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High levels of serum antibodies to merozoite surface protein 2 of *Plasmodium falciparum* are associated with reduced risk of clinical malaria in coastal Kenya

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Abstract

The merozoite surface protein (MSP) 2 is a vaccine candidate antigen of *Plasmodium falciparum* that is polymorphic in natural populations. In a prospective cohort study in two coastal populations of Kenya using recombinant proteins derived from the two major allelic types of MSP2, high serum levels of IgG to MSP2 were associated with protection from clinical malaria. This protection was independent of that associated with antibodies to another vaccine candidate antigen (AMA1) in these populations. However, low antibody levels to MSP2 appeared to be associated with increased susceptibility to malaria within people who were parasite negative at the time of serum collection. These data suggest that an MSP2 based vaccine should be designed to induce high level antibody responses against the different MSP2 types present globally in *P. falciparum* populations and that MSP2 could be combined with other *P. falciparum* antigens to form a multi-component malaria vaccine.

Keywords: Kenya; Malaria; Epidemiology

1. Introduction

There is good evidence that antibodies against blood stages of *Plasmodium falciparum* offer protection from malaria in naturally exposed human populations, given that parasitaemias in *P. falciparum* infected non-immune individuals are greatly reduced by the passive transfer of purified immunoglobulin G (IgG) from semi-immune adults [1,2]. The challenge facing vaccine designers is to determine against which of the many *P. falciparum* antigens these protective antibodies are directed. In this quest, prospective cohort studies are useful to identify the association of human antibodies to specific *P. falciparum* antigens (present prior to malaria transmission) with a reduced incidence of clinical disease (during subsequent transmission). Such prospective cohort studies have included defined antigens representing different regions of the merozoite surface protein 1 (MSP1) [3–7], merozoite surface protein 2 (MSP2) [8,9,10], RESA [8], Apical Membrane Antigen 1 (AMA1) [11], as well as crude whole parasite (schizont) extract [12].

The MSP2 protein is located on the surface of the merozoite. A central region of the protein is highly polymorphic with sequences containing a series of variable repeat motifs. The polymorphic region is flanked by dimorphic sequences of one or the other of two major types, either the IC1 or FC27 type (also known as type A and type B, respectively) [13,14].

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Studies using a range of recombinant antigens representing both major types of MSP2 have shown that human antibodies can recognise epitopes located on all proteins of a given type (type specific antibodies) or on only a subset of proteins within that type (subtype specific antibodies) [15–18]. If these type/subtype specific immune reactivities are protective then they may maintain allelic diversity in parasite populations due to frequency-dependent selection, and the frequency distribution of MSP2 alleles in populations appears to reflect this [19].

Analysis of human immunoglobulin G (IgG) against one of the two types of MSP2 in Papua New Guinea using a 3D7 (type A) recombinant protein showed an association with decreased incidence of clinical disease [8]. This association was shown to be independent of antibodies against the polymorphic central repeat motif. Two studies in The Gambia showed significant associations of anti-MSP2 IgG3 antibodies with reduced morbidity, measured using another type A recombinant protein [9] and also proteins of both allelic types [10]. Since a vaccine is needed in areas of different malaria endemicity and for different ethnic peoples throughout the world, it is important to determine whether anti-MSP2 antibodies are involved in acquired immunity in different populations. In addition, in order to determine the protective effects of anti MSP2 antibodies compared to antibodies against other *P. falciparum* surface accessible proteins then comparative analyses must be done within the same cohorts.

Here, we present a study in two Kenyan populations in which high levels of naturally acquired human antibodies to specific MSP2 antigens were significantly associated with decreased risk of malaria, although low but positive levels were apparently associated with increased risk in parasite negative individuals. The protection associated with antibodies to MSP2 was independent of that associated with antibodies to the Apical Membrane Antigen 1 (AMA1), another leading vaccine candidate and a target of protective antibodies in these populations [11]. These results suggest that MSP2 could form part of an effective *P. falciparum* vaccine in combination with other *P. falciparum* antigens such as AMA1 if high anti-MSP2 antibody levels can be induced.

2. Materials and methods

2.1. Study area and population

The study was undertaken in Chonyi and Ngerenya, two villages approximately 40 km apart in Kilifi district near the coast in eastern Kenya. Inhabitants of these villages (predominantly of the Mijikenda ethnic group) are exposed to biannual peaks of malaria transmission in November to December and May to July (with the latter generally being the most intense period of transmission). The entomological inoculation rate (EIR) is an estimate of the number of infectious mosquito bites a person receives in a given period, and is taken here to indicate exposure to *P. falciparum* infections. For Chonyi and Ngerenya the EIR has been calculated at 20–100 and 10 infectious bites per year, respectively, indicating a higher transmission rate of malaria amongst people in Chonyi than those in Ngerenya [20]. Whole blood samples were collected from 534 individuals from each location on October 2000. Individuals ranged in age from 7 weeks to 85 years in Chonyi and from 3 weeks to 85 years in Ngerenya. The two cohorts were followed for malaria weekly with active and passive case detection over 28 weeks, which included the lower of the two annual peaks of malaria transmission (November to January) as part of the mild malaria surveillance program. A malaria episode was defined as a febrile episode (axillary temperature >37.5 °C) together with a *P. falciparum* parasitaemia of greater than 2500 μl^{-1} blood, except for infants under one year of age when any *P. falciparum* parasitaemia plus fever was counted as malaria. These levels of parasitaemia have been shown to be the most sensitive measure for malaria case detection in these populations [20]. The presence of *P. falciparum* in the blood stream at the time of sample collection was determined by microscopic observation of thick blood smears.

2.2. Antigens

Serum IgG to two MSP2 recombinant antigens was assayed for all individuals. The two recombinant MSP2 antigens used represented residues 1–184 and 22–247 of the CH150/9 and Dd2 proteins, respectively [21]. CH150/9 is a type A MSP2 protein (EMBL accession number DQ059548), whilst Dd2 is a type B protein (EMBL accession number U91678). The proteins were expressed as GST fusion proteins using the pGEX-2T vector [22]. The GST protein on its own was also expressed from the pGEX-2T vector as a negative control antigen. IgG subclass reactivity to the two MSP2 proteins and four additional antigens were also studied for a limited number of sera. These additional antigens were: AMA1 Pf14-0 (a full length ectodomain from the FVO AMA1allele) [23], MSP1–19 (the C-terminal fragment from MSP1) [24] and MSP1 block 2 Palo Alto and MSP1 block 2 RO33 (two of three polymorphic types from the N terminal block 2 region of MSP1) [25]. These antigens were chosen because antibody reactivities against them have been shown to be highly skewed towards either IgG1 (AMA1 and MSP1–19) or IgG3 (MSP1 block 2).

2.3. Enzyme linked immunosorbent assay

Fifty nanograms of MSP2 CH150/9, MSP2 Dd2 and GST were used to coat individual wells of Dynex 4HBX plates (Dynex Technologies Inc.) in 100 μl carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.3). Schizont extract of cultured A4 strain of *P. falciparum* was coated onto plates in PBS following published methods [26]. Plates were incubated overnight at 4 °C, washed in PBS with 0.05% Tween 20, and blocked in the same buffer containing 1% skimmed

milk for 5 h at room temperature. Plates were washed again and 100 μl diluted human sera (sera were diluted 1/500 in blocking buffer) was incubated (in duplicate) overnight at 4 °C in antigen-coated wells. Sera were recovered from whole blood samples, which had been spun and stored in serum separator collectors. These coating concentrations and serum dilutions had been determined by chequer board titrations to give the best balance between maximum analytical sensitivity and maximal specificity [21]. The wells were washed, and then incubated with 100 μl of HRP-conjugated rabbit anti-human IgG (at 1/5000 in blocking buffer) (Dako Ltd.) before detection with *O*-phenylenediamine/H_2O_2 (Sigma). Optical density (OD) values were measured at 594 nm wavelength. Means were calculated for the duplicate assays of each serum-antigen combination. For each serum the mean OD value for the GST negative control antigen was subtracted from the mean OD value for each MSP2 antigen to correct for any background antibody binding to GST. Where the subtraction resulted in a residual OD value that was less than zero, the OD was changed to zero (an OD value of less than zero is biologically meaningless). Sera were scored as positive for serum IgG to an MSP2 antigen if the mean corrected OD value was higher than the mean corrected OD plus three standard deviations of sera from 20 malaria naïve UK donors.

IgG subclass specific ELISAs were performed with 96 sera that had tested positive for antibody to schizont extract, in order to determine the subclasses of IgG antibody to MSP2, MSP1 block 2, MSP1–19 and AMA1. The initial screening of sera with whole schizont extract ensured a higher probability of sera having detectable IgG subclass antibody to the different antigens. Antigens were coated at 50 ng per well for these subclass specific ELISAs that were performed exactly as above, except that human sera were diluted at 1/4000 for the AMA1 antigen (this has been shown to be the optimal dilution for signal strength and specificity with this antigen [11]). Subclass specific antibodies were HRP-conjugated polyclonal sheep antibodies specific for human IgG1, IgG2, IgG3 or IgG4 (The Binding Site, Birmingham, UK) and 100 μl was used of a 1/3000 dilution. These second antibodies were also incubated with plate bound purified human myeloma proteins IgG1, IgG2, IgG3 and IgG4 (coated at two fold dilutions from 10 to 0.0049 μg ml^{-1}) before detection with *O*-phenylenediamine/H_2O_2. The resulting standard curves were used to convert the raw OD readings for each serum IgG subclass—antigen combination into an estimate of antigen-specific antibody concentration. Although probably linearly related to the quantity of human myeloma protein in each dilution plated out, the exact quantity of human myeloma protein that bound to the plate well in each dilution was unknown and it could therefore be misleading to quote antibody levels in μg ml^{-1} serum. Therefore, the deduced μg antibody ml^{-1} serum reading was converted to an arbitrary standardised unit of concentration by dividing each reading for a given antigen by the highest single reading recorded for that antigen (out of all four subclasses). Thus, all readings were on a scale 0–1, comparable and linearly proportional to the concentration of antibody.

2.4. Analysis of the relative levels of type A and type B MSP2 alleles in infections in each population and their association with antibody levels

Given that antibodies to MSP2 can bind to type specific motifs, it was of interest to see if there was a difference in the relative frequencies of type A and type B MSP2 alleles in infections in the two villages. Such a difference could conceivably alter any association of type specific antibodies with protection. Cell pellets from October 2000 blood samples that were *P. falciparum* positive by microscopy were retrieved from serum separator tubes by inversion of the tubes and centrifugation. DNA was extracted using the Qiamp DNA mini kits (Qiagen, UK) for 107 and 74 such blood samples from Chonyi and Ngerenya, respectively. MSP2 specific DNA products were amplified by a nested PCR method to produce type A and type B specific products in separate tubes [27]. Products were visualised on 2% agarose gels and photographed using the VisonWorks software (Ultra-Violet Products Ltd., Cambridge, UK). Each photograph was scored by eye and the MSP2 allelic type of the dominant (brightest) and minority bands recorded for each sample. A Chi-squared analysis was carried out to assess differences in the relative proportions of the two MSP2 allelic types within infections at the two locations. Only the dominant allelic product for each PCR positive sample was included in the analysis. Where a single dominant allelic product could not be determined that sample was excluded from the analysis. A Chi-squared analysis was also carried out to look for differences in the frequency of mixed clone infections (where both type A and type B alleles were clearly present in a blood sample) to single clone infections (where alleles were only type A or type B) in the two villages.

An analysis was also performed to test whether the MSP2 allele(s) present in each blood sample were associated with the presence of appropriate type-specific anti-MSP2 antibody in that sample. The single dominant allelic MSP2 product in the 181 DNA samples from the two villages was typed and samples that could not have a dominant infection assigned to them were excluded as above. Antibody levels against type A MSP2 were compared between the sera samples from individuals with a clear dominant type A allelic infection and the sera samples from individuals with a clear dominant type B allelic infection using the Wilcoxon rank sum test. An identical analysis was performed for antibody levels against type B MSP2.

2.5. Analysis of association of anti-MSP2 antibody with protection

Antibody data (both OD values and antibody positivity data) were merged with clinical follow up data in Stata 8.2 (StataCorp, Texas, USA). The data was first tested using

univariate logistic regression for an association of antibody positivity with whether an individual presented with a clinical case of malaria. Clinical immunity to malaria and the presence of IgG to different malarial antigens are known to be associated with both age and previous exposure to malaria in endemic locations. In order to account for the confounding factors of age and previous exposure to malaria, multivariate analyses were performed in which the following variables were adjusted for: age (in years), the level of serum IgG to whole parasite (schizont extract) and a previous episode of malaria in the twelve months prior to serum collection. The first two were included as continuous variables, the last as a dichotomous variable.

Study of the same cohorts in Kilifi district have revealed that parasite slide positivity at the time of pre-transmission season serum collection had a significant effect on the association of pre-transmission season antibodies against the Apical Membrane Antigen 1 with protection from subsequent malarial morbidity [11]. In order to investigate whether the association of anti-MSP2 antibodies with protection was significantly affected by the presence of pre-transmission season parasites an interactions test was performed. An interaction variable was constructed for each MSP2 antigen where those individuals who were positive for both *P. falciparum* parasites and anti-MSP2 antibodies at the time of serum collection were scored as positive. This interaction variable was included as a co-variable along with the variables parasite positivity and positivity for anti-MSP2 antibody in an analysis to calculate the residual association with clinical malaria that was specific to each one. The resulting P value for the interaction variable represented the probability that the association of positivity for anti-MSP2 antibody with clinical malaria was significantly modified by the presence of circulating *P. falciparum* at the time of serum collection.

In addition to antibody positivity, the association of relative antibody levels and clinical outcome was tested by dividing the positive antibody levels (OD values) into quartiles. Quartiles boundaries were determined by the 25th, 50th and 75th centiles of serum OD values that were above the cut off for each antigen within a given village, such that each quartile contained *n*/4 OD values (where *n* was the number of antibody positive values). The number of people presenting with clinical malaria in each quartile was plotted and the odds ratio of presenting with malaria calculated for each quartile relative to the antibody negative group using multivariate logistic regression analyses, adjusting for parasite positivity (the presence of malaria parasites in the blood stream at time of serum sampling) as a co-variable in the analysis. In addition to parasite positivity, age, reactivity to schizont extract and an episode of malaria in the twelve month prior to serum collection were also adjusted for as predictors of clinical outcome in a second analysis. Using quartiles of antibody OD values as categorical data within a logistic regression makes no assumption of a linear association between antibody level and clinical outcome, unlike analyses that would use antibody level as a continuous variable.

2.6. Multivariate analysis of association of anti MSP2 and anti AMA1 reactivities with protection

Sera of 1068 individuals from Chonyi and Ngerenya screened here for IgG antibodies to MSP2 had previously been studied for IgG antibodies to the *P. falciparum* antigen AMA1 [11]. In that study anti-AMA1 antibodies were significantly associated with reduced risk of malaria, especially reactivities against a full length FVO allelic product in parasite positive people from Chonyi. In order to determine if the association of antibodies to AMA1 with protection from malaria was independent of any protective effect conferred by antibodies to MSP2, a multivariate analysis was used to calculate residual associations with protection that were antigen specific. In order to simplify calculations and retain maximum power in a multivariate analysis, which encompassed data from both MSP2 and AMA1, antibody (OD) levels to the AMA1 and MSP2 antigens were recoded into binary variables. For MSP2, only high levels of antibody were associated with protection in this study, therefore OD values were recoded into high (top 50% of OD values) and low/negative (bottom 50% of OD values) values. By contrast, high levels of antibody to the AMA1 full-length ectodomain antigen appeared to confer no greater level of protection than low (but positive) levels of antibody within the Kenyan cohorts (data not shown). Therefore, antibody levels to AMA1 were encoded as antibody positive and antibody negative for logistic regression analysis (as in the published analysis [11]). This had the effect of maximising the association with protection of antibodies to each antigen. Odds ratios were calculated using reactivities to either a single antigen or pairs of antigens in multivariate analyses (adjusting for reactivity to schizont extract and age), in order to compare the residual protective effect of antibody specific to each antigen.

3. Results

3.1. Development of human IgG to MSP2 with age

The frequency of individuals with positive IgG levels to each MSP2 recombinant antigen increased with age for people in Chonyi (Fig. 1A and C) and Ngerenya (Fig. 1B and D) ($P < 0.000001$ for each antigen, Chi-square test for trend). This increase with age was apparently more rapid in Chonyi, where 78% and 84% of children aged 2–3 years were antibody positive to type A (CH150/9) and type B (Dd2) MSP2 antigens, respectively and 99% of individuals aged 16 and over had antibody to both types. When considering antibody levels a gradual increase was apparent over a broader age range than that seen with simple antibody positivity (Fig. 2A and C). In 2–3 year olds from Chonyi the median IgG level (OD) to type A and type B was 0.73 and 0.96, respectively, about half the median levels recorded for adults aged 16 and over (OD 1.62 to type A and OD 1.56 to type B). The apparent age-dependant increase in antibody levels occurred at a

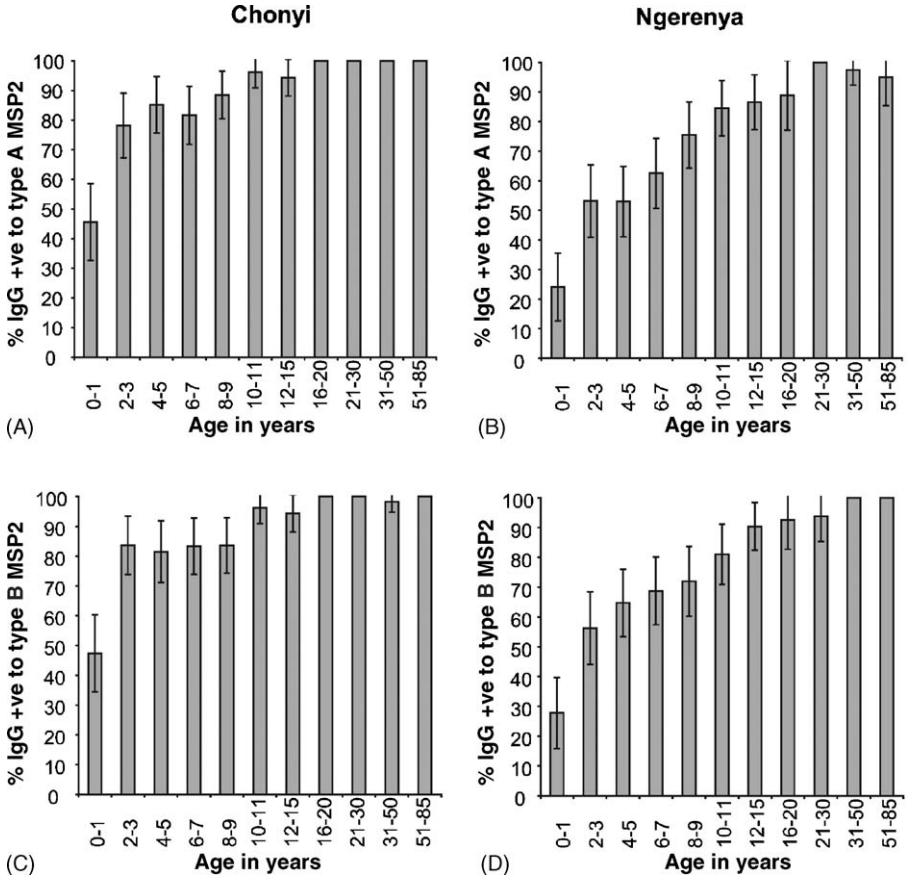

Fig. 1. Percentage of Kenyan individuals of defined age groups from Ngerenya and Chonyi who were serum IgG positive to MSP2 antigens in October 2000. The MSP2 antigens used were CH150/9, a type A antigen (A and B) and Dd2, a type B antigen (C and D). 95% CI are shown for each percentage. Any level of serum IgG that was greater than mean + 3SD of sera from 20 malaria naive donors was deemed positive (the cut off OD for type A antigen was 0.173 and 0.221 for type B antigen).

slower rate for people in Ngerenya (Fig. 2B and D), where the median antibody levels in children aged 2–3 years were 0.29 to type A and 0.23 to type B. These were four times lower than those found in adults aged 16 and over (OD 1.33 and 1.36 to type A and type B, respectively).

There appears to be a high degree of type-specific immune reactivities in both populations (Fig. 2E and F), where sera bind strongly to only one of the two allelic forms of MSP2. The Spearman rank (r) correlation was 0.72 for ELISA OD levels against the two recombinant antigens in both locations.

3.2. Antibody subclasses to MSP2 and other merozoite antigens

IgG antibody subclasses were analysed in sera from 96 individuals who were positive for antibody to schizont extract. A strong predominance of IgG3 was observed for antibodies binding with both type A (Fig. 3A) and type B (Fig. 3B) MSP2 antigens. This skew towards IgG3 was also seen with antigens representing the block 2 of MSP1 (Fig. 3E and F). A radically different subclass skew was detected with antigens representing the ectodomain of AMA1 (Fig. 3C) and the C terminal 19 kDa fragment of MSP1 (MSP1–19) (Fig. 3D), antibodies being predominantly of the IgG1 subclass in the same 96 individuals.

3.3. Frequency of MSP2 genotypes in the two villages and their association with antibody levels

DNA was extracted from blood samples of 107 people from Chonyi and 74 people from Ngerenya who were *P. falciparum* slide positive at the time of sera collection in October 2000. Positive PCR results were obtained for MSP2 genotypes in 97/107 of the Chonyi infected individuals: 40 individuals had a majority of PCR products that were type A MSP2 alleles, 29 had a majority that were type B alleles and 28 had both type A and type B alleles of approximately equal visual intensity. Overall there were 57 mixed allele infections amongst the Chonyi samples (where a sample yielded allelic products of more than one type, regardless of whether there was a single dominant allelic product). Of 74 DNA samples from infected individuals in Ngerenya, 56 gave a positive PCR result. Of these individuals, 25 had a majority of type A MSP2 allele(s) and 22 had a majority of type B MSP2

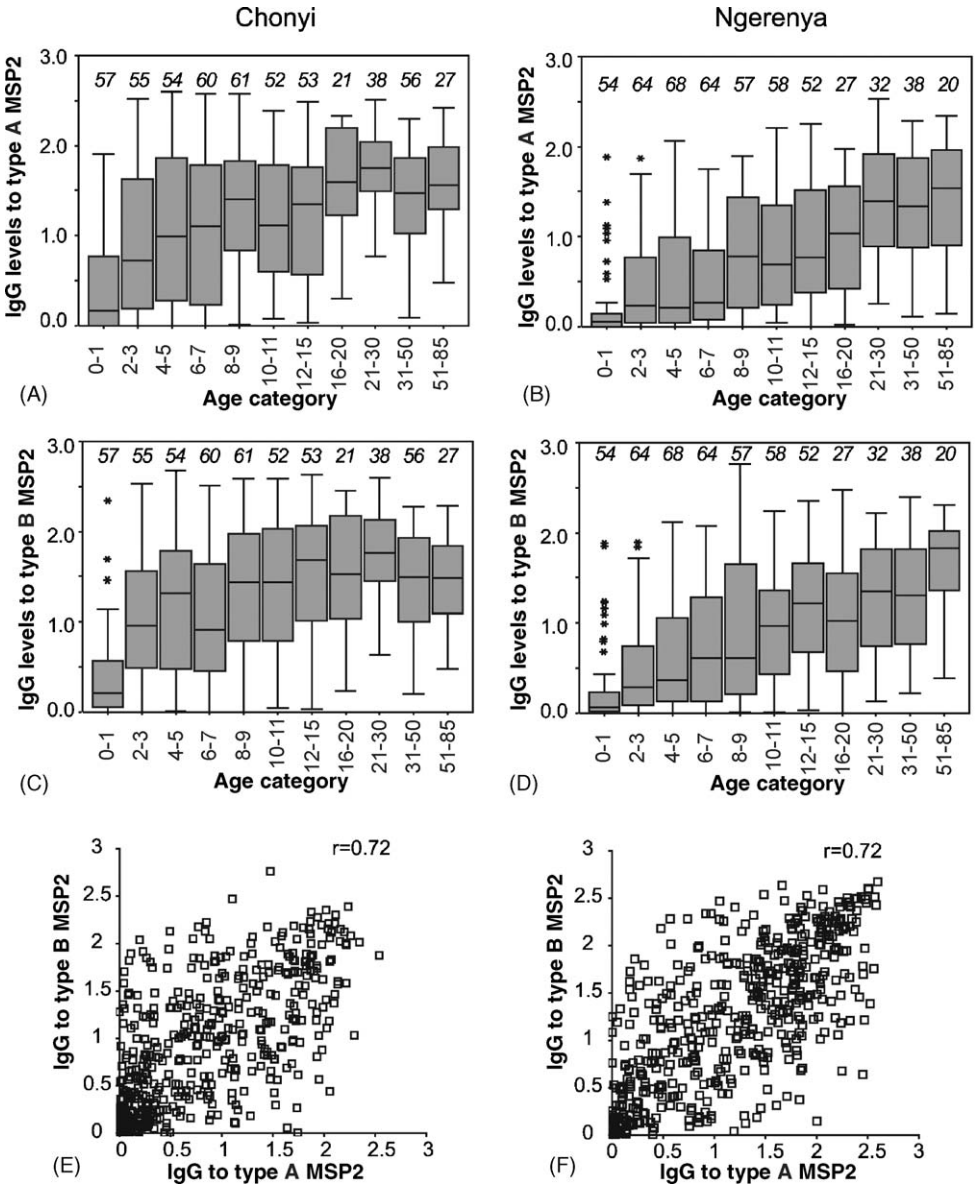

Fig. 2. Increase in levels of antibodies to MSP2 with age in Ngerenya and Chonyi. Box plots (A–D) show the median (black horizontal bar), lower (Q25) and upper (Q75) quartiles (grey boxes) and minimum and maximum values (whiskers) excluding outliers (asterisk) of antibody (OD) levels to type A and type B MSP2 for October 2000 serum samples from different age groups. Any value lying further away from the median than 2.5 times the distance between the median and the quartile is shown here as an outlier (asterisk). Numbers above the box plots indicate the number of individuals in each age category Scatter plots show pair wise reactivities to type A and type B MSP2 for Chonyi (E) and Ngerenya (F), with the spearmans ranks correlation (r) at the top right of the scatterplot.

allele(s). Nine samples from Ngerenya had infections where type A and type B alleles were amplified to equal intensities, whilst a further six contained both type A and type B MSP2 alleles which amplified to different intensities (thus there were fifteen mixed allele infections determined). There was no significant difference in the proportion of MSP2 type A and B infections in Chonyi and Ngerenya ($P = 0.75$, Chi-squared analysis with Yates correction). However, there was a significant difference between the two sites in the proportion of mixed allele infections ($P < 0.001$), with 59% in Chonyi and 26% in Ngerenya.

Paired samples of PCR-genotyped parasites and sera analysed for anti-MSP2 antibodies were obtained from a total of 146 individuals from the two locations (the remaining seven PCR positive samples had inadequate serum amounts and were not included in the antibody studies). The 146 paired samples were divided into three groups containing those samples with a majority of parasites with type A alleles, those with of a majority of parasites with type B alleles and samples with equal intensities of type A and type B infections. Sera from individuals who had infections with a majority of type A allele(s) had significantly lower antibody OD levels

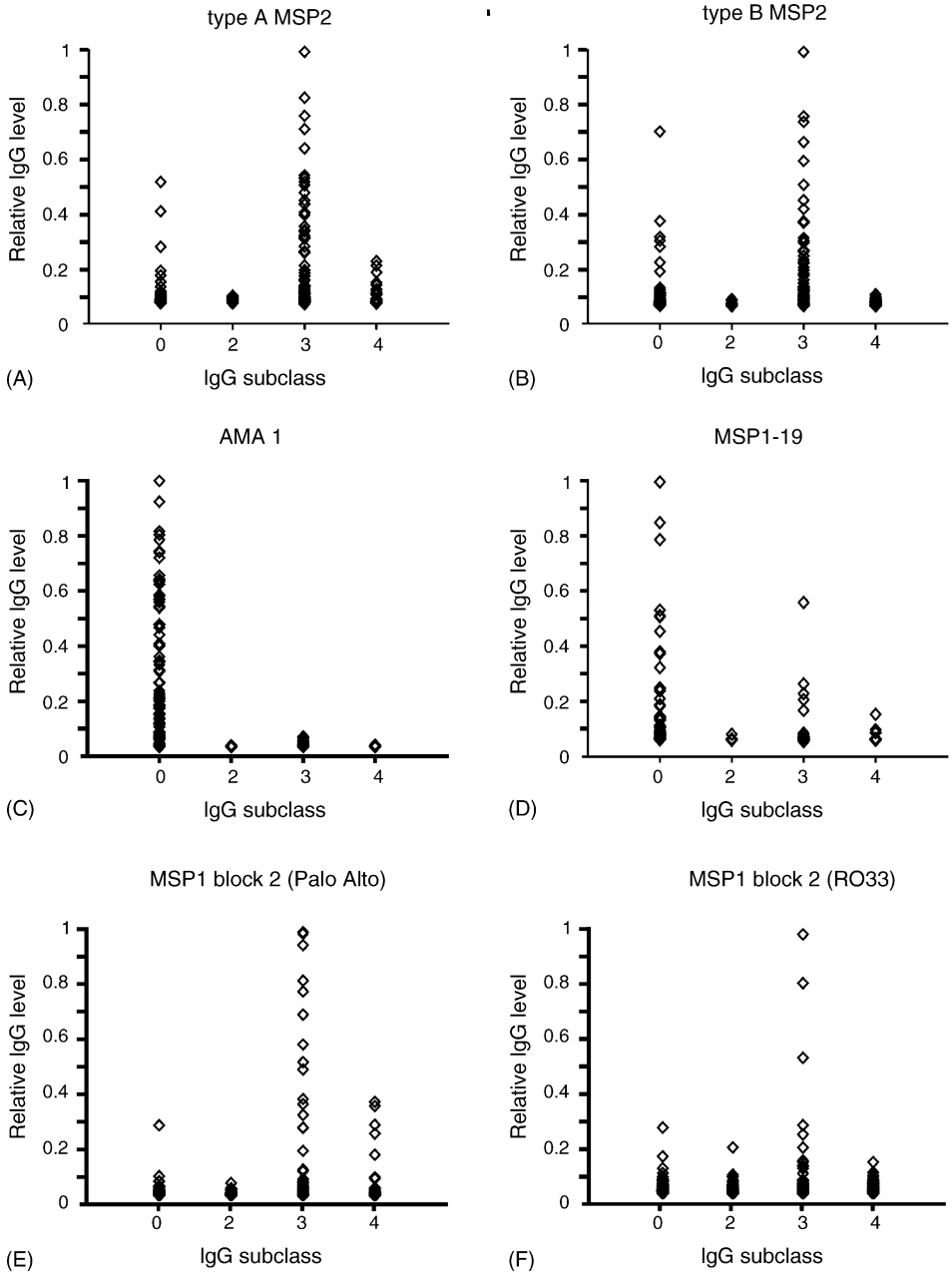

Fig. 3. Relative IgG subclass levels to different merozoite antigens in human sera from Ngerenya and Chonyi. Relative IgG subclass levels to: (A) type A MSP2; (B) type B MSP2; (C) full length ectodomain of AMA1; (D) MSP1–19; (E) MSP1 block 2 Palo Alto and (F) MSP1 block 2 RO33 were calculated from standard curves using purified human myeloma proteins IgG1, IgG2, IgG3 and IgG4. The amount of subclass IgG antibodies recorded for each serum-antigen combination (calculated from standard curves of plate bound human myeloma proteins) were then divided by the highest single such value recorded for each antigen, hence the relative concentrations values vary between 0 and 1 for each antigen.

to the type B MSP2 antigen compared to sera from those individuals who had infections with a majority of type B alleles ($z = 3.130$, $P = 0.002$). Conversely, the sera from individuals who had infections with a majority of type A allele(s) had higher antibody OD levels to the type A MSP2 antigen compared to sera from those individuals who had infections with a majority of type B alleles, the difference between the levels being of borderline significance ($z = -1.799$; $P = 0.072$).

3.4. IgG against MSP2 and protection from subsequent infection for people in Chonyi

When the Chonyi cohort was considered as a whole, serum IgG to neither type A nor type B MSP2 antigens was significantly associated with risk of malaria in the subsequent transmission season (OR, 1.84; 95% CI, 0.81–4.16 and OR, 0.80; 95% CI, 0.42–1.54, respectively in univariate analysis) (Table 1). In order to adjust for a possible age related

Table 1
Association of detectable serum IgG antibodies to MSP2 in October 2000 with risk (Odds ratio, OR) of subsequent clinical malaria during the period October 2000 to March 2001 in Chonyi village

	Univariate analysis		Multivariate analysis[a]	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
All ($n=534$)				
Type A	1.84 (0.81–4.16)	0.144	3.68 (1.39–9.78)	0.009**
Type B	0.80 (0.42–1.54)	0.505	1.19 (0.53–2.70)	0.667
P. falciparum slide negative[b] ($n=338$)				
Type A	4.35 (1.02–18.64)	0.048*	8.94 (1.79–44.55)	0.008**
Type B	0.64 (0.28–1.45)	0.285	0.76 (0.27–2.12)	0.599
P. falciparum slide positive[b] ($n=196$)				
Type A	0.22 (0.06–0.85)	0.028*	0.47 (0.08–2.87)	0.414
Type B	0.35 (0.09–1.37)	0.131	0.83 (0.17–4.00)	0.818

Odds ratio (OR) for people with anti-MSP2 antibodies (optical density (OD) greater than the mean + 3S.D. of sera from 20 malaria-naive donors) were calculated by comparison to people who were antibody negative. 95% confidence intervals (95% CI) are shown in brackets.Significant association of detectable antibodies with disease is shown as follows. *$P<0.05$. **$P<0.01$.

[a] In multivariate analyses, age, reactivity to crude schizont extract and an episode of malaria in the 12 months prior to serum collection were adjusted for in logistic regression.

[b] Parasite positivity/negativity refers to detectable parasites at time of serum sampling.

increase in the level of serum IgG to other blood stage malaria antigens a multivariate analysis was done using age, IgG reactivity to parasite schizont extract and a clinical episode of malaria in the twelve months prior to serum collection as co-variables. In such multivariate analysis, IgG to type A MSP2 (CH150/9) was significant association with increased risk of malaria (OR, 3.68; 95% CI, 1.39–9.78). However, in the same cohort no significant association with subsequent morbidity was seen for IgG to type B MSP2 (Dd2) although a trend towards increased risk was also seen (OR, 1.19; 95% CI, 0.53–2.70) in multivariate analysis.

Within the Chonyi cohort here, 196 out of 534 individuals were slide positive at the time of serum collection. An interactions test showed that parasite positivity significantly modified the association between antibodies to type A MSP2 and clinical outcome ($P<0.001$), so a stratified analysis was performed on the data set. Among parasite-positive individuals, antibody positivity to type A MSP2 was significantly associated with subsequent protection from malaria in univariate but not multivariate analysis (Table 1). In these same individuals antibody to type B MSP2 was not associated with protection in either univariate or multivariate analysis, although a non-significant trend towards protection was seen. Among parasite-negative individuals, IgG to type A MSP2 was associated with increased risk in both univariate and multivariate analysis, whereas for antibody to type B MSP2 there was a non-significant trend towards protection for serum IgG.

From these multivariate analysis comparing clinical outcome for antibody positive and antibody negative individuals it would appear that antibody to MSP2 did not protect people in Chonyi from malaria morbidity. However, the following analysis of antibody levels (rather than antibody positivity/negativity) proved more informative. When the antibody positive individuals were divided into quartiles according to OD values (Fig. 4) it was obvious that for the cohort as a whole people who had low levels of antibody (Quartiles 1 and 2) were more at risk from malaria than those people who were antibody negative. This result was most apparent for antibody levels to type A MSP2. By contrast, people with the highest levels of antibody (Quartile 4) appeared to be protected from malaria. Examining the cohort as a whole, the odds ratio of presenting with clinical malaria was calculated for people in each quartile compared to those people who were antibody negative (Table 2), taking into account the effect of parasite presence at the time of serum collection. People whose antibody levels fell into the lowest quartile (Q1) had an increased risk of malaria compared to those who were antibody negative, a result which was highly significant only for antibody to type A MSP2 (OR, 4.27; 95% CI, 1.58–11.59) after correction for parasite positivity, reactivity to schizont extract, age and an episode of malaria in the twelve months prior to serum collection. By contrast, people whose antibody levels fell into the highest quartile (Q4) had a decreased risk of malaria, a result that was highly significant for antibody to type B MSP2 (OR, 0.19; 95% CI, 0.05–0.70 in multivariate analysis). It would appear therefore that the increased risk of malaria in people who had low but detectable levels of anti-MSP2 antibodies was responsible for the association of simple antibody positivity with increased risk of subsequent malaria in the initial analysis and masked the protective associations seen with the higher levels of anti-MSP2 IgG. When the cohort was divided according to the presence of parasites at the time of bleed, it could be seen the increased risk of malaria associated with low levels of anti-MSP2 IgG was most pronounced within the slide negative group (Fig. 4). Within the slide positive group people who had low but positive levels of anti-MSP2 antibodies actually had less probability of presenting with malaria than those individuals who had undetectable levels of anti-MSP2 antibodies.

3.5. IgG against MSP2 and protection from subsequent *P. falciparum* malaria in Ngerenya

When simple antibody positivity/negativity was considered for the Ngerenya cohort as a whole, serum IgG to neither type A or type B MSP2 was significantly associated with protection from malaria in the subsequent transmission season in either univariate (OR, 0.84; 95% CI, 0.56–1.27 and OR, 1.12, 95% CI, 0.72–1.73, respectively) or multivariate analyses (OR, 0.87; 95% CI, 0.49–1.55 and OR, 1.23; 95% CI, 0.69–2.20) (Table 3). In contrast to the Chonyi cohort, stratification for parasite presence at the time of sample collection for antibody assessment had no significant effect on the association of anti-MSP2 antibody with disease in Ngerenya ($P=0.164$ and 0.152 for anti-type A and type B MSP2, respectively).

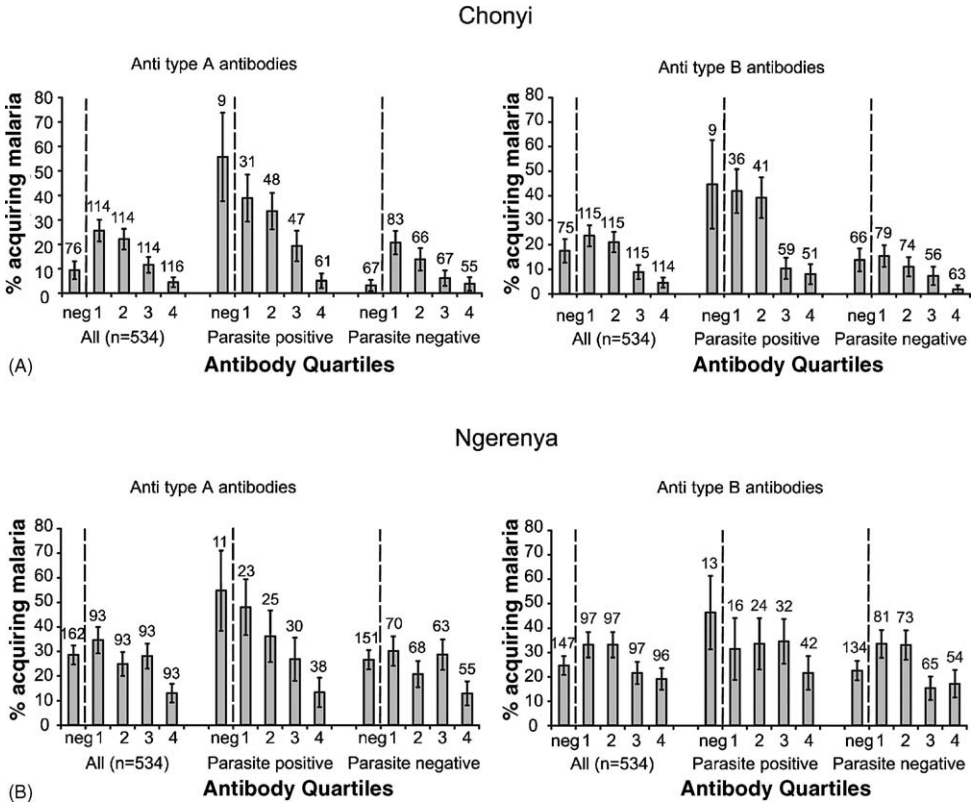

Fig. 4. Proportion of people acquiring at least one episode of malaria in October 2000 to March 2001 categorised by serum IgG levels to MSP2 antigens and parasite (slide) positivity in October 2000. Antibody reactivities have been divided into antibody negative (to left of dotted line and less than or equal to the mean + 3S.D. of antibody reactivities of 20 malaria naïve individuals) and antibody positive (to right of dotted line). Each set of antibody positive reactivities have been further divided into quartiles where the quartile boundaries are delineated by the 25th, 50th and 75th centiles of antibody (OD) levels. Quartile 1 contains people with OD values between the positive cut off and the 25th centile value, quartile 2 people with OD values between the 25th and 50th centile, quartile three people with OD values between the 50th and 75th centile and quartile four people with OD values above the 75th centile. For Chonyi the cut off, 25th, 50th and 75th centiles were 0.173, 0.810, 1.44 and 1.868 for type A MSP2 and 0.221, 0.872, 1.454 and 1.949 for type B MSP2. For Ngerenya these values were 0.173, 0.492, 0.997 and 1.630 for type A MSP2 and 0.221, 0.614, 1.108 and 1.679 for type B MSP2. The numbers above each bar show the number in that quartile, whilst the 95% confidence intervals of the number of individuals experiencing clinical episodes of malaria are shown by the error bars. The individuals in each quartile group have been further stratified according to whether people were parasite positive or negative by microscopy at the time of serum collection.

Table 2
Association of serum IgG levels to MSP2 and the presence of circulating *P. falciparum* in October 2000 with risk (Odds ratio, OR) of subsequent clinical malaria during the period October 2000 to March 2001 in Chonyi village

	Analysis of antibody levels and parasite positivity[a]		Multivariate analysis of antibody levels[b]	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Anti-type A				
Type A Q1	2.79(1.13–6.89)	0.026*	4.27 (1.58–11.59)	0.004**
Type A Q2	1.84 (0.72–4.65)	0.200	3.76 (1.27–11.07)	0.016*
Type A Q3	0.82(0.30–2.23)	0.692	2.36 (0.74–7.51)	0.145
Type A Q4	0.24(0.07–0.83)	0.024*	0.56 (0.14–2.26)	0.418
Anti-type B				
Type B Q1	1.08(0.50–2.33)	0.848	1.31 (0.56–3.08)	0.530
Type B Q2	0.85(0.39–1.88)	0.696	1.19 (0.47–3.01)	0.705
Type B Q3	0.23(0.09–0.60)	0.003**	0.41 (0.13–1.21)	0.116
Type B Q4	0.12 (0.04–0.37)	<0.001***	0.19 (0.05–0.70)	0.013*

Odds ratio (OR) for people in each quartile of positive antibody levels in the entire cohort ($n = 534$) was calculated by comparison to those people who were antibody negative. Quartiles (Q 1–4) are the same as those in Fig. 4. Significant associations with outcome are shown as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

[a] The presence of *P. falciparum* as detected by slide positivity at the time of bleed was included as a co-variable in these analyses, and was significantly associated with clinical malaria, OR: 2.95 (1.64–5.31) for analysis with type A MSP2 and 3.97 (2.31–6.80) with type B MSP2.

[b] In addition to parasite positivity, age, reactivity to crude schizont extract and an episode of malaria in the 12 months prior to serum collection were also adjusted for as continuous variables. In these analyses the association (OR) of parasite positivity with clinical malaria was 2.77 (1.53–5.01) with type A and with 2.90 (1.61–5.25) type B MSP2.

Table 3
Association of detectable serum IgG antibodies to MSP2 in October 2000 with risk (Odds ratio, OR) of subsequent clinical malaria during the period October 2000 to March 2001 in Ngerenya village

	Univariate analysis		Multivariate analysis[a]	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
All ($n=534$)				
Type A	0.84 (0.56–1.27)	0.411	0.87 (0.49–1.55)	0.642
Type B	1.12 (0.72–1.73)	0.617	1.23 (0.67–2.20)	0.484
P. falciparum slide negative[b] ($n=407$)				
Type A	0.85 (0.53–1.35)	0.490	0.67 (0.34–1.30)	0.223
Type B	1.20 (0.73–1.95)	0.474	0.98 (0.50–1.92)	0.951
P. falciparum slide positive[b] ($n=127$)				
Type A	0.33 (0.09–1.16)	0.084	0.96 (0.22–4.20)	0.957
Type B	0.48 (0.15–1.52)	0.210	1.82 (0.40–8.27)	0.440

Odds ratio (OR) for people with anti-MSP2 antibodies were calculated by comparison to people who were antibody-negative. 95% confidence intervals (95% CI) are shown in brackets.

[a] In multivariate analyses age, reactivity to crude schizont extract and an episode of malaria in the 12 months prior to serum collection were adjusted for in logistic regression.

[b] Parasite positivity/negativity refers to detectable parasites at time of serum sampling.

As for the Chonyi cohort, however, there was a difference in the percentage of people suffering from malaria when quartiles were used to describe antibody levels instead of simple antibody positivity/negativity (Fig. 4B). When the cohort was considered as a whole, people with low level antibodies to MSP2 were more at risk from malaria than those who were antibody negative (type A and type B Q1 OR: 1.11; 95% CI, 0.58–2.13 and 1.37; 95% CI, 0.71–2.64, respectively after adjustment for age, reactivity to schizont extract and an episode of malaria in the twelve months prior to serum collection). In contrast, people whose antibody levels to type B MSP2 were in the highest quartile showed no such increased risk in multivariate analysis (Q4: OR 0.78; 95% CI, 0.31–1.81) (Table 4), whilst people in the highest quartile for antibody levels to type A MSP2 had a significantly reduced risk of presenting with malaria in multivariate analysis (Q4: 0.39; 95% CI, 0.16–0.98).

When the cohort was divided into people who were parasite negative and parasite positive at the time of bleed (Fig. 4), it could again be seen that the increased risk associated with low levels of antibody to MSP2 was only evident in the parasite slide negative individuals. Within the slide positive group people with low but positive levels of anti-MSP2 antibody had less probability of presenting with malaria than those individuals who had undetectable levels.

3.6. The association with protection against clinical malaria specific for IgG against MSP2 and AMA1

Within the Chonyi dataset, multivariate analyses (adjusting for reactivity to schizont extract, age and a clinical episode of malaria in the twelve months prior to serum collection) of antibodies to the two types of MSP2 and the AMA1 antigen studied showed that antibodies to all three antigens were associated with a significant decrease in malaria within the parasite-positive group when each was analysed singly. For MSP2 type A and type B the odds ratios for high versus low levels of antibody were: 0.37 (95% CI 0.16–0.89, $P=0.027$) and 0.33 (95% CI 0.14–0.78, $P=0.012$). For AMA1 the odds ratio for positive versus negative antibody levels was 0.34 (95% CI 0.13–0.91, $P=0.031$). When high level reactivities to the type B MSP2 antigen and positive reactivities to the AMA1 antigen were analysed together as co-variables high levels of antibody to type B MSP2 remained significantly associated with protection within the parasite-positive group

Table 4
Association of serum IgG levels to MSP2 and the presence of circulating *P. falciparum* in October 2000 with risk (Odds ratio, OR) of subsequent clinical malaria during the period October 2000 to March 2001 in Ngerenya village

	Analysis of antibody levels and parasite positivity[a]		Multivariate analysis of antibody levels[b]	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
Anti-type A				
Type A Q1	1.20 (0.69–2.10)	0.516	1.11 (0.58–2.13)	0.740
Type A Q2	0.74 (0.41–1.34)	0.323	0.67 (0.32–1.39)	0.281
Type A Q3	0.85 (0.47–1.53)	0.592	0.82 (0.39–1.74)	0.610
Type A Q4	0.31 (0.15–0.64)	0.001**	0.39 (0.16–0.98)	0.044*
Anti-type B				
Type B Q1	1.47 (0.83–2.59)	0.188	1.37 (0.71–2.64)	0.347
Type B Q2	1.41 (0.79–2.50)	0.242	1.40 (0.69–2.84)	0.350
Type B Q3	0.76 (0.40–1.42)	0.382	0.93 (0.43–2.02)	0.853
Type B Q4	0.60 (0.31–1.16)	0.130	0.78 (0.34–1.81)	0.561

Odds ratio (OR) for people in each OD quartile of antibody level from the entire cohort ($n=534$) were calculated by comparison to those people who were antibody-negative. Quartiles (Q 1–4) are the same as those in Fig. 4. Significant associations with outcome are shown as follows: $^{*}P<0.05$, $^{**}P<0.01$.

[a] The presence of *P. falciparum* as detected by slide positivity at the time of bleed together with a malarious episode in the previous 12 months were included as a co-variable in these analyses. The association (OR) of parasite positivity with clinical malaria was 1.66 (1.04–2.67) for analysis with type A MSP2 and 1.58 (0.99–2.53) with type B MSP2.

[b] In addition to parasite positivity, age and reactivity to crude schizont extract were also adjusted for as continuous variables. In these analyses the association of parasite positivity with clinical malaria was 1.19 (0.71–2.01) with type A and 1.18 (0.70–2.01) with type B MSP2.

Table 5
Residual association with risk of subsequent clinical malaria specific to high serum IgG levels to MSP2 and detectabe IgG levels to AMA1 antigens in October 2000, for the period October 2000 to March 2001 in Chonyi village

	Residual OR specific to MSP2 antigen		Residual OR specific to AMA1 antigen	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
All ($n=534$)				
MSP2 type A and AMA1	0.52 (0.27–0.99)	0.047*	1.47 (0.77–2.80)	0.239
MSP2 type B and AMA1	0.33 (0.17–0.64)	0.001**	1.54 (0.82–2.90)	0.177
Slide negative[a] ($n=338$)				
MSP2 type A and AMA1	0.54 (0.20–1.46)	0.224	2.52 (1.01–6.31)	0.048*
MSP2 type B and AMA1	0.25 (0.09–0.72)	0.009**	2.57 (1.04–6.35)	0.041*
Slide positive[a] ($n=196$)				
MSP2 type A and AMA1	0.44 (0.15–1.01)	0.066	0.40 (0.15–1.09)	0.075
MSP2 type B and AMA1	0.40 (0.16–0.98)	0.045*	0.40 (0.15–1.06)	0.065

Individuals were assigned as having high levels (top 50% of OD values) and low/negative levels (bottom 50% of OD values) of anti-MSP2 antibodies, and positive (greater than the mean + 3S.D. of sera from 20 malaria-naive donors) and negative levels of AMA1 antibodies for these analyses. Odds ratios (OR) for these two co-variables were adjusted for reactivity to whole parasite extract and age in years, 95% confidence intervals (95% CI) are given in brackets. Significant associations with outcome are shown as follows: $^{*}P<0.05$, $^{**}P<0.01$.

[a] Parasite positivity/negativity refers to detectable parasites at time of serum sampling.

Table 6
Residual association with risk of subsequent clinical malaria specific to high serum IgG levels to MSP2 and positive IgG levels to AMA1 in October 2000, for the period October 2000 to March 2001 in Ngerenya village

	Residual OR specific to MSP2 antigen		Residual OR specific to AMA1 antigen	
	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
All ($n=534$)				
MSP2 type A and AMA1	0.63 (0.37–1.06)	0.083	0.67 (0.39–1.14)	0.141
MSP2 type B and AMA1	1.13 (0.66–1.91)	0.430	0.57 (0.33–0.98)	0.045*
Slide negative[a] ($n=407$)				
MSP2 type A and AMA1	0.66 (0.35–1.23)	0.191	0.83 (0.45–1.55)	0.565
MSP2 type B and AMA1	0.81 (0.44–1.52)	0.519	0.92 (0.43–1.49)	0.479
Slide positive[a] ($n=127$)				
MSP2 type A and AMA1	0.42 (0.14–1.25)	0.120	0.34 (0.10–1.11)	0.073
MSP2 type B and AMA1	2.69 (0.79–9.15)	0.113	0.23 (0.07–0.78)	0.018*

Individuals were assigned as having high levels (top 50% of OD values) and low/negative levels (bottom 50% of OD values) of anti-MSP2 antibodies, and positive (greater than the mean + 3S.D. of sera from 20 malaria-naive donors) and negative levels of AMA1 antibodies for these analyses. Odds ratios (OR) for these two co-variables were adjusted for reactivity to whole parasite extract and age in years, 95% confidence intervals (95% CI) are given in brackets.

[a] Parasite positivity/negativity refers to detectable parasites at time of serum sampling.

of people, whilst the protection seen with antibody to AMA1 was of borderline significance (Table 5). A similar outcome was seen in an analysis of high level antibodies to the type A MSP2 antigen and any positive reactivities to AMA1. Within the cohort as a whole it was only high levels of antibodies to MSP2 antigens that remained significantly associated with protection when included as a co-variable with antibody to AMA1 in multivariate analysis.

In the Ngerenya cohort, a non-significant trend towards protection was observed with high levels of antibody to type A MSP2 (OR 0.38; 95% CI 0.13–1.11, $P=0.076$) and a significant association with protection was seen with positive levels of antibody to AMA1 (OR 0.28; 95% CI 0.09–0.92, $P=0.035$) in the parasite-positive group of people when these variables were analysed separately in multivariate analyses adjusting for age, IgG reactivity to schizont extract and an episode of malaria in the twelve months prior to serum collection. When the reactivities to type A MSP2 and AMA1 were analysed together in multivariate analysis the residual protection seen with each was not significant, however a trend towards protection was also seen with both (Table 6). No trend towards protection was detected with antibodies to type B MSP2 in either the parasite-positive or parasite-negative groups even before adjusting for antibody to AMA1 (OR 2.65; 95% CI 0.63–6.61 and 0.69; 95% CI 0.37–1.27, respectively).

4. Discussion

Multivariate data analyses in this study have shown that high antibody levels against MSP2 can be associated with

protection against malaria even after a protective effect of antibody to another antigen (AMA1) is adjusted for. This suggests that the protection conferred by anti MSP2 antibodies is to some extent independent of that conferred by anti-AMA1 antibodies. Testing of antibodies to additional antigens within the same cohort would allow further multivariate comparative analyses with the aim to narrow down the number of candidate antigens to be included in a malaria vaccine to those most strongly associated with protection in naturally exposed populations.

Within Chonyi, only high levels of antibodies to type B MSP2 were significantly associated with protection (considering the cohort as a whole), whilst only high level antibodies to type A were associated with protection in Ngerenya. Data in this study complement previous data showing that many individuals possess antibodies that bind differentially to the type A and type B allelic products of MSP2 [8–10,15,17,18]. Given that the predominant immunoglobulin subclass to MSP2 was IgG3, which has the shortest half life of all the IgG subclasses [28], it is not surprising therefore that among the parasite positive individuals there is a strong positive relationship between the predominant MSP2 type of the parasite in the infection and the type-specificity of the antibody present at the same time. Whether this association between antigen type of the current infection and antibody reactivity is more exaggerated for MSP2 than other surface antigens on the merozoite due to the subclass bias towards IgG3 resulting in a limited persistence of the antibody is as yet undetermined.

Could differences in the distribution of MSP2 types among parasites infecting individuals at each location explain the different associations with protection in Ngerenya and Chonyi of antibodies to type A and type B MSP2 with protection? The intensity of malaria infectious mosquito bites experienced by people appeared to be very different in the two locations, as shown here by the different proportion of mixed clone infections in samples from each population, but the ratios of type A to type B alleles were similar in blood samples from both cohorts. However, there could have been differences in allele frequencies at the level of subtype variability. As well as type-specific antibodies, antibodies that discriminate between different subtypes of MSP2 are known to occur in response to natural infections [17,18]. The current study was not designed to investigate the role of anti-MSP2 subtype-specific antibodies in protection, which is as yet unknown.

Possibly the most surprising result from this study was the increased risk of malaria in people with low level antibodies to MSP2 compared with those who were antibody-negative. In vitro invasion assays have shown an increase in the percentage of multiple invasion events (where more than one parasite enters a single red blood cell) in the presence of anti-MSP2 antibodies suggesting that the parasites have been cross-linked by antibody, yet are still capable of invading red blood cells [29]. Could such multiple invasion events increase the likelihood of malaria morbidity? An alternative explanation is that the people with low titres of antibody have been exposed to more infective bites than those with undetectable levels due to micro-epidemiological differences and cannot control the subsequent malaria infection. There is known to be great heterogeneity in the risk of malaria infections among individuals, partly explained by household effects on exposure [30], and it is likely that a proportion of antibody negative people were continuously at low risk. Given that low levels of antibody were only associated with increased risk of malaria in people who were parasite negative at the time of serum collection, a heterogeneity in the level of exposure would seem the most likely explanation. Both parasite slide positivity and an episode of malaria in the previous twelve months were significantly associated with increased risk of malaria, re-enforcing such a hypothesis. In contrast to people with low yet positive levels of anti-MSP2 antibodies, individuals with higher levels of anti-MSP2 antibodies appeared able to control parasite infections.

The use of logistic regression to calculate the associations of antibody levels with protection relies on a linear association of the predictor with outcome (where a unit increase in antibody level is associated with a unit increase in protection) if antibody levels are considered as continuous data. The skewed nature of antibody levels in a population means that log adjustment should be applied to the data before they are considered as continuous data, but the lower risk of malaria seen here in the antibody negative group compared to some of the antibody positive group would clearly have violated such a linear association even after log adjustment of the data. Therefore, such data are better analysed by categorical quartiles of antibody levels if the association of antibody levels with clinical disease is to be determined.

Overall, this study provides strong evidence that high levels of anti MSP2 IgG antibodies can protect individuals in eastern Kenya from malaria, whilst low levels of antibody are either not protective or may even increase the risk of malaria morbidity. As shown here, the type specificity of anti-MSP2 antibodies generally reflects the allelic types of the parasites within an infection. Could high level type-specific antibodies that would protect against all *P. falciparum* infections be induced by a vaccine? A field tested MSP2 based vaccine containing only one of the two MSP2 types was relatively ineffective against preventing disease from a full repertoire of wild type *P. falciparum* isolates [31], but it did have an effect in that it appeared to produce a selective pressure on MSP2 allele frequencies. Thus, a potentially successful MSP2 vaccine should include a minimum of two allelic products to protect against parasites with type A or type B alleles. Logically, it is only by analysing the role of extensive sub-type variation in anti-MSP2 mediated immunity that the optimal number of products required for a efficacious vaccine could be predicted. This study shows that at a population level natural exposure to AMA1 and MSP2 can induce antibodies, which independently protect people in a malaria endemic location against clinical disease. Thus, both AMA1 and MSP2 would appear

suitable for inclusion within a multi-component malaria vaccine.

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