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Acquisition of functional antibodies that block the binding of erythrocyte binding antigen 175 and protection against *Plasmodium falciparum* malaria in children

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A 40-word summary of the article's main point.

Antibodies that inhibit binding of the merozoite invasion ligand EBA-175 are acquired by children exposed to *P. falciparum* and associated with protection from malaria. This identifies an important target and mechanism of antibody-mediated immunity to malaria, relevant to vaccine development.

Abbreviations

BIA: Binding Inhibition Assay

DBL: Duffy Binding Like

EBA: Erythrocyte Binding Antigen

HR: Hazard Ratio

MFI: Mean Fluorescence Intensity

PNG: Papua New Guinea.

RII: Region II

RT: Room Temperature

Abstract

Background: The targets and mechanisms of human immunity to malaria are poorly understood, which poses a major barrier to malaria vaccine development. Antibodies play a key role in human immunity and may act by inhibiting receptor-binding functions of key merozoite invasion ligands. Antibodies to the major invasion ligand and vaccine candidate, EBA-175, have been linked with protection, but how these antibodies function has not been established.

Methods: We developed two new assays that quantify the ability of antibodies to inhibit binding of EBA-175 to its erythrocyte receptor, glycophorin A, using either native or recombinant EBA-175. Binding-inhibitory antibodies were evaluated in a longitudinal cohort study of Papua New Guinean children and related to risk of malaria, age, infection status, and markers of parasite exposure.

Results: Binding-inhibitory assays were reproducible and the two assays had a high level of agreement. Inhibitory antibodies were common among children and were acquired in association with markers of increasing parasite exposure and were high in those children with active infection. Inhibitory antibodies correlated with total IgG levels to the EBA-175 binding domain (region II). Importantly, binding-inhibitory antibodies were significantly associated with protection from symptomatic malaria when measured using either binding inhibition assay.

Conclusion: Findings suggest that naturally-acquired binding-inhibitory antibodies are an important functional mechanism that contributes to protection against malaria and further supports the potential of EBA-175 as a vaccine candidate. Identifying vaccines and approaches that induce potent binding-inhibitory antibodies may be a valuable strategy in the development of highly efficacious malaria vaccines.

Introduction

Decreasing the burden of malaria and protecting susceptible populations remains a global health priority; an effective vaccine would greatly advance this goal [1]. The blood-stage of the parasite lifecycle causes symptomatic malaria, hence malaria vaccine development has had a strong focus on blood-stage vaccine candidates to prevent clinical illness and death [2]. Protective immunity develops after repeated natural exposure, and antibodies play a key role in immunity [3, 4]. This provides a strong rationale that the development of effective blood-stage vaccines are achievable and defining protective targets and molecular mechanisms of acquired immunity is valuable [5]. Acquired human immunity appears to act by controlling and inhibiting replication of blood-stage parasites, preventing the development of high-density parasitaemias and symptomatic illness [2, 3]. Merozoites are important targets of acquired antibodies and these antibodies probably act in part by inhibiting erythrocyte invasion, opsonizing merozoites for phagocytosis, and fixing complement [2, 6-8]. However, the specific antigenic targets and protective effector mechanisms for an effective blood-stage vaccine remain elusive.

Currently, there are a lack of established functional assays that assess molecular interactions between antibodies and specific merozoite antigens, which has limited identification of targets of protective antibodies. Growth inhibition assays are commonly used, but do not reliably predict protective immunity [9], are usually not antigen specific, and measure the cumulative effect of antibodies on the entire growth cycle without specifying whether antibodies inhibit merozoite invasion, schizont development or rupture [5]. There is a strong need for antigen-specific functional assays that determine antibody activity at a molecular level.

We hypothesized that antibodies that inhibit the receptor-binding function of key merozoite invasion ligands would contribute to protective immunity by limiting parasite replication. Erythrocyte-binding antigen (EBA)-175 is a major invasion ligand and binds to the prominent erythrocyte molecule, glycophorin A. EBA-175 is a good model antigen to study binding-inhibitory antibodies as it is one of few antigens that has a defined role in invasion and a known binding receptor [10, 11]. Human antibodies to EBA-175 have been associated with protective immunity [12, 13] and can inhibit parasite invasion [14, 15] and the inhibit binding of erythrocytes to COS-7 cells expressing EBA-175 [16]. EBA-175 is a leading vaccine candidate that was recently evaluated in phase I trials [17], which demonstrated vaccine safety and immunogenicity, and the induction of *in vitro* growth inhibitory activity [17]. Understanding the role of binding inhibitory antibodies to EBA-175 is necessary to understand its potential as a vaccine candidate. Evaluating binding-inhibitory antibodies for EBA-175 would allow similar approaches for its paralogues, EBA-140, EBA-181, EBL-1 [18], as well as other antigens that have receptor-ligand interactions. EBA-175 is a type-1 transmembrane protein with six extra-cellular regions [19]. Region II (RII) is the functional binding domain that comprises two cysteine-rich DBL domains (F1 and F2) [10]. During invasion, EBA-175 is released from micronemes [20] and RII binds glycophorin A on the erythrocyte to stimulate rhoptry protein release and tight junction formation [21]. EBA-175 is then cleaved from the merozoite surface by *Pf*ROM4 and released into the intravascular space; cleavage is thought to be important to enable other interactions and subsequent merozoite entry into the erythrocyte [22]. The function of region III-V (RIII-V) remains unknown, although antibodies to it can inhibit invasion [23].

We aimed to study functional binding-inhibitory antibodies to EBA-175, to quantify these responses in populations acquiring immunity, investigate the potential role of these antibodies in protective immunity, and evaluate assays that could be applied to EBA-175 vaccine trials.

To achieve this, we developed two binding-inhibition assays and applied these in a longitudinal cohort of children in Papua New Guinea (PNG).

Methods

Details of human samples

Plasma samples were obtained from a treatment re-infection cohort study involving 206 malaria exposed children from PNG (5-14 years of age), described previously [24]. At enrolment, demographic details were collected and venous blood samples were drawn. All children were treated with artesunate for 7 days to clear parasitaemia. They were actively followed up fortnightly for 6 months to determine episodes of asymptomatic reinfection and symptomatic malaria. During this time, 95.3% of participants became reinfected with *P. falciparum* and 39.3% developed clinical disease (fever >37°C and *P. falciparum* parasitaemia >5000/µl).

Plasma from enrolment was tested using both BIAs for binding inhibition and a standard ELISA protocol [25] for IgG to schizont lysate [12, 26] and EBA-175 Region II (1µg/ml in PBS). Six months of prospective clinical follow up was used to test for associations with protection. Samples from PNG adults were used as positive controls. Negative control samples were obtained from malaria naïve Australian blood donors.

Ethics approval

Ethics approval was obtained from PNG Institute of Medical Research Institute (Goroka) and Alfred Hospital Human Research Ethics Committee. Informed consent was obtained from subjects and their guardians.

Native binding-inhibition assay

In brief, erythrocytes (0.4% haematocrit) were incubated with parasite culture supernatant at room temperature (30 min RT). EBA-175 binding was detected using polyclonal EBA-175 Region III-V rabbit Ab (1/1000; 30 min RT), followed by anti-rabbit Alexa-488-conjugated Ab (1/1000; 30 min RT, Invitrogen). Mean Fluorescence Intensity (MFI) was measuring on the FACSCalibur (BD). For binding inhibition, plasma (1/500) was incubated with the parasite supernatant prior to the binding step (30 min; RT). Further details in Supplementary Methods.

Recombinant binding-inhibition assay

In brief, native glycophorin A (8µg/ml) was adsorbed onto F96 Maxisorp plates (overnight; 4^{o}C; Nunc), then blocked (1% w/v BSA; 2 hours RT). Recombinant EBA-175 Region II was incubated to allow binding (2µg/ml; 2 hours RT) and this binding was detected using polyclonal EBA-175 Region II rabbit sera (1/1000; 2 hours RT) [27], anti-rabbit HRP-conjugated Ab (1/500; 2 hours RT, Millipore), and ABTS liquid substrate (1 hour RT, Sigma). Optical density was measured at 405 nm. For binding inhibition, plasma (1/20) was incubated with EBA-175 RII prior to the binding step (30 min; RT). Further details in Supplementary Methods.

Statistical analysis

Individuals with binding-inhibitory antibodies were defined as those with binding responses lower than 3 standard deviations of the mean binding in the presence of malaria-naïve controls (n=12). BIA responses were not normally distributed; therefore non-parametric statistical analyses were performed using StataSE 11 (StataCorp) and Prism 6 (GraphPad) software (details in Supplementary Methods).

Results

Development of quantitative EBA-175 binding-inhibition assays

To investigate the acquisition of EBA-175 binding-inhibitory antibodies and their potential role in immunity, we developed a quantitative BIA using native EBA-175 protein and intact human erythrocytes (Figure S1). Supernatants from *in vitro* parasite cultures were collected as the source of native EBA-175, and parasites lacking EBA-175 (3D7ΔEBA-175) were used as a control. This new assay used flow cytometry to demonstrate the binding of native EBA-175 protein to the erythrocyte surface (Figure 1A). Binding of EBA-175 was further confirmed by western blotting of proteins eluted from the surface of erythrocytes (Figure 1B). Specificity of binding was demonstrated by absence of binding with supernatants from 3D7ΔEBA-175 parasites (Figure 1A and B) and by the expected pattern of binding-inhibition following enzyme treatment of erythrocytes to cleave surface receptors; there was substantially reduced binding with neuraminidase or trypsin, but not chymotrypsin (Figure 1C) [10, 28]. A range of conditions were explored to optimise the assay, including concentration of parasite proteins, haematocrit (data not shown), and antibodies for detection of bound EBA-175 (Figure S2). Human plasma antibodies were then tested for inhibition of EBA-175 binding across a range of concentrations. Samples from malaria-naïve adults did not inhibit EBA-175 binding, whereas samples from malaria-exposed PNG adults effectively inhibited binding in a concentration-dependent manner (Figure 1D).

While the native EBA-175 BIA with erythrocytes best represents *in vivo* binding, a cell-free BIA would be ideal for application to large clinical studies and vaccine trials and allow standardization of reagents. Therefore, a second assay was developed and optimised using recombinant EBA-175 RII and immobilized glycophorin A, in a 96-well ELISA-based format (Figure S1C). Conditions were explored to optimise the assay for application to clinical studies (Figure S3A-E), and we confirmed binding of recombinant EBA-175 RII to

erythrocytes (Figure S3F). Specificity of binding was demonstrated by the absence of binding following enzyme treatment of glycophorin A with neuraminidase, and other negative controls (Figure 2A). Following optimization of assay conditions (Supplementary Methods), human antibodies were tested for their ability to inhibit EBA-175 RII binding. As seen with the native BIA, samples from malaria-exposed PNG adults effectively inhibited binding in a concentration-dependent manner, whereas samples from malaria-naïve adults did not (Figure 2B). Both BIAs showed low intra-assay and inter-assay variability (Figure 2C)[29]. Individuals responded similarly in BIAs with native (full-length) and recombinant (RII only) EBA-175 suggesting that both assays could be used to evaluate EBA-175 binding-inhibition. We confirmed that purified IgG from PNG donors, but not malaria-naïve Melbourne residents, are able to inhibit EBA-175 binding (Figure S4).

PNG children develop binding-inhibitory antibodies to EBA-175

The prevalence of EBA-175 binding inhibitory antibodies was assessed in a longitudinal prospective cohort of 206 PNG children. Plasma at enrolment was tested in both BIAs and showed a wide range of responses (Figure S5). The majority of individuals tested in native (67%) and recombinant (59%) BIAs had binding-inhibitory antibodies (Table 1). When responses of each assay were compared, there was a high level of agreement (Table 1; 86.0% agreement, Kappa = 0.7017, $p<0.0001$) and a significant correlation in levels of inhibitory activity (Table 1; Spearman's rho = 0.7122, $p<0.0001$).

EBA-175 binding-inhibition by antibodies was strongly related to EBA-175 IgG levels (measured to the RII binding region by ELISA); inhibition was highest among EBA-175 IgG high responders (defined as the upper tertile of responses), and lowest among the low responder group (Figure 3). This is also reflected in the strong correlation between EBA-175

binding inhibition and IgG to EBA-175 RII (native BIA: Spearman's rho = -0.853, p<0.0001; recombinant BIA: Spearman's rho = -0.704, p<0.0001).

Relationship between EBA-175 binding inhibitory antibodies and age, infection and exposure

The magnitude of EBA-175 binding inhibition was assessed relative to markers of the acquisition of immunity: age, concurrent parasitaemia, and antibodies to schizont protein extract (widely used as a broad marker of antibodies to blood-stage antigens, reflecting cumulative exposure and recent infection)[12, 30]. There was no significant association with age using a dichotomous variable of older versus young children (Figure 4A) or with age as a continuous variable (native BIA: Spearman's rho = - 0.07, p= 0.3216; recombinant BIA Spearman's rho = 0.01, p= 0.9258). At enrolment, 67.5% of children were positive for *P. falciparum* infection by PCR [24]. EBA-175 binding inhibition was significantly higher in the parasitaemic group (Figure 4B) suggesting that inhibitory antibodies were boosted or induced by active infection. This trend was also observed when stratified by age (Figure S6). EBA-175 binding inhibition was also significantly higher among children who were high responders to schizont protein extract (Figure 4C). Taken together, previous and active exposure appeared to contribute to the development of EBA-175 binding inhibitory antibodies, but it is not closely linked with children's age.

EBA-175 binding inhibitory antibodies were associated with protection from symptomatic malaria

The design and longitudinal structure of the cohort study combined treatment to clear parasitaemia at enrolment with 6 months of active follow-up to detect reinfection and clinical malaria cases (Figure 5A). This design enabled us to prospectively assess the relationship between EBA-175 binding-inhibitory antibodies and symptomatic malaria, and test the hypothesis that binding-inhibitory antibodies may contribute to protective immunity or act as

valuable biomarkers of such immunity. Children with EBA-175 binding inhibitory antibodies had a significantly reduced risk of developing symptomatic malaria compared to children without EBA-175 binding inhibitory antibodies (Figure 5B-D), and this association was found when using results from the native and recombinant BIAs (native BIA: unadjusted hazard ratio (HR) = 0.52, 95%CI [0.32, 0.85], p=0.009; recombinant BIA: unadjusted HR = 0.52, 95%CI [0.32, 0.85], p=0.008). Previous analyses in this cohort identified age and residential location as potential confounders [12]. Adjusting for these factors had a minimal effect on the hazard ratios and results remained statistically significant (Figure 5B). Other studies have reported that parasitaemia status at baseline can affect antibody levels and malaria risk [31]. While parasitaemic individuals in this study had higher antibody levels at enrolment, parasitaemia itself was not associated with malaria risk and is therefore not a significant confounder; adjusting analyses for parasitaemia at baseline did not substantially affect HRs (Table S1). Individuals who were positive for inhibition in both BIA assays had a significantly reduced risk of malaria compared to those who were negative in both assays (Table S2).

Discussion

Merozoite antigens are important targets of acquired immunity and have significant potential as vaccine candidates. However, there remain substantial challenges in identifying targets and mechanisms of protective antibody responses to guide vaccine development. There are currently no established antigen-specific functional assays that have been shown to correlate with protective immunity and could be used for vaccine evaluation [5]. In this study, we have developed assays to measure binding-inhibitory antibodies to the major merozoite invasion ligand and vaccine candidate, EBA-175. We show that binding-inhibitory antibodies to EBA-175 are acquired by children following exposure to malaria, and appear to be boosted

by active infection. Importantly, we demonstrate for the first time that binding-inhibitory antibodies are associated with protection from malaria in children.

To evaluate binding-inhibitory antibodies, we first developed an assay that would best represent physiological conditions using native EBA-175 and whole human erythrocytes. Quantifying binding by flow cytometry was a significant advantage over established western blot approaches [28, 32, 33] especially for assessing large numbers of clinical samples (using <3 µl of plasma for each sample). The use of native EBA-175 enabled the entire ectodomain of the antigen to be used, increasing confidence in achieving correct conformational structure of the protein *in vitro*. This is important because until recently it has not been possible to recombinantly express full length EBA-175 mostly because of its large size (approximately 1500 amino acids) [34]. A further benefit of using full length native EBA-175 interacting with the dynamic bi-lipid membrane of the erythrocyte surface was to increase the ability to assess responses that inhibit more complex binding interactions, such as the predicted dimerisation of EBA-175 with glycophorin A [35, 36]. Additionally, the recent finding that recombinant full-length EBA-175 binds glycophorin A with a ten-fold higher affinity than recombinant RII alone highlights the importance of developing two assays to compare inhibition between native (full length) and recombinant (RII) binding [34]. We developed our second assay using an ELISA-based approach with recombinant EBA-175 to facilitate standardization and quality control for application across future clinical studies and vaccine trials. The EBA-175 RII construct was recently used in phase I vaccine trials; it should be possible to adapt this assay to use full-length recombinant protein that has recently been expressed [34].

Both binding-inhibition assays performed well with a high level of precision, and good agreement between the assays. The correlation between the assay results suggests that most of the binding inhibitory activity is mediated by antibodies targeting RII epitopes.

Results suggest that the recombinant BIA accurately predicts inhibition of native protein binding and would be sufficient for use in future clinical studies and vaccine trials. It is likely that the ability of human antibodies to inhibit EBA-175 is due to either direct targeting of epitopes required for glycophorin A binding, epitopes that enable dimer formation, or epitopes outside of these regions but close enough that steric hindrance of the antibody effectively blocks these interactions [37]. We also observed a strong correlation between total IgG EBA-175 RII responses (by standard ELISA) and binding-inhibitory activity. This suggests that there is an antibody concentration-dependent effect with binding inhibition, highlighting the importance of maintaining (or rapidly boosting) a memory response to obtain a high concentration of EBA-175 antibodies to mediate a binding inhibitory effect to contribute to immunity. Further studies of these responses are needed to determine whether antibodies to EBA-175 measured by standard ELISA are a good surrogate measure of inhibitory activity.

This study is the first to demonstrate an association between binding-inhibitory antibodies to EBA-175 and protection from clinical malaria. The longitudinal study design was used to prospectively determine the relationship between antibodies and subsequent clinical illness [24]. Significant associations with protection remained after adjusting for potential confounders. It seems likely that binding-inhibitory antibodies act to limit *P. falciparum* invasion of erythrocytes, and blood-stage replication, thereby preventing clinical illness. The functional activity of the antibodies and their prospective association with protection provides strong evidence for a role for antibodies to EBA-175 in protective immunity, as previously proposed [12]. We believe these findings are particularly significant because there are very few functional antibody responses that have been associated with protective immunity in children, and there is a lack of antigen-specific functional assays that are predictive of immunity. Although EBA-175 was the focus of this study, the approaches in

this study also provide a basis to evaluate antibody-mediated binding inhibition for other merozoite antigens and advance the identification of key targets of protective human immunity. A smaller previous study examined EBA-175 binding-inhibitory antibodies in a longitudinal study of adults (n=81) in a high transmission area of Kenya where levels of immunity in adults are high [16]. Their assay assessed the ability of antibodies to inhibit binding of erythrocytes to COS-7 cells expressing EBA-175 RII. No association was found between symptomatic parasitaemia and antibodies, but higher levels of binding-inhibitory antibodies were seen in adults who did not develop parasitaemia compared to those who did. Our study differs significantly from this in that we evaluated immunity in children, not in adults. We also used a parasitaemia density threshold for the diagnosis of malaria (as used by others [38, 39]) and PCR-based detection of parasitaemia, neither of which was performed by the prior study.

In conclusion, our findings provide important evidence that EBA-175 binding-inhibition by antibodies contributes to acquired human immunity and further supports the potential of EBA-175 as a vaccine candidate. Assays developed here have an obvious application in vaccine development of EBA-175 and possibly other antigens. Identifying vaccines and approaches that induce potent binding-inhibitory antibodies may be a valuable strategy in the development of highly efficacious malaria vaccines.

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Conflict of interest

The authors have declared that no competing interests exist.

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Figure legends

Figure 1. Binding of native EBA-175 to erythrocytes and inhibition of binding by human antibodies. The degree and specificity of native EBA-175 binding to the surface of intact human erythrocytes was assessed by flow cytometry and western blot. (A) Native EBA-175 from the 3D7 wild-type parasite supernatant (solid line) and 3D7ΔEBA-175 negative controls (dashed line) were assessed with higher fluorescence (x axis) indicating higher EBA-175 binding. (B) Western blots were used to confirm the presence or absence of native EBA-175 from parasite culture supernatants using 3D7 wild-type (Lane 1) and 3D7ΔEBA-175 (Lane 2), respectively. The ability of EBA-175 to bind to human erythrocytes was then assessed by co-incubating the parasite supernatants with human erythrocytes, eluting off bound EBA-175 and detecting the presence of EBA-175 by western blot using a rabbit anti-EBA-175 polyclonal antibody. Binding of EBA-175 was detected with a 175 kDa band when using 3D7wt supernatant (Lane 3) but not with 3D7ΔEBA-175 supernatant (Lane 4). A faint spectrin band can be seen at 250 kDa. (C) The specificity of EBA-175 binding to erythrocytes was assessed following enzyme treatment of the erythrocytes with neuraminidase, trypsin, or chymotrypsin. These enzymes differentially cleave off sialic acids and glycophorin A. Culture supernatants from 3D7 wild-type (black bars) and 3D7ΔEBA-175 (white bars) parasites were used to detect the mean fluorescence intensity (MFI, y axis) of EBA-175 binding to human erythrocytes using the flow cytometry assay described earlier. Error bars show range for samples tested in duplicate. (D) Human sera samples were used to assess the ability to inhibit the binding of EBA-175 to human erythrocytes in the flow cytometry assay described earlier with binding inhibitory samples yielding a low MFI (y axis). Representative samples included adults resident in malaria-endemic Papua New Guinea

(PNG; n=2), and samples from malaria-naïve blood donors resident in Melbourne, Australia (Melb; n=2). The line graph shows a titration of sera (x axis) tested singly.

Figure 2. Binding of recombinant EBA-175 to glycophorin A and inhibition of binding by human antibodies. The degree and specificity of recombinant EBA-175 (Region II) binding to glycophorin A was assessed in an ELISA-like assay that measures the degree of EBA-175 binding with an anti-EBA-175 rabbit polyclonal antibody with optical density (OD) measured at 405 nm. (A) The specificity of recombinant EBA-175 binding to glycophorin A (Gly A) was assessed following neuraminidase treatment and compared to other negative controls (no glycophorin A, no EBA-175, no rabbit antibody for detection). Error bars show range for samples tested in duplicate. (B) Human sera samples were used to assess the ability to inhibit the binding of EBA-175 to glycophorin A in the recombinant binding assay described earlier with binding inhibitory samples yielding a low OD (y axis). Representative samples included adults resident in malaria-endemic Papua New Guinea (PNG; n=2), and samples from malaria-naïve blood donors resident in Melbourne, Australia (Melb; n=2). The line graph shows a titration of sera (x axis) tested singly. (C) Intra-assay variability (within plate) and inter-assay variability (across plates) for both the native binding inhibition assay (BIA) and recombinant BIA are shown as the average co-efficient of variation (CV) for inhibitory and non-inhibitory samples.

Figure 3. Binding inhibitory responses of PNG children and relationship with IgG to EBA-175 RII. Plasma samples from a cohort of Papua New Guinean children were used to determine the relationship between EBA-175 binding inhibition and total IgG response to EBA-175 Region II as determined by ELISA. The continuous values for IgG responses were used to divide the cohort into 3 equal categories reflecting low, medium and high EBA-175 Region II IgG responders (x axis). This relationship between these antibody levels and absolute binding inhibition was assessed using the (A) native binding inhibition assay (BIA)

and (B) recombinant BIA. Greater degrees of binding inhibition were indicated by low mean fluorescent intensity (MFI) and low optical density (OD) respectively. Differences across all tertiles were first tested using a Kruskal-Wallis test (p=0.0001) and further differences between groups (low, intermediate or high) were tested using a Wilcoxon-Rank Sum test (p<0.0001 for all tests). Bar graphs indicate the median binding activity and whiskers indicate the interquartile range.

Figure 4. Binding inhibitory responses of PNG children and relationship with age, parasitaemia, and schizont protein extract IgG levels. Plasma samples from a cohort of Papua New Guinean children were used to determine the relationship between EBA-175 binding inhibition and (A) age, (B) concurrent parasitaemia at the time of sample collection determined by PCR, and (C) IgG reactivity to schizont protein extract as a broad marker of antibodies to blood-stage antigens, reflecting cumulative exposure and recent infection. This relationship between these antibody levels and absolute binding inhibition was assessed using the native binding inhibition assay (BIA) and recombinant BIA; top and bottom panels respectively. Greater degrees of binding inhibition were indicated by low mean fluorescent intensity (MFI) and low optical density (OD), respectively. Difference between groups was tested using a Wilcoxon-Rank Sum test. Bar graphs indicate the median binding activity and whiskers indicate the interquartile range. There was a significant association between increasing age group and schizont extract reactivity ($p < 0.0001$); this relationship remained significant when analysis was stratified by parasitaemia status (parasitaemic p= 0.0026, n=67; and non-parasitaemic p= 0.0054, n=139).

Figure 5. Binding inhibitory responses of PNG children and risk of symptomatic malaria. (A) A longitudinal cohort of 206 PNG children was treated to clear parasitaemia after enrolment. Children were actively followed up for 6 months to detect reinfection. (B) Plasma from enrolment was tested in the native and recombinant binding inhibition assays

(BIA). 'Inhibitors' were defined as those with binding responses lower than three standard deviations of the malaria naïve control in each respective assay. Hazard ratios indicate the relationship between EBA-175 binding inhibitors and non-inhibitors and risk of symptomatic malaria over the 6 month active follow up period. Kaplan-Meier curves show the relationship between groups of inhibitors and non-inhibitors from the (C) native BIA and (D) recombinant BIA and the time to first case of symptomatic malaria (fever >37°C and *P. falciparum* parasitaemia >5000/µl). Differences between groups were tested using the Log rank test.

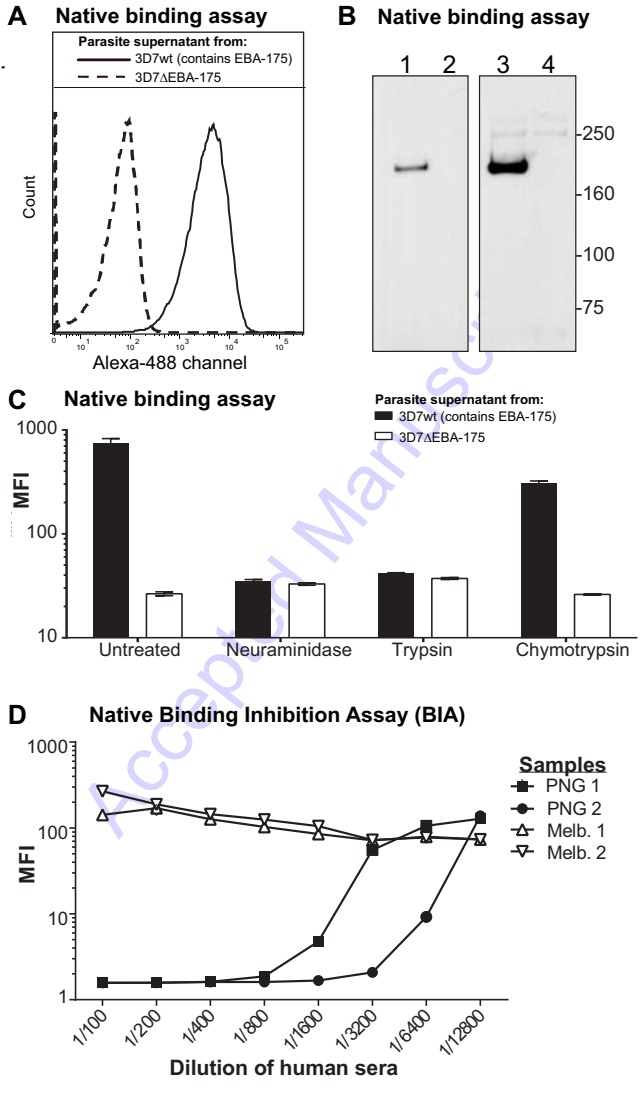
Table 1. Agreement between PNG cohort responses tested with native and recombinant BIA

		Native BIA		
		Inhibitors[#]	**Non-inhibitors**[#]	**Total** *(% of total)*
Recombinant BIA	**Inhibitors**	112	6	**118** *(59%)*
	Non-inhibitors	22	60	**82** *(41%)*
	Total *(% of total)*	**134** *(67%)*	**66** *(33%)*	**200*** *(100%)*

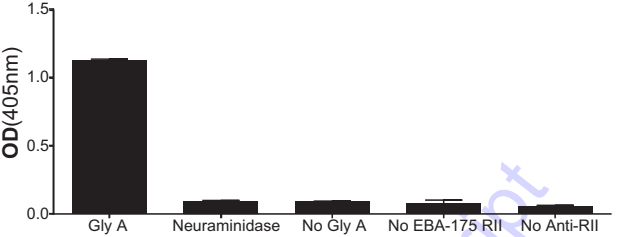
Cohen's Kappa test: Agreement: 86.0%; Kappa :0.7017; p<0.0001

[#]Inhibitors were defined as binding responses lower than three standard deviations of the malaria naïve control

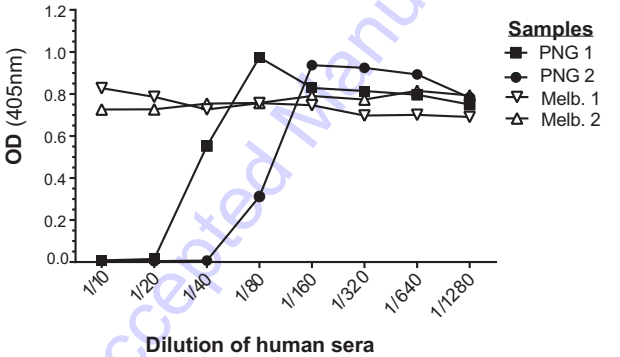
*The total cohort included 206 children. All 206 samples were tested in the Native BIA and one outlier was removed (Native BIA analysis n=205), however this sample was included in the recombinant BIA analysis (1 non-inhibitor). Only 201 samples were tested in the recombinant BIA as 5 samples were depleted, however these 5 samples were included in the Native BIA analysis (3 inhibitors and 2 non-inhibitors in native BIA). These 6 samples were not present in this table due to the nature of the analysis.



A **Recombinant binding assay**



B **Recombinant Binding Inhibition Assay (BIA)**

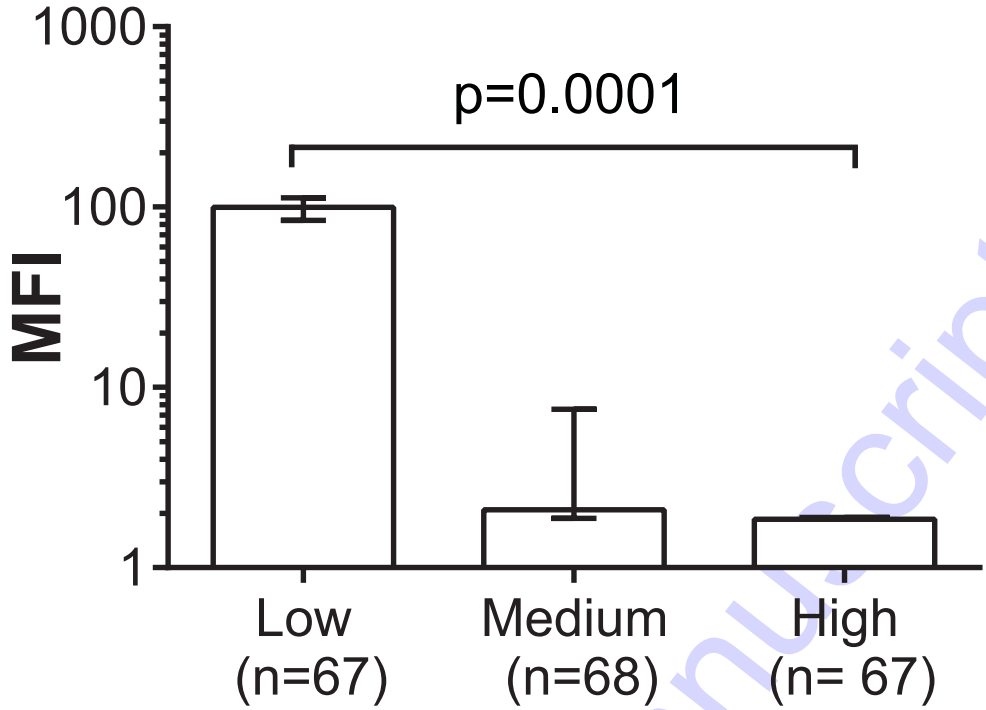


C **Intra and Inter-assay variation for the BIA**

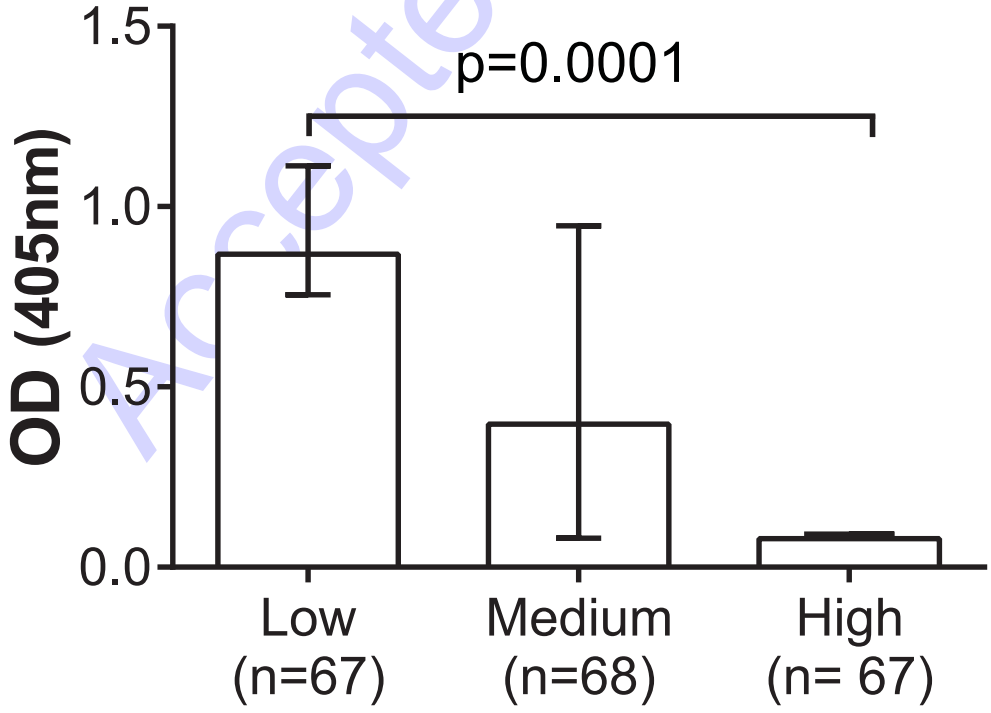
	Intra-assay variability (CV*)	Inter-assay variability (CV*)
Native BIA	5.4%	12.7%
Recombinant BIA	9.6%	9.7%

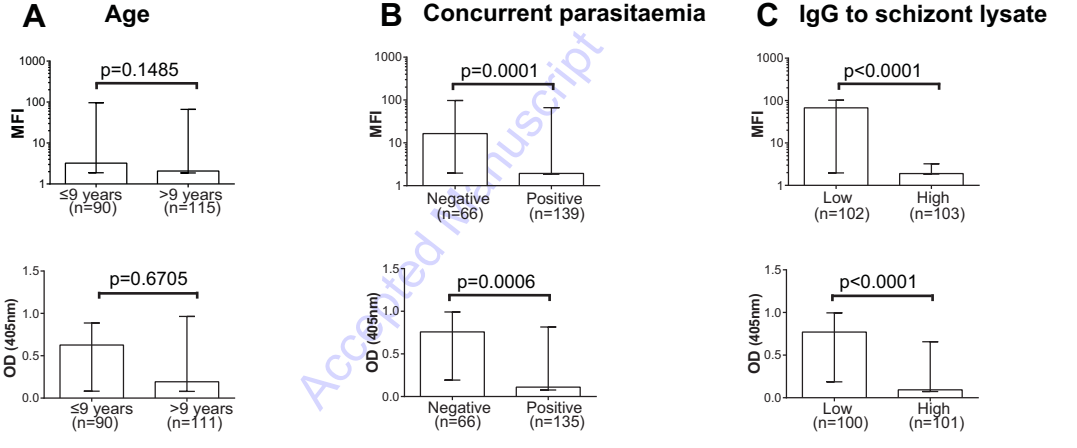
**CV : Coefficient of variation ((Standard Deviation/Mean)*100)*

A Native BIA: IgG to EBA-175 RII

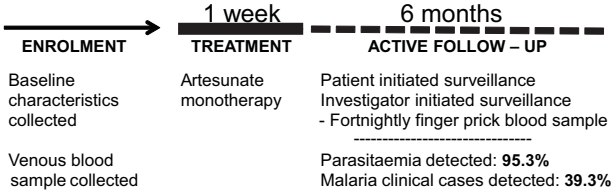


B Recomb. BIA: IgG to EBA-175 RII





A **Schematic of the PNG cohort**

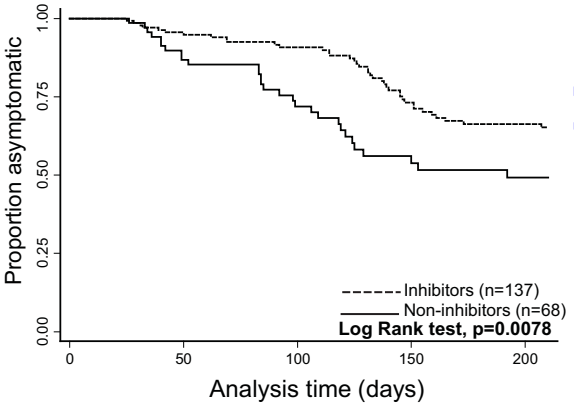


B **Binding inhibition and risk of clinical malaria**

	Unadjusted analysis		Adjusted* analysis	
	HR *(95% CI)*	*p*	HR *(95% CI)*	*p*
Native BIA *(n=205)*	0.52 *(0.32 - 0.85)*	0.009	0.60 *(0.37- 0.99)*	0.048
Recombinant BIA *(n=201)*	0.52 *(0.32 - 0.85)*	0.008	0.56 *(0.34 - 0.91)*	0.019

*Adjusted for age and distance from the sea.

C **Native BIA : Symptomatic malaria**



D **Recombinant BIA : Symptomatic malaria**