**Author's response to reviews**

**Title:** Age-adjusted Plasmodium falciparum antibody levels in school-aged children are a stable marker of microgeographical variations in exposure to Plasmodium infection

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**Author's response to reviews:** see over
Dear Editor,

Thank-you for considering the manuscript “Age-adjusted *Plasmodium falciparum* antibody levels in school-aged children are a stable marker of microgeographical variations in exposure to *Plasmodium* infection” (ms: 1975196841346808).

We have found the reviewers’ comments very useful and now submit an improved manuscript, revised according to these comments. Point-by-point replies to the reviewers’ questions are given below.

Yours faithfully,

Shona Wilson
We have tried to divide comment 1 into subsections to facilitate a point-by-point reply. The use of a serological marker for exposure over PCR as the outcome measure of choice, and the use of prevalence on a household basis are addressed separately.

1a. Outcome measure of choice:

The referee suggests that PCR would be a better marker for recent exposure than serology. This is a very interesting point, however, we have found in a small-scale study of school-aged children, from within the same study area, at a time point intermediate between transmission seasons (prevalence by microscopy was 27.8%), that 74.7% were positive for *Plasmodium* infection by PCR. This high level of prevalence could result in failure to detect microgeographical variations in exposure at many times of the year and therefore, although more sensitive, is constrained by same caveat of time of sampling in relation to transmission season as prevalence by microscopy. We accept that using PCR would be a useful tool in spatial epidemiology studies, where the longitudinal dynamics of binary conversion rates of household negative to positive and back again (similar to that suggested by the referee for prevalence by microscopy) would provide important information on patterns of transmission in seasonal endemic areas. However, we believe this important area of research is beyond the scope of the present manuscript, in which we attempt to define a marker for levels of chronic exposure that could be used at any time during the annual transmission cycle, and reflect accumulative exposure, rather than present exposure. Particularly as we are interested in defining a marker that could be used for associations with chronic morbidities, which do not to regress during the low transmission seasons.

1b. “Prevalence” amongst household samples:

The referee’s comment that there would be high statistical error in prevalence on a household basis is true. However, in order to counter this, two different statistical approaches were taken. One was to group individuals together in an ordinal variable, according to how far the house they resided in was from the water body in question, rather than on a household basis. The second approach was to use Kulldorff Scan to assess whether or not there was any spatial clustering. This method uses the data from the population as a whole to detect “hot spots” of cases, with household used to assign spatial position to each individual.

The maps generated are on a household basis, so this visual representation of the data collected, will contain a degree of error, however, the software used to generate the maps, along with the concept of spatial auto-correlation, allowed us to smooth the maps to a degree, as the average value is taken whenever the 0.5km radius circles representing each household overlap. A statement relating to this has been added to the second paragraph of the methods section, sub-section study site.
2. There is a mix of *A. funestus* and *A. gambiae* vector species in the study area. Although no full-scale systematic trapping has been carried out in the study site, preliminary indications are that in the east of the study area the two vectors are present in almost equal numbers, while to the west, *A. gambiae* is the predominant vector. A statement about the vectors has been added to the first paragraph of the methods section, sub-section study site.

3. Rainfall data has been collected at Kambu, a town approximately 15km from the study area. The data collected for 2003 and the first few months of 2004, indicate that the rainfall pattern for this period of time was typical concerning the low transmission time point, with the previous rains falling in May 2003. The high transmission time point was due to the early arrival of the rains in 2004, when there was substantial precipitation in January, with a steady decrease through to April 2004. However, the difference in prevalence between the two time points, indicates that there was substantial differences in transmission between the two time points and therefore, we believe the conclusions reached are still appropriate, even though the rains in 2004 could be considered atypical. A statement about the timing of the study in relation to rainfall that year is now in methods section, sub-section study population.

A water body was considered to be seasonal if completely dry towards the end of the long dry season, as was the case during the low transmission time point. The permanent streams to the east of the study area are not known to dry out and even continued to flow during the long period of drought that hit the area after the completion of the study. How the rains affect the discharge levels of these streams was not assessed. To address this revision, we have added a statement about seasonal water bodies drying up during the long dry season to the first paragraph of the methods section, sub-section study site.

4. The referee suggests that the manuscript is rich in data and would benefit from either shortening or dividing into 2 separate papers. However, we think that the present manuscript would be weakened by the removal of the Pfs-IgG3 subsets. The study was designed to validate Pfs-IgG3 as a marker and these subsets do describe the potentially useful approach of using age-adjusted serological markers to determine relative accumulative exposure. The second referee agrees this is a more useful than the use of sero-conversion rates, which has previously been reported for macrogeographical scale mapping. In terms of validation of the marker, we are aware that this would have strengthened by entomology, but as discussed, previous studies, carried out by specialists in the field of entomology, do indicate that the distances over which our serological marker varies is comparable with the majority of host seeking behaviour of the vector. We therefore, in agreement with the referee’s first suggestion, and in concordance with the second referee, have significantly shortened the paper but have kept the Pfs-IgG3 subsets.
Reviewer 2 Chris Drakeley

1. Reference for the Pf antigen preparation and laboratory strain have been added to Methods, sub-section “Antigen preparations”.

2. A correlation from a pre-study comparison of antibody levels using plasma samples and spotted whole blood, from the same individuals and time point, is added to the first paragraph of methods sub-section “Dried Blood Spot Elution and Antibody ELISA”. The word diameter is added after “6mm” in the same paragraph. The final dilution factors for the elute are indicated in the second paragraph of this section.

3. No controls for positivity were used. Although a selection of European plasma samples were on each plate, they were insufficient in number (the same samples are added to each plate) and were only used to correct for any between plate variation in readings. This is now indicated in the first paragraph of methods sub-section “Dried Blood Spot Elution and Antibody ELISA”.

4. Fifteen fields of each blood smear were read before a slide was recorded as negative. This is now stated in the methods sections, sub-section study population.

5. We have measured Pfs-IgG1 levels for this study population. Results were very similar to those for Pfs-IgG3. Considering the similarity of the results between Pfs-IgG1 and Pfs-IgG3, it was decided that the manuscript would be too cumbersome if both responses were presented and as we have previously discussed Pfs-IgG3 as a potential marker for exposure, we decided that it would be more appropriate to present the results for this sub-class. This similarity is now discussed in the second last paragraph of the discussion. For the interest of the reviewer the main IgG1 results are outlined below.

The cross-sectional profiles of Pfs-IgG1 levels with age, did not reach as apparent a plateau as Pfs-IgG3 levels, however, the β co-efficient of the regression line with age, amongst adults was less than that of the children, although whether or not the slopes were significantly different was not assessed. There were no significant increases in Pfs-IgG1 levels found during the high transmission season, for any of the age groups. The generated maps for age-adjusted Pfs-IgG1, and the graphs of mean levels with distances from either the permanent streams or the seasonal ponds were also similar to those generated for age-adjusted Pfs-IgG3. This is due to the very high correlation co-efficients that were found for the levels of Pfs-IgG1 and Pfs-IgG3 ($r=0.773$ during the low transmission season and $r=0.764$ during the high transmission season). The small increases in Pfs-IgG1 levels that were observed to occur with age, during adulthood, did not result in a significant relationship between age-adjusted Pfs-IgG1 levels and distance from nearest water body. The one difference found between the results for Pfs-IgG1 and Pfs-IgG3 levels was that levels of age-adjusted Pfs-IgG1, although lower by 0.5-1.5km from the nearest
water body, were not significantly lower until >1.5km from the nearest water body.

6. The conclusion is based upon school-aged children due to the low numbers of <5-yrs olds. It is probable that children <5-yrs old could be included in similar analysis, though the narrow age range would probably limit the approach if only the <5-yrs olds were involved. However, this may vary depending on transmission intensities and is something that could potentially be explored in future studies. A statement relating to why the conclusion is based on school-aged children is now added to the second last paragraph of the discussion.

7. The discussion is now 25% shorter than in the original manuscript. Large parts of the first two paragraphs have been removed and what remains has been compacted.

8. There are several points in this useful assessment of the original discussion, so these will be addressed separately.

8a. Organisation and content from other studies

We agree with the referee that the literature on serological responses to malaria antigens is difficult to interpret due to lack of standardisation of techniques. We have, therefore, adjusted the discussion as requested to include fewer references to studies that use different end points, particularly clinical, and the extent to which the response to other different malaria antigens is discussed, is also reduced.

8b. Use of technique in other areas – mixed Ag v recombinant Ag

We agree with the referee that using the technique laid out in the manuscript, that standardisation between studies in difference areas, would not be achieved and that the approach of using recombinant Ag is more likely in the long-term to yield a maker that could be used for standardising approaches across studies. However, we think that the approach taken is novel, particularly in the use of age-adjusted, rather than actual Ab levels, in the context of microgeographical variations in exposure, and would be applicable to other studies in which internal variations in exposure levels could provide information on the aetiology of morbidity and influence of other pathogens. To make it clear that this is what we propose from our results, we have changed the sentence “Mapping school-aged children’s serological responses is, therefore, likely to be an approach that is applicable to other endemic areas” to “Mapping school-aged children’s serological responses is, therefore, likely to be an approach that is applicable to studies in other endemic areas, where internal comparisons of relative exposure to *Plasmodium* infections would be informative”.

9. Sample numbers have been added to each of the figure legends.