33 - 2.43)	
October 2002[b]	N[c]	37	110	108	36		36	105	99	31		1	5	9	5	
	Median	0.04	0.05	0.14	0.14	**<0.001**[d]	0.04	0.04	0.13	0.12	**<0.001**[d]	0.01	0.62	0.21	1.65	0.193
	IQR	(0.01 - 0.1)	(0.02 - 0.09)	(0.06 - 0.34)	(0.07 - 0.47)		(0.02 - 0.1)	(0.02 - 0.08)	(0.06 - 0.33)	(0.07 - 0.34)		(0.01 - 0.01)	(0.04 - 0.77)	(0.14 - 0.54)	(0.5 - 2.43)	
May 2003[b]	N[c]	27	102	105	51		26	93	88	40		1	9	17	11	
	Median	0.09	0.04	0.13	0.13	**<0.001**[d]	0.10	0.04	0.10	0.14	**<0.001**[d]	0.06	0.12	0.73	1.16	**0.048**[d]
	IQR	(0.01 - 0.23)	(0.01 - 0.1)	(0.05 - 0.43)	(0.07 - 0.72)		(0.01 - 0.23)	(0.01 - 0.09)	(0.04 - 0.31)	(0.06 - 0.31)		(0.06 - 0.06)	(0.04 - 0.26)	(0.2 - 1.39)	(0.2 - 2.56)	
October 2003[b]	N[c]	23	103	110	58		23	93	91	45		10	18	13	0	
	Median	0.08	0.06	0.13	0.13	**<0.001**[d]	0.08	0.06	0.10	0.18	**<0.001**[d]	0.43	0.49	1.77	0.00	0.426
	IQR	(0.04 - 0.19)	(0.02 - 0.25)	(0.05 - 0.41)	(0.08 - 0.56)		(0.04 - 0.19)	(0.01 - 0.16)	(0.04 - 0.28)	(0.07 - 0.44)		(0.29 - 1.03)	(0.32 - 0.75)	(0.32 - 2.17)	(0 - 0)	
May 2004[b]	N[c]	21	93	86	79		21	88	84	69		0	5	2	10	
	Median	0.01	0.04	0.08	0.08	**<0.001**[d]	0.01	0.03	0.08	0.17	**<0.001**[d]	0.00	0.22	0.49	0.43	0.809
	IQR	(0 - 0.02)	(0 - 0.1)	(0.04 - 0.24)	(0.06 - 0.6)		(0 - 0.02)	(0 - 0.09)	(0.04 - 0.23)	(0.04 - 0.47)		(0 - 0)	(0.2 - 1.53)	(0.25 - 0.72)	(0.17 - 2.14)	

October 2004[b]	N[c]	15	84	87	87		15	80	87	81		0	4	0	6	
	Median	0.02	0.04	0.07	0.07	**<0.001**[d]	0.02	0.03	0.07	0.13	**<0.001**[d]	0.00	0.45	0.00	1.63	0.088
	IQR	(0.01 - 0.06)	(0.01 - 0.08)	(0.03 - 0.14)	(0.05 - 0.45)		(0.01 - 0.06)	(0.01 - 0.07)	(0.03 - 0.14)	(0.05 - 0.37)		(0 - 0)	(0.13 - 1.33)	(0 - 0)	(1.17 - 2.31)	

Notes

[a] *P*-values calculated using a Kruskal Wallis test (*P*-values ≤ 0.05 indicated in bold type).

[b] Cross sectional survey.

[c] Number of samples tested by ELISA. Data on antibodies to the FC27 allele of MSP2 is provided in the Supplementary Material

[d] *P*-values ≤ 0.05 when comparing antibody levels between children 1-3 years and children 7-10 years using a Mann Whitney U test (*P*-values not shown).

Table 5. Maintenance of antibodies to AMA1 and MSP2 between October 2003 and October 2004

	Seropositive (n)[1]		
	October 2003	October 2004	Maintained seropositivity[2]
AMA1(W2mef)	45.3% (124)	36.5% (100)	73.4% (91)
AMA1(HB3)	44.5% (122)	36.1% (99)	73.8% (90)
AMA1(3D7)	43.8% (120)	34.7% (95)	69.2% (83)
MSP2(3D7)	15.3% (42)	9.1% (25)	52.4% (22)
MSP2(FC27)	19.0% (52)	9.5% (26)	42.3% (22)

Notes

[1] All children present at October 2003 and October 2004 cross sectional bleeds (n=274).

[2] Percentage of seropositive individuals in October 2003 who maintained their seropositive status in October 2004.

Figure 1

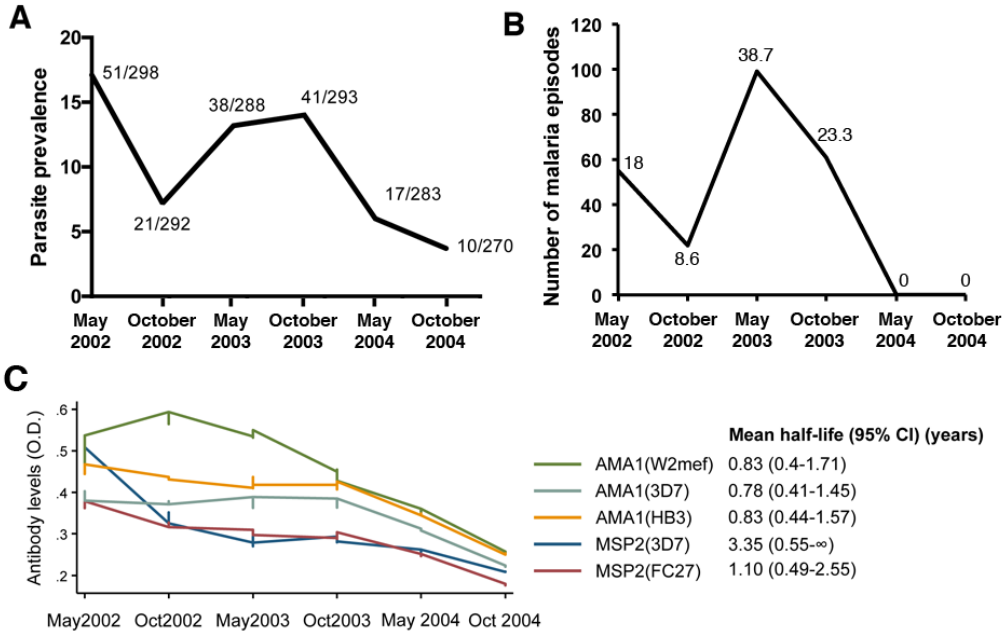

Figure 2