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Patterns of protective associations differ for antibodies to *P.falciparum*-infected erythrocytes and merozoites in immunity against malaria in children

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Abbreviations:

Ab: Antibody

AMA1: apical membrane antigen 1

CI: confidence intervals

DC: domain casettes

EPCR: endothelial protein C receptor

EBA175: Erythrocyte binding antigen 175

HR: hazard ratio

IE: Plasmodium falciparum-infected erythrocytes

ICAM-1: Intercellular adhesion molecule 1

$_{mol}$FOI: molecular force of infection

MSP1: merozoite surface protein 1

MSP2: merozoite surface protein 2

PCR: polymerase chain reaction

PfEMP1: Plasmodium falciparum erythrocyte membrane protein 1

PNG: Papua New Guinea

RIFIN: repetitive interspersed family proteins

STEVOR: subtelomeric variable open reading frame proteins

SURFIN: surface-associated interspersed gene family proteins

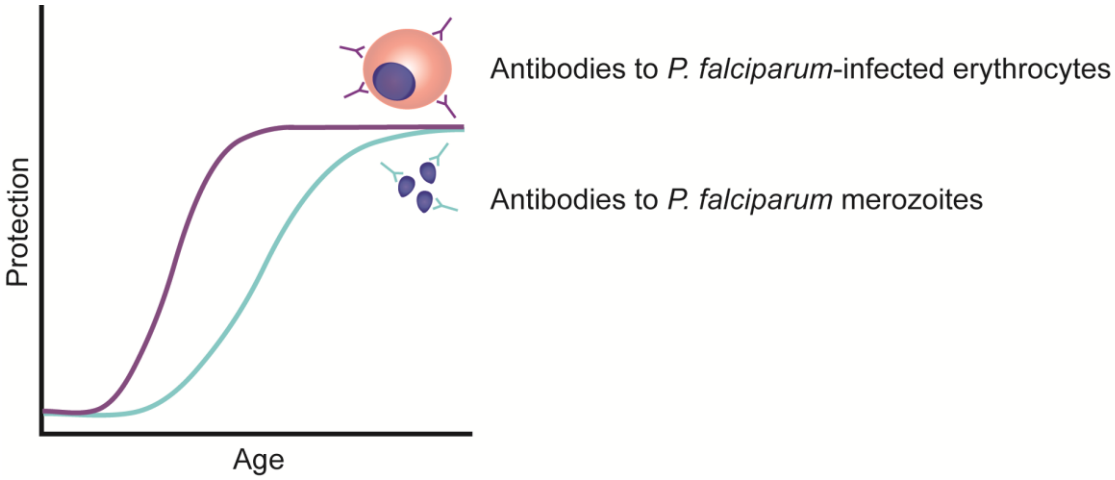
vpkd: *var* promoter knock-down

ABSTRACT

Acquired antibodies play an important role in immunity to *P. falciparum* malaria and are typically directed towards surface antigens expressed by merozoites and infected erythrocytes (IEs). The importance of specific IE surface antigens as immune targets remains unclear. We evaluated antibodies and protective associations in two cohorts of children in Papua New Guinea. We used genetically-modified *P. falciparum* to evaluate the importance of PfEMP1 and a *P. falciparum* isolate with a virulent phenotype. Our findings suggested that PfEMP1 was the dominant target of antibodies to the IE surface, including functional antibodies that promoted opsonic phagocytosis by monocytes. Antibodies were associated with increasing age and concurrent parasitemia, and were higher among children exposed to a higher force-of-infection as determined using molecular detection. Antibodies to IE surface antigens were consistently associated with reduced risk of malaria in both younger and older children. However, protective associations for antibodies to merozoite surface antigens were only observed in older children. This suggests that antibodies to IE surface antigens, particularly PfEMP1, play an earlier role in acquired immunity to malaria, whereas greater exposure is required for protective antibodies to merozoite antigens. These findings have implications for vaccine design and serosurveillance of malaria transmission and immunity.

Antibodies to surface antigens of *P. falciparum*-infected erythrocytes were associated with protection against symptomatic malaria in younger and older children, while antibodies to merozoite antigens were only protective in older children.

PfEMP1 is the major target of antibodies to the surface of infected erythrocytes among children from Papua New Guinea



INTRODUCTION

Despite recent declines in prevalence, malaria remains a global health burden. Mortality from *P. falciparum* infection was estimated at 438 000 deaths worldwide in 2015 [1]. In humans, protective immunity against symptomatic malaria develops relatively slowly after repeated exposure to *P. falciparum* (reviewed in [2]). In areas of moderate to high malaria transmission, the burden of severe, life-threatening disease falls mostly on non-immune, young children [3]-[6]. Naturally acquired antibodies represent an essential component of protective immunity for residents in malaria-endemic regions and these antibodies primarily target *P. falciparum* blood stage antigens [2]. These include merozoite antigens and antigens expressed on the surface of *Plasmodium falciparum*-infected erythrocytes (IEs) (reviewed in [7],[8]).

During intra-erythrocytic development of *P. falciparum*, numerous parasite-derived antigens are expressed on the IE surface including *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) [9], repetitive interspersed family proteins (RIFIN) [10],[11], subtelomeric variable open reading frame proteins (STEVOR) [12], surface-associated interspersed gene family proteins (SURFIN) [13] and possibly others [14]. Of all the IE surface antigens, PfEMP1 is the best characterised and mediates adhesion and sequestration of IEs in the microvasculature of various organs, which contributes to disease pathogenesis. PfEMP1 is encoded by the *var* multigene family, which undergoes clonal antigenic variation due to selective immune pressure [7]. The switching of *var* genes facilitates evasion of acquired antibody responses and results in various adhesive phenotypes of IEs [15]-[17]. Members of the *var* multigene family can be classified into three main groups (groups A, B, C) based on their chromosome location and upstream promoter regions [18]. The transcription of different *var* gene subgroups has been linked to various IE phenotypes and disease severity, and recent data suggests that a subset of *var* gene types with more virulent phenotypes may be responsible for causing the bulk of

disease. Group A *var* genes have been associated with rosetting parasites [19] and severe malaria in African children [20]-[23]. Group B/A *var* genes encode PfEMP1 variants capable of binding to endothelial protein C receptor (EPCR) [24] expressed by human brain endothelial cells and may be important in the pathogenesis of severe malaria, particularly involving sequestration in the cerebral vasculature [20],[21],[25]. Despite undergoing a high rate of *var* gene recombination, certain tandem domain cassettes (DCs) of PfEMP1 appear to be functionally conserved. High transcript levels of *var* genes containing DC8 (group B/A) and DC13 (group A) were associated with severe malaria syndromes, including severe anaemia and cerebral malaria in African children [20],[26]. These findings suggest that antibodies against PfEMP1 variants encoded by key *var* gene subgroups could be important in protective immunity to malaria.

Due to technical limitations, published studies on antibodies to native IE surface antigens typically measure the collective antibody response to all antigens expressed on the IE surface (i.e. PfEMP1, RIFIN, STEVOR, SURFIN and others) (reviewed in [7]). The dissection of the relative importance of each antigen as antibody targets has relied on the recombinant expression of these proteins. However, the selection of relevant domains to study and the folding of recombinant proteins may not reflect the tertiary and/or quaternary structure of the native antigen. This could be why the association between antibodies to recombinant PfEMP1 domains and protection against malaria in naturally exposed human populations has been inconsistent [7]. We have recently used genetically-modified *P. falciparum* with suppressed PfEMP1 expression to quantify the relative importance of PfEMP1 and other IE surface antigens as targets of acquired immunity [7],[27],[28]. In an African population, we found that antibodies to PfEMP1 were significantly associated with protection, whereas antibodies to other IE surface antigens were not [27]. It is not known if this association is also found in populations outside Africa; currently, there are few published studies on the role of immune responses to IE surface

antigens in non-African populations (reviewed in [7]). Furthermore, only a handful of studies have been published on the protective role of antibodies to other IE surface antigens. For example, antibodies to RIFIN were associated with rapid parasite clearance in Gabonese children presenting with severe malaria [29], suggesting that RIFIN antibodies may contribute to protective immunity. It remains unknown if antibodies to STEVOR are important in protective immunity.

Antibodies to various merozoite surface antigens also play an important role in protective immunity to malaria [8],[30]-[33]. However, the relative contribution of antibodies to IE surface antigens and merozoite surface antigens in acquired immunity remains unclear. In young children or in children with limited malaria exposure, the levels of antibodies to merozoite surface antigens may be below the threshold required to provide sufficient protective immunity [34],[35]. This has not yet been evaluated for antibodies to IE surface antigens. Understanding this acquisition of immunity relative to exposure is particularly important at present, given the global epidemiologic transition in malaria, with many regions reporting significant reductions in malaria burden in the face of reduced yet persisting transmission. Furthermore, while there is a growing evidence base from studies in sub-Saharan Africa for immune targets and their role in acquisition of immunity, data from populations elsewhere is much more limited.

In the present study, we evaluated the acquisition of antibodies to surface antigens of IEs in two longitudinal cohorts of children in Papua New Guinea (PNG), and compared these to antibody responses against merozoite surface antigens. The cohorts differed in their age structures and histories of malaria exposure; we studied a cohort of young children aged 1 to 4 years (Ilaita region) and a cohort of older children aged 5 to 14 years (Mugil region), who were actively acquiring immunity to *P. falciparum* malaria [34],[36],[37]. We evaluated the influence of age,

active *P. falciparum* infection, and spatial heterogeneity in exposure levels on the protective associations of antibodies to surface antigens of IEs and related these to the acquisition and protective associations for antibodies to merozoites antigens. Using genetically-modified *P. falciparum* with substantially reduced PfEMP1 expression, we were able to directly quantify the importance of PfEMP1 as a target of antibodies and their association with protective immunity. We used a *P. falciparum* 3D7 line expressing PfEMP1 variants that have been associated with virulent phenotypes and disease pathogenesis, to test the hypothesis that antibodies to these virulent phenotypes may be important in childhood immunity. We determined whether antibodies to surface antigens of IEs and merozoites have different roles in the acquisition of protective immunity. Additionally, we used a novel measure of malaria exposure, the molecular force of infection ($_{mol}$FOI) [34],[38] in the cohort of younger PNG children, to gain a better understanding of the relationship of heterogeneity of malaria exposure to antibody acquisition and risk of symptomatic malaria illness.

RESULTS

Identification of a *P. falciparum* isolate expressing *var* genes associated with disease pathogenesis

To evaluate antibodies to IE surface antigens, we identified a laboratory-passaged *P. falciparum* 3D7 isolate that was commonly recognized by antibodies among children in PNG and analysed its *var* expression profile (Supplemental Fig S1). The most abundant *var* transcript identified was PF11_0521, which encodes a PfEMP1 variant belonging to group A *var* genes (Table 1). PF11_0521 also contains the DC13 structure (PfEMP1 domain architecture of DBLα1.7 and CIDRα1.4, followed by a DBLβ3 and DBLβ6). DC13 expression has been associated with severe malaria [20],[21],[25],[39] and the tandem CIDRα1.4-DBLβ3 domains in other DC13 *var* genes mediates dual adhesion to EPCR and ICAM-1 on brain endothelial cells [40]. Two other group A

var genes were also upregulated (PFD1235w and PFA0015c). PFD1235w is widely recognised by antibodies from semi-immune children in Africa [23], contains a DC4 structure (DBLα1.4-CIDRα1.6-DBLβ3) [41] and binds ICAM-1 and EPCR [41],[42]. Other prominent *var* genes include PFF1580c (group B/A) and PFD0625c (group C). Group B/A *var* genes have also been associated with severe disease in some studies [22],[43].

PfEMP1 is a major target of naturally acquired antibodies to the IE surface among children

We investigated whether PfEMP1 was the dominant target of acquired antibodies to the IE surface in the Mugil cohort of PNG children, as we had previously reported for African children [7],[27]. We used the *var* promoter 'knock-down' (vpkd) transgenic parasites with suppressed PfEMP1 expression, as we have previously published [27], and the 3D7 isolate described above that expresses *var* genes associated with virulent phenotypes. We studied responses among malaria-exposed children aged 5-14 years old (n=181) who were enrolled in a longitudinal treatment-reinfection cohort study, conducted in the Mugil area of PNG. We measured their levels of antibodies to the surface of erythrocytes infected with 3D7 parental and 3D7vpkd parasites. The overall IgG binding to 3D7vpkd, which has greatly reduced PfEMP1 expression [27], was substantially reduced by 88% compared to 3D7 parental (Fig 1A; $p<0.0001$). Analysis of antibody labeling of IEs using flow cytometry among samples that were classified as antibody positive indicated that the whole IE population was generally labeled rather than minor sub-populations (Supplemental Fig S2). This indicates that antibody reactivity to 3D7 IEs largely reflect antibodies to PfEMP1 types encoded by *var* genes associated with severe malaria. Plasma from all children showed a marked reduction in IgG binding to 3D7vpkd compared to 3D7 parental (Fig 1B). A similar pattern of reduced IgG binding to 3D7vpkd IEs was seen across all age groups in the cohort (Supplementary Fig S3). In addition, we tested samples from young children and adults and found a similar pattern of reactivity to 3D7 parental and 3D7vpkd.

These findings suggest that PfEMP1 is the dominant target of antibodies to the IE surface in this cohort (defined as IgG binding to 3D7 parental minus 3D7vpkd; referred to as 3D7-PfEMP1).

PfEMP1-specific antibodies mediate the opsonic phagocytosis of IEs

To understand the functional importance of acquired antibodies, we tested serum samples for the ability to promote opsonic phagocytosis of IEs, which is thought to be an important mechanism for immunity and parasite clearance [7]. We used an established assay with the THP-1 monocyte cell line and included a selection of samples from young children (aged 0-4 years; n=19), older children (aged 5-10 years, n=11) and adults (n=9) residing in the same area of PNG, and exposed to similar levels of transmission to children in the Mugil cohort (due to insufficient sample availability, it was not possible to test samples from the Mugil cohort). Substantial levels of opsonic phagocytosis activity were observed among samples against 3D7 IEs; in contrast, opsonic phagocytosis activity was markedly reduced for 3D7vpkd (Fig 2; $p<0.001$). This was observed in samples of all age groups. Among samples that were positive for opsonic phagocytosis of 3D7 parental IEs, there was a 77.1% reduction in opsonic phagocytosis of 3D7vpkd IEs, indicating that PfEMP1 is the major target of these functional antibodies. Our results suggest that naturally acquired antibodies function by opsonising IEs for phagocytic clearance. We also measured the level of total IgG binding to the IE surface of 3D7 parental and 3D7vpkd parasites among these children (Supplemental Fig S3). There was reduction in IgG binding to 3D7vpkd compared to 3D7 parental parasites (Supplemental Fig S3A, 3B), which corresponds to results from opsonic phagocytosis assays. When comparing total IgG binding and opsonic phagocytosis activity, there was a strong positive correlation for 3D7 parental and no correlation for 3D7vpkd (Fig 2E, 2F). Thus, our results suggest that PfEMP1-specific antibodies mediate the opsonic phagocytosis activity of IEs.

Abs to surface antigens of IEs and merozoites are associated with age and baseline parasitaemia

In the Mugil cohort of older children (5-14 years), the seroprevalance of antibodies to IE surface antigens was not significantly higher in older (≥9 years, ~55%) compared to younger children (<9 years, ~45%, all p>0.4; Table 2). This suggests that in this cohort, antibodies to IE surface antigens were acquired relatively early in life and are not greatly increased by subsequent malaria exposure. Antibody levels to IE surface antigens were significantly higher in children who were infected with *P. falciparum* at enrolment (diagnosed by PCR) compared to those who were not (p<0.001, Table 2). This suggests that asymptomatic *P. falciparum* infection boosts antibody responses to IE surface antigens. Similar associations with age and infection status were generally found for antibodies to several merozoite surface antigens (Table 2). MSP1, MSP2, AMA1, and EBA175 were used as representative merozoite surface antigens (previously published data on merozoite antibody responses was reanalyzed here for comparison) [30],[44].

Abs to surface antigens of IEs and merozoites are associated with protection in the Mugil cohort

The association between antibodies to IE surface antigens and prospective risk of symptomatic *P. falciparum* infection (fever plus >5000 *P. falciparum* parasites/μL) was investigated in the Mugil cohort. Children had active surveillance and access to early diagnosis and treatment for malaria and therefore, it was not possible to evaluate the association between antibodies and risk of severe malaria. We adjusted hazard ratios (HR) for the predetermined confounders of age (<9 years and ≥9 years) and location of residence [36] (Table 3), using the Cox proportional hazards model. We used the age groups (<9 years and ≥9 years) previously established [36],[44] as we did not find other age groupings to be more informative (data not shown). Initially, children were stratified into three equal groups (tertiles) reflecting high, medium and

low antibody levels. We found that the risk of symptomatic malaria was very similar between medium and high antibody responders. These two groups were, therefore, merged into one group to maximize statistical power and compared to the low antibody responders (Table 3, Fig 3). Children with high-medium antibodies to surface antigens of 3D7 parental IEs had a significant (51%) reduction in the risk of malaria after adjusting for confounding factors (adjusted HR=0.49, 95% CI: [0.28-0.84], p=0.01). Furthermore, antibodies to 3D7-PfEMP1 (represented as antibody reactivity to 3D7 parental minus 3D7vpkd) were also significantly associated with a reduced risk of malaria in adjusted and unadjusted analyses. There was also protective association for antibodies to 3D7vpkd IEs that was of weak statistical significance in the unadjusted analysis but had stronger statistical significance in the adjusted analysis. However, the protective association observed with 3D7vpkd parasites should be interpreted with caution given the relatively low levels of antibodies to 3D7vpkd, and prior studies suggesting that some of the antibody reactivity with 3D7vpkd is due to low levels of residual PfEMP1 [27]. Collectively, these associations were not impacted by infection status at enrolment (data not shown). These results suggest that antibodies to the IE surface, predominantly PfEMP1, play an important role in mediating protective immunity against malaria. In this same cohort we have previously reported that antibodies to a range of merozoite surface antigens, which are also vaccine candidates (e.g. MSP1, MSP2, AMA1, and EBA175), were significantly associated with protection from symptomatic malaria [30]. This suggests that responses to both surface antigens of IEs and merozoites contribute to immunity in these children.

Associations between antibodies and age, parasitaemia and force of infection among young children

We further investigated the association between antibody levels to IE surface antigens and protection against malaria, in a cohort of younger children (1-4 years old) from the Ilaita area of

PNG. These younger children have had less prior malaria exposure than children in the Mugil cohort, which is reflected in having significantly lower levels of antibodies to merozoite antigens compared to children from the Mugil cohort [34],[37]. The children (n=183) were enrolled in a longitudinal cohort study with active surveillance for malaria symptoms over 16 months [34],[37].

We analysed antibody levels to the IE surface of 3D7 parental parasites and stratified them by age and infection status (as determined by PCR) (Fig 4A). IgG levels to 3D7 parental were significantly associated with age among infected children (Fig 4A; p=0.001), but not in uninfected children who had low levels of antibodies overall (Fig 4A; p=0.23). There were higher antibody levels to 3D7 in children who were classified as parasite positive (p=0.05). We further examined antibody responses to IE surface antigens in relation to a novel molecular marker of malaria exposure, the molecular force of infection ($_{mol}$FOI) as previously described [34],[38]. Previous studies have validated $_{mol}$FOI as the best parameter to adjust for *P. falciparum* exposure in this cohort [34],[38]. Briefly, $_{mol}$FOI is defined as the number of new *P. falciparum* clones acquired during the study follow-up period as measured by molecular detection and genotyping [34],[38]. Increasing $_{mol}$FOI was associated with higher levels of antibodies to IE surface antigens in infected children (Fig 4B; p=0.03), but not in uninfected children (Fig 4B; p=0.22). We have previously reported similar broad associations with age, parasitemia and $_{mol}$FOI for antibodies to merozoite surface antigens in this cohort [34].

Abs to IEs but not merozoite surface antigens are associated with protection among young children

We evaluated the association between antibodies (high-medium vs low) at baseline and the subsequent incidence of symptomatic malaria (fever plus >2500 *P. falciparum* parasites/µl)

during the follow-up period, using negative binomial regression analyses. After adjusting for exposure with $_{mol}$FOI and conventional confounding factors (age, location of residence, parasitemia at enrolment), individuals with high levels of antibodies to 3D7 parental parasites had a significant reduction in the rate of symptomatic malaria compared to children with low antibody levels (Table 4; IRR=0.64, 95% CI: [0.49-0.83], p=0.001); there was also some reduced risk of malaria for the medium group, but this was less strong and not statistically significant. In contrast, antibodies to merozoite antigens (MSP1-19, MSP2-3D7, AMA1-3D7, and EBA175) showed no protective associations with the rate of symptomatic malaria after adjusting for $_{mol}$FOI (Table 4), or in unadjusted analyses, as we have previously reported [34]. This suggests that antibodies to IE surface antigens contribute to protective immunity in young children, whereas antibodies to merozoite antigens do not significantly contribute at this age.

Correlation between antibodies to surface antigens of IEs and merozoites

Given the different patterns of associations with protection for antibodies to surface antigens of IEs and merozoites in the cohort of young children, we examined the relatedness between these responses (Table S1). In the Ilaita cohort of young children, there was only a weak positive correlation between antibodies to IE surface antigens and antibodies to each of the merozoite antigens, except MSP1. In the Mugil cohort of older children, antibodies to IE surface antigens were weakly negatively correlated with antibodies to MSP2 and EBA175. This indicates that the antibodies to surface antigens of IEs and merozoites are not strongly co-acquired in either cohort and may have different contributions to immunity.

DISCUSSION

We examined the acquisition and protective associations of antibodies to surface antigens of IEs compared to merozoite antigens in two cohorts representing older and younger children, and used a *P. falciparum* isolate that expressed PfEMP1 variants associated with a virulent phenotype. We first demonstrated that PfEMP1 is the major target of acquired antibodies to the IE surface by comparing reactivity to parental parasites versus genetically-modified parasites with inhibited PfEMP1 expression. Second, we established that PfEMP1 is also the major target of antibodies that promote opsonic phagocytosis of IEs by THP-1 monocytes. Third, higher antibody levels were associated with asymptomatic infection in both cohorts, and with $_{mol}$FOI in the cohort of younger children (Ilaita). Broadly similar associations were seen for levels of antibodies to merozoite surface antigens. Fourth, an important finding was that high levels of antibodies to IE surface antigens were significantly associated with protection from malaria in both the older and younger children's cohorts. In contrast, high levels of antibodies to merozoite surface antigens were only associated with protection in the older children's cohort. Our results point towards different roles in the acquisition of immunity for antibodies to different blood stage antigens. Antibodies to IE surface antigens appear to contribute to the early development of malaria immunity in young children, whereas antibodies to merozoite surface antigens appear to play a role later in the further development and possibly maintenance of immunity to uncomplicated malaria in older children. This may be because the development of protective antibodies to merozoite surface antigens requires substantially more exposure for the development of sufficiently high antibody levels, above a protective threshold [34],[35], than the acquisition of protective antibodies to IE surface antigens. In our earlier studies, we found that antibody levels to merozoite antigens in the younger children's cohort were generally much lower than antibody levels among protected children in the Mugil cohort [34]. This supports a conclusion that there may be a high threshold level of antibodies to merozoite antigens required

for protective immunity. There was limited correlation between antibodies to IE and merozoite surface antigens among children, indicating that these responses are not strongly co-acquired and quantitatively, may have different contributions to protective immunity, depending on age and recent exposure history.

We measured antibodies to the surface of 3D7 parental IEs and compared this to IgG binding to 3D7vpkd IEs, a genetically modified parasite line that has previously been characterized to express little to no PfEMP1, based on the absence of *var* transcripts by Northern blot, the lack of detectable PfEMP1 in IE membrane extracts by Western blot and markedly reduced cytoadhesion to vascular receptors [27]. Notably, RIFIN and STEVOR remained expressed by these parasites, suggesting that protein expression and trafficking was not affected [27]. The overall IgG binding to 3D7vpkd was markedly reduced compared to 3D7 parental parasites. This suggests that naturally acquired antibodies to the IE surface are predominantly directed towards PfEMP1. We confirmed that this pattern is observed across a broad age range of children and among adults. These results complement our previous findings in Kenyan children and adults [27]. We recently provided further evidence that PfEMP1 is a major target of naturally acquired antibodies by using a parasite line with inhibited PfEMP1 surface expression due to a disruption of PfEMP1 trafficking [28]. Collectively, these findings demonstrate the importance of PfEMP1 as a dominant antibody target in different populations. Findings from our study also provide a better understanding of humoral immunity to IE surface antigens in human populations outside of Africa, which is currently limited. In our studies to investigate the role of antibodies to IE surface antigens with protection from malaria in children, we used a *P. falciparum* 3D7 isolate that expressed specific *var* genes that are associated with virulent phenotypes and severe malaria pathogenesis. The 3D7 isolate expressed *var* genes that encode cytoadhesive domains for binding EPCR and ICAM-1 receptors, both of which have recently been implicated in the binding of IEs to brain microvascular endothelium [24],[40],[45]. Our

results suggest that antibodies to these virulent PfEMP1 variants may play a role in immunity to malaria in children.

In the Mugil cohort of older children, individuals with high or medium antibody levels to 3D7 parental and 3D7-PfEMP1 had a significantly reduced risk of symptomatic malaria, compared to those with low antibody levels. In contrast, antibody levels to other antigens expressed by 3D7vpkd IEs were quite low and the statistical evidence of protective associations was less strong. Furthermore, IgG reactivity to 3D7 parental IEs correlated with opsonic phagocytosis activity, and there was greatly reduced opsonic phagocytosis of 3D7vpkd IEs, indicating PfEMP1 is that major target of these functional antibodies. Our findings suggest that antibodies to non-PfEMP1 antigens are a less important component of the protective response, as we observed in a Kenyan population [27]. Our prior study in a Kenyan population showed that antibodies to 3D7vpkd were not associated with protection and there was a low level of functional antibodies to 3D7vpkd parasites [27]. However, these studies do not exclude a contributing role for other IE surface antigens as targets of protective immunity. For example, we previously found many Kenyan serum samples were able to promote opsonic phagocytosis of 3D7vpkd parasites [27], suggesting that surface antigens other than PfEMP1 may also be targets of functional antibodies. Others have reported some association between recombinant RIFIN proteins and immunity [29]. Further studies are necessary to better understand the significance of these IE surface antigens as targets of antibodies and the protective and functional importance of these antibodies. In the Mugil cohort, antibodies to recombinant merozoite antigens quantified by ELISA were also associated with protection from malaria, suggesting that these antibodies also play a role in immunity. Prior studies have identified antibodies that fix and activate complement [46], and promote opsonic phagocytosis of merozoites [47] as functional immune responses to merozoites that are associated with protective immunity.

It is possible that a limited subset of PfEMP1 variants, similar to those expressed by the 3D7 parasites used in this study, is sufficient to induce a significant protective response to malaria, as proposed before [48],[49]. The parasite line used in our study abundantly expressed a DC4 PfEMP1, which contains an unusually conserved DBLβ3 domain. This DC4 PfEMP1 can bind ICAM-1 and EPCR [41],[42] and thus has been proposed to play a role in disease pathogenesis [41]. This DC4 PfEMP1 was preferentially recognised by antibodies from semi-immune children in Africa [23], consistent with the model that PfEMP1 variants that cause severe disease or disease in young children are commonly expressed [50],[51]. This could explain why antibodies to these PfEMP1 variants are prevalent in the general population and particularly in children who are still acquiring immunity [50],[51]. The parasite line used in this study also expressed a DC13 *var* gene that contains CIDRα1.4 and DBLβ3 domains that bind to EPCR and ICAM-1 [40]. Other DC13 PfEMP1 variants also mediate IE adhesion to brain endothelium and their expression is upregulated in severe malaria [20],[25]. Antibodies to the 3D7 parasite line may recognize conserved epitopes, or shared epitopes that are expressed by a range of isolates causing malaria in PNG children.

In conclusion, our study has provided a unique insight on antibodies to surface antigens of IEs and merozoites and their relative importance in mediating protective immunity against symptomatic malaria in a non-African population. We found that PfEMP1 is the main target of protective antibodies to the IE surface in PNG children, and antibodies that promote the opsonic phagocytosis of IEs. Antibodies to the surface of IEs expressing virulent PfEMP1 variants appear to play a role in immunity acquired by both younger and older children, whereas antibodies to merozoite antigens appear to play a significant role in older children or following much greater malaria exposure after higher levels of antibodies are acquired. These results have significant

implications for measuring and monitoring clinical immunity in malaria endemic populations [52], and raise the possibility that malaria vaccines based on the surface antigens of IEs and merozoites may be helpful as a public health tool in an era of declining but persisting malaria transmission in highly endemic areas within and outside Africa.

MATERIALS AND METHODS

Study population and ethics statement

Samples were collected as part of a prospective treatment-reinfection study of 206 children (age 5-14 years) conducted from June to December 2004 at the Mugil and Megiar Elementary Schools, Madang Province, PNG [36]. This cohort has been extensively described elsewhere [36]. A clinical episode of *P. falciparum* malaria was defined as the presence of fever and parasitemia >5000/μL. Some samples from the original cohort could not be included due to insufficient sample volumes available for testing. Samples were also collected as part of another longitudinal study of 264 children (age 1-4 years) from the Ilaita area of Maprik District, PNG [34],[37]. In this cohort, a clinical episode of *P. falciparum* malaria was defined as the presence of fever and parasitemia >2500/μL [34],[37]. Plasma samples were also available from a cross-sectional study in Madang Province, PNG, which were collected in 2007. These samples included 118 individuals; 49 adults (median age 28 years) and 69 children (median age 6 years) [53]. From this study, we selected samples for testing in opsonic phagocytosis assays and assays to quantify total IgG binding to the IE surface.

Ethics approval was obtained from the Medical Research Advisory Council PNG (MRAC# 05/20; 98/03; 14/12 – Amendment 2005), the Walter and Eliza Hall Institute Human Research and

Ethics Committee and the Alfred Hospital Human Research and Ethics Committee. Written informed consent was obtained from all study participants or their parents or legal guardians.

P. falciparum culture and isolates

P. falciparum isolates were maintained in continuous culture and synchronized as previously described [27]. 3D7vpkd parasites (*var* promoter 'knock-down' transgenic parasites with suppressed PfEMP1 expression) were generated as previously described [27],[54].

Measuring antibodies to the IE surface by flow cytometry

Measuring IgG binding to the IE surface of pigmented trophozoites was performed with an established flow cytometry-based assay as previously described [27]. Levels of IgG specific to IE surface antigens in each plasma sample were expressed as the geometric mean fluorescence intensity (MFI; arbitrary units) for IEs, after subtracting that for uninfected erythrocytes. The seropositivity threshold was defined as the mean MFI plus 3 standard deviation of the reactivity of non-exposed control serum from non-exposed Melbourne residents. Plasma from malaria-exposed adults was used as positive controls. The use of cryopreserved IEs at the pigmented trophozoite stage [27] have been previously validated (Beeson et al, unpublished) [55].

Measuring antibodies to merozoite surface antigens

Antibody levels to merozoite surface antigens that were measured by ELISA had been previously published for both the Mugil [44],[56] and the Ilaita cohort [34]. In the present study, the data were re-analysed for comparison to antibody responses from IE surface antigens.

Opsonic phagocytosis assays

Measuring the level of opsonic phagocytosis activity using undifferentiated THP-1 monocytes was performed as previously described [57]. The level of phagocytosis was expressed as a percentage of the positive control (Abcam, rabbit antibody raised against human erythrocytes). Negative controls from non-exposed Melbourne residents were included in all assays.

Quantitative RT-PCR

RNA from pelleted IEs was extracted with 10 pellet volumes of TRIzol (Invitrogen) and purified as previously described [58]. cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen) and subjected to quantitative PCR as described previously [58]. Briefly, relative quantitation of the 3D7 *var* repertoire by $2^{-\Delta\Delta Ct}$ analysis was performed using a previously described primer set [59] supplemented with previously described additional primers [60]-[62]. Analysis was performed using ring stage expressed gene SBP1 as a normaliser [63] and 3D7 parental gDNA as a calibrator.

Statistical analyses

Non-parametric analytical methods were used to evaluate assay results from cohort studies, as the data obtained were not normally distributed. Differences in antibody levels between parental and transgenic parasites were assessed using a paired Wilcoxon signed rank test or Kruskal Wallis test (>2 groups). Correlations between antibody responses were examined using Spearman's rho (r_s) and comparison of proportions was calculated using the χ^2 test. Statistical analyses were performed using Prism version 5 (GraphPad Software Inc) or STATA 12.0

(STATACorp). To determine the association between antibody levels and subsequent episodes of symptomatic malaria, children were classified according to seroprevalence in the cohort or stratified into three equal groups (tertiles) reflecting low, medium and high antibody responders. This approach was previously used for the analyses of antibodies to merozoite antigens and their association with protective immunity in this cohort [44],[56],[64]. For the Mugil cohort, Cox regression was used to calculate hazard ratios (HR) for the risk of symptomatic malaria between antibody responder groups [56],[64]. Assumptions of proportional hazards were assessed using statistical methods and visual inspection of graphical methods. For antibody variables that showed non-proportional hazards, an interaction term between the antibody response and time (3 categories: t=0–100, t=100–150, and t>150 days) was included in the analysis [56],[64]. All other analyses were performed without the interaction with time. For the IIaita cohort, a negative binomial model with generalized estimating equations (GEE) (based on an XTNBREG procedure) with an exchangeable correlation structure and a semirobust variance estimator was used to calculate incidence rate ratios (IRR) for the risk of symptomatic malaria between antibody responder groups [34].

In this study, only age and location of residence were identified as being significantly associated with levels of antibodies and malaria outcomes [36],[44]. Baseline parasitaemia was not associated with symptomatic outcomes and did not affect the multivariate regression models. Therefore, multivariate analysis was used to calculate adjusted hazard ratios using only covariates of age and location of residence. Age was used as a binary variable (<9 years, ≥9 years), as previous studies indicated that this stratification was the most informative approach for assessing the effect of age [36]. No other confounders were identified. Parasitaemic status at enrolment and erythrocyte polymorphisms (that may impact on PfEMP1 expression as described by [65]) were not associated with the risk of malaria in the Mugil cohort [36],[37]. Additionally, it was previously reported that common erythrocyte polymorphisms such as

alpha-thalassemia, Southeast Asian ovalocytosis, Gerbich negativity and CR1 polymorphisms were not associated with the risk of *P. falciparum* infections in the Mugil cohort [37]. The first episode of symptomatic *P. falciparum* infection is defined as fever plus *Pf* density of >5000 parasites/μL.

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TABLES

Table 1 Predominant *var* genes expressed by our 3D7 parental isolate

Gene ID	ups[1]	DC type[2]
PF11_0521	A	13
PFF1580c (MAL6P1.4)	B/A	14
PFD0625c	C	-
PFA0015c	A	var3
PFD1235w (MAL7P1.1)	A	4

[1]ups refers to the upstream promoter regions of *var* genes

[2]DC type refers to the tandem domain cassettes of PfEMP1

Table 2 Association between antibodies to *P. falciparum* surface antigens and age and baseline parasitaemia in the Mugil cohort

Pf isolates	n[1]	Age[2]			Baseline *Pf* parasitaemic status[3]		
		<9 y[4]	**≥9 y[5]**	***p***	**PCR-[6]**	**PCR+[7]**	***p***
3D7 parental IE	148/181	16/67	17/81	0.74	18/44	15/104	0.007
	81.8%	80.7%	82.7%		71%	87.5%	
3D7vpkd IE	62/181	55/28	64/34	0.89	51/11	68/51	0.001
	34.3%	33.7%	34.7%		17.7%	42.9%	
3D7-PfEMP1 IE	140/181	21/62	20/78	0.43	23/39	18/101	0.001
	77.3%	74.7%	79.6%		62.9%	84.9%	
MSP1-19	199/206	6/85	1/114	0.02	5/62	2/137	0.03
	96.6%	93.4%	99.1%		92.5%	98.6%	
MSP2-3D7	182/206	17/74	7/108	0.005	16/51	8/131	<0.001
	88.4%	81.3%	93.9%		76.1%	94.2%	
AMA1-3D7	205/206	1/90	0/115	0.26	1/66	0/139	0.15
	99.5%	98.9%	100%		98.5%	100%	
EBA175 RIII-V	184/206	16/75	6/109	0.004	17/50	5/134	<0.001
	89.3%	82.4%	94.8%		74.6%	96.4%	

[1]Proportion of seropositive individuals and the total number of samples tested

[2]<9 y indicates children less than 9 years of age; ≥9 y indicates children 9 years and older.

[3]PCR-, *P. falciparum* not detected by PCR; PCR+, *P. falciparum* detected by PCR.

[4]n=83 for IE antigens, n=91 for merozoite antigens

[5]n=98 for IE antigens, n=115 for merozoite antigens

[6]n=62 for IE antigens, n=67 for merozoite antigens

[7]n=119 for IE antigens, n=139 for merozoite antigens

p values were calculated using the χ^2 test for comparison of proportions.

Table 3 Association between antibodies and risk of symptomatic malaria among high and medium versus low antibody responders in the Mugil cohort

Pf isolates/antigens	Unadjusted analysis		Adjusted analysis	
	HR [95% CI]	***p***	**HR [95% CI]**	***p***
3D7 parental	0.49 [0.29-0.82]	0.007	0.49 [0.28-0.84]	0.01
3D7vpkd	0.63 [0.37-1.07]	0.09	0.53 [0.31-0.91]	0.02
3D7-PfEMP1	0.53 [0.32-0.90]	0.02	0.56 [0.33-0.96]	0.04

Children were stratified into high, medium, and low antibody responders based on tertiles. The high and medium groups were merged into one group as initial analysis indicated that these two groups had the same risk of malaria during follow-up. The risk of symptomatic malaria during follow-up was compared between the merged high-medium groups and the low responders. Hazard ratios (HR) were calculated using Cox regression; unadjusted data and data adjusted for the predetermined confounders of age and location of residence are presented.

Table 4 Association between antibodies among young children to *P. falciparum* surface antigens and risk of symptomatic malaria in the Ilaita cohort

Pf isolates/antigens	Unadjusted analysis		Adjusted+$_{mol}$FOI	
	IRR [95% CI]	***p***	**IRR [95% CI]**	***p***
3D7 parental				
IgG (M)	0.81 [0.59-1.11]	0.19	0.83 [0.64-1.08]	0.16
IgG (H)	0.88 [0.65-1.19]	0.41	0.64 [0.49-0.83]	0.001
MSP1-19				
IgG (M)	1.27 [0.93-1.73]	0.13	0.95 [0.74-1.22]	0.67
IgG (H)	1.11 [0.81-1.52]	0.52	0.91 [0.72-1.16]	0.47
MSP2-3D7				
IgG (M)	1.34 [0.95-1.89]	0.09	1.21 [0.92-1.58]	0.18
IgG (H)	1.72 [1.28-2.33]	0.001	1.13 [0.86-1.48]	0.38
AMA1-3D7				
IgG (M)	1.49 [1.08-2.07]	0.02	1.28 [0.97-1.69]	0.08
IgG (H)	2.15 [1.56-2.96]	0.001	1.29 [0.97-1.72]	0.08
EBA175 RIII-V				
IgG (M)	1.61 [1.22-2.13]	0.001	1.35 [1.02-1.78]	0.04
IgG (H)	1.61 [1.23-2.13]	0.001	1.19 [0.87-1.64]	0.21

Children were stratified into high, medium, and low antibody responders to each antigen based on tertiles. The association between risk of symptomatic malaria and antibody responses was compared between high (H) and medium (M) antibody levels versus low antibody levels. IRR was determined by negative binomial with generalized estimating equations (GEE) model, comparing antibody responses with protection from malaria. IRR were adjusted for $_{mol}$FOI and conventional confounding factors (age, location of residence, parasitemia at enrolment).

FIGURE LEGENDS

Figure 1 PfEMP1 is the major target of naturally acquired antibodies

(A) Antibody levels to surface antigens expressed by erythrocytes infected with 3D7 parental and 3D7vpkd parasites. Levels of total IgG binding in each plasma sample were determined by an established flow cytometry-based assay. Data represent results from 2 independent assays; IgG binding levels are expressed as geometric mean fluorescence intensity (MFI); bars represent median and interquartile ranges of samples tested in duplicate (n=181); *p* value was calculated using a paired Wilcoxon signed rank test. (B) A representative selection of plasma samples tested for antibodies to surface antigens expressed by 3D7 parental and 3D7vpkd parasites. Samples were from the Mugil cohort (M1-M11) and from non-exposed Melbourne residents (Control). Assays were performed twice independently; bars represent mean and range of samples tested in duplicate.

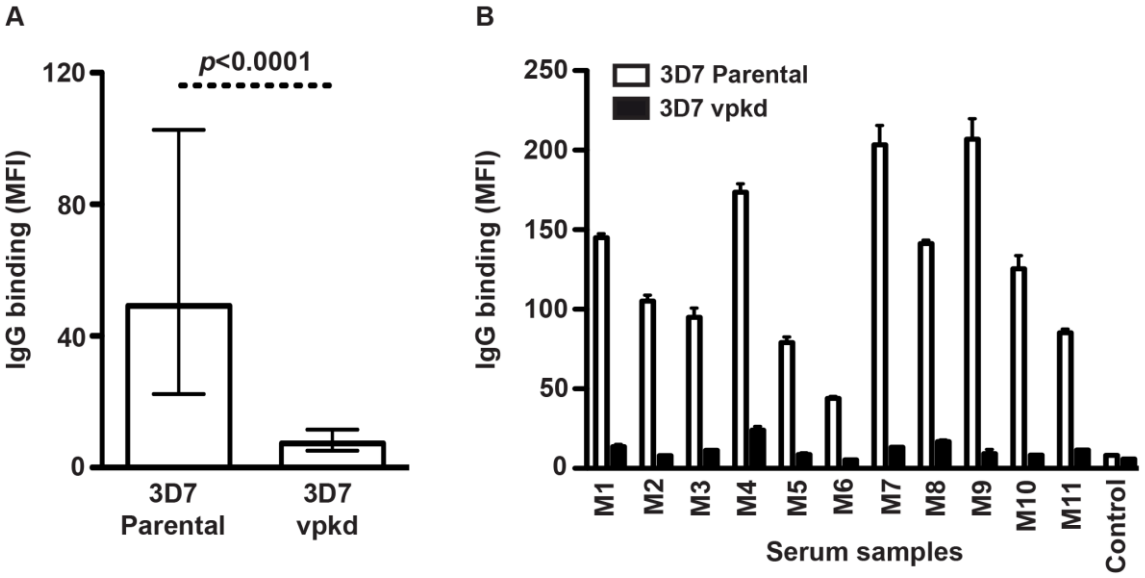

Figure 2 PfEMP1-specific antibodies mediate the opsonic phagocytosis of IEs

The level of opsonic phagocytosis activity with IEs from 3D7 parental and 3D7vpkd in serum from young children (**A**) and in serum from older children and adults (**C**). Data represent results from a single assay (n=19 for young children, n=11 for older children, n=9 for adults); bars represent median and interquartile ranges of samples tested in duplicate; *p* values were calculated using a paired Wilcoxon signed rank test; the level of phagocytosis is expressed as a percentage of the positive control (rabbit anti-human erythrocytes).

A representative selection of samples from young children (B) and older children and adults (D) tested for opsonic phagocytosis activity to 3D7 parental and 3D7vpkd parasites. Samples were from individuals residing in PNG and from non-exposed Melbourne residents (Control). Assays were performed once independently; bars represent mean and range of samples tested in duplicate.

Correlations between the level of opsonic phagocytosis activity and total IgG binding to the IE surface of (E) 3D7 parental parasites and (F) 3D7vpkd parasites. Correlations were evaluated in samples from older children, using Spearman's rho (r_s).

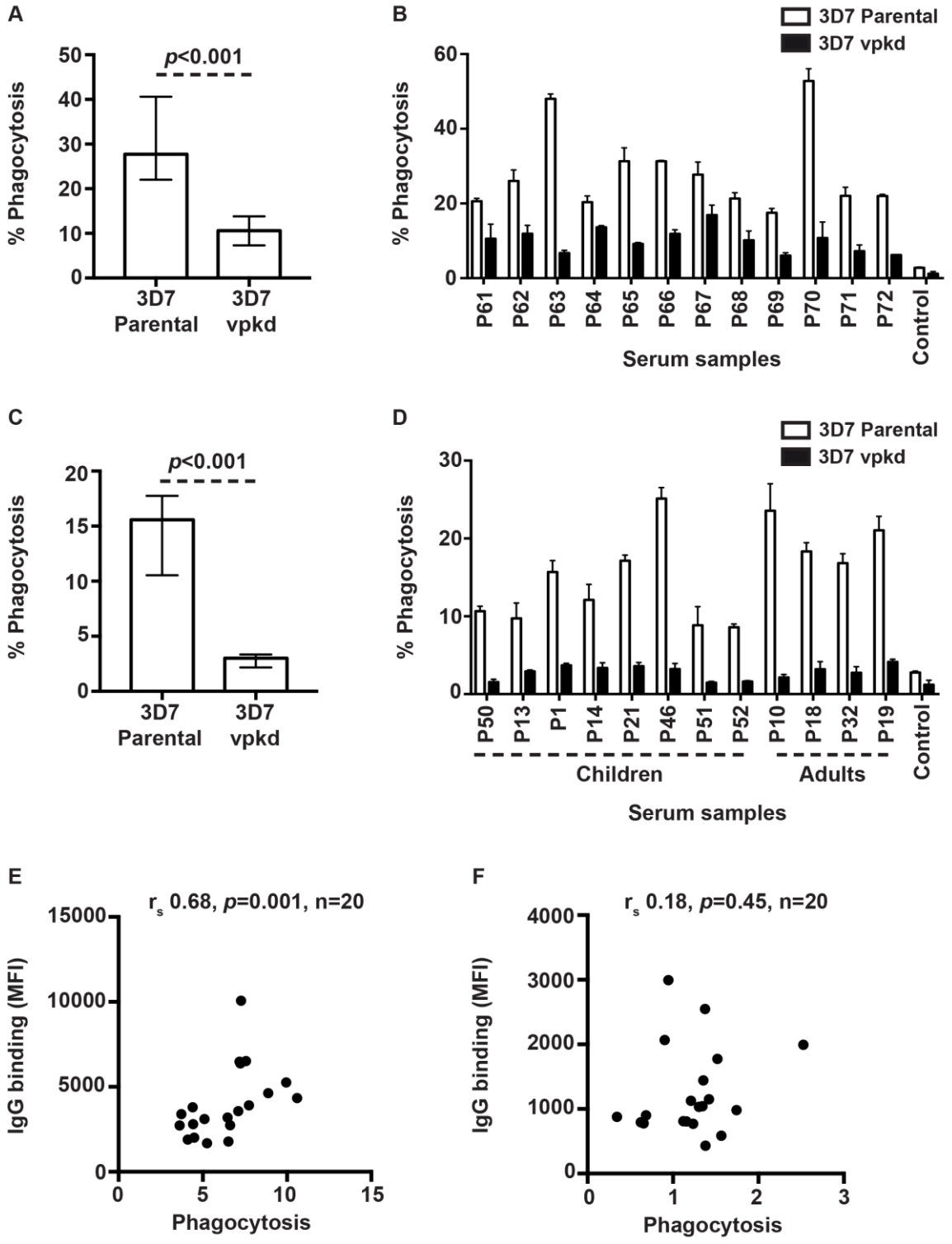

Figure 3 Antibodies to PfEMP1 are associated with a reduced risk of malaria

Kaplan-Meier survival curves show the proportion of children who remained free of malaria episodes over time, for IgG responses to IE surface antigens of (A) 3D7 parental parasites, (B) 3D7vpkd parasites and (C) 3D7-PfEMP1 (defined as IgG to 3D7 parental minus 3D7vpkd). Antibody responses were divided into three equal response groups and are presented as high and medium (merged, black line) versus low (grey line). Hazard ratios (HR) were calculated using Cox regression; 95% confidence intervals are presented in square brackets; data adjusted for the predetermined confounders of age and location of residence are shown.

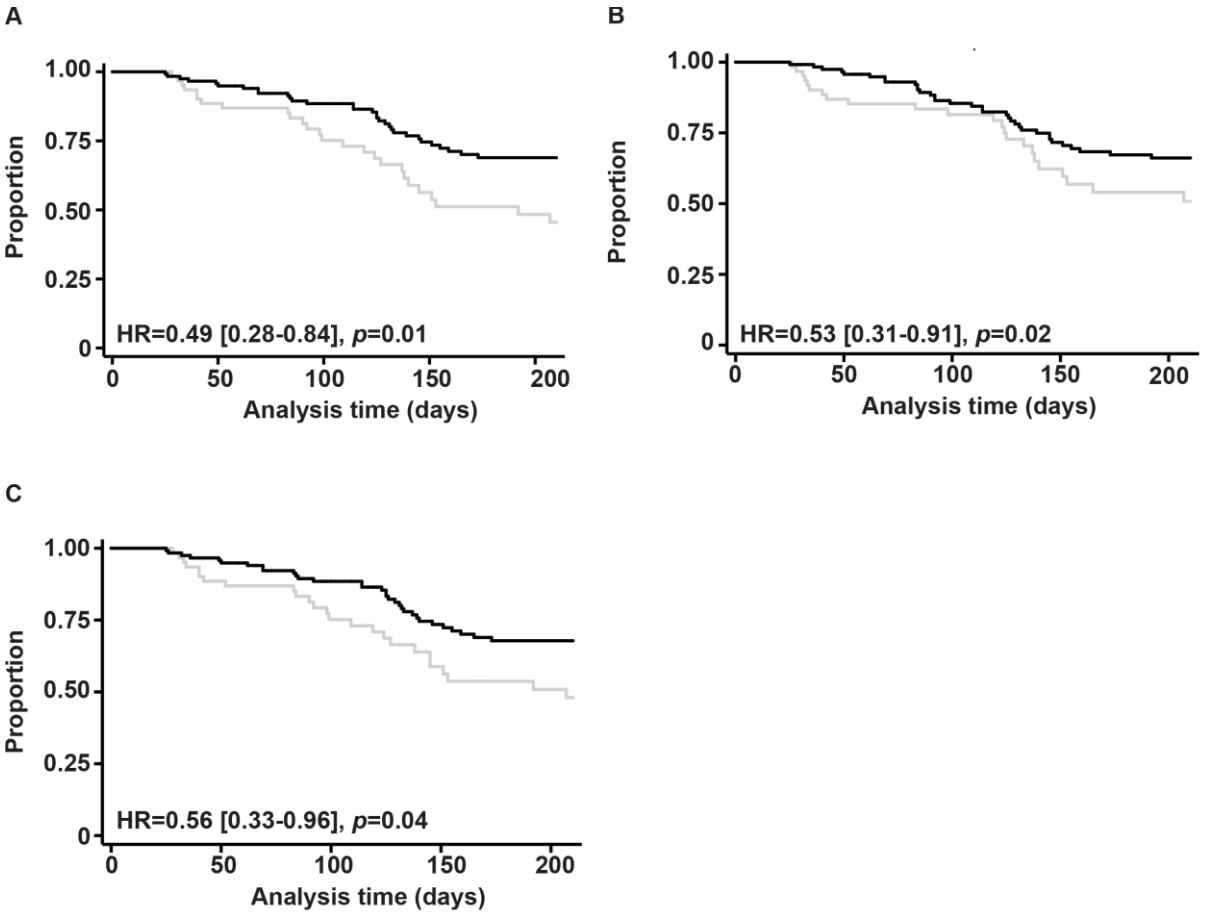

Figure 4 Association between antibodies to *P. falciparum* IE surface antigens and age, infection status and force of infection in the IIaita cohort

(A) IgG levels to IE surface antigens in relation to age and *P. falciparum* infection status as determined by PCR. Children were divided into 4 age groups to examine associations with age (n=183 children, each sample tested once [37]); *p* values were calculated using the Kruskal-Wallis test.

(B) IgG levels to IE surface antigens in relation to $_{mol}$FOI and *P. falciparum* infection status as determined by PCR. Children were divided into 3 groups (low, medium and high $_{mol}$FOI levels) to examine associations between antibody levels and $_{mol}$FOI as a marker of exposure (n=183 children; samples for PCR were collected every 2-months for 15 months; each sample was tested once in PCR for parasite detection and once for genotype [38]). For both graphs, IgG binding levels are expressed as geometric mean fluorescence intensity (MFI) and data are presented as box and whisker plots (boxes represent medians and interquartile ranges; error bars represent 95% confidence intervals).