Author's response to reviews

Title: Increased deposition of C3b on red cells with low CR1 and CD55 in a malaria endemic region of western Kenya: Implications for the development of severe anemia

Authors:

Collins O Odhiambo (codhiambo@wrp-ksm.org)
Walter Otieno (wotieno@wrp-ksm.org)
Christine Adhiambo (cadhiambo@wrp-ksm.org)
Michael M Odera (modera@wrp-ksm.org)
Jose A Stoute (jose.stoute@us.army.mil)

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Author's response to reviews: see over
23 April 2008

Dr Lolu da-Silva
Senior Assistant Editor, BMC-series journals

Dear Dr. da-Silva,

I would like to thank you and the reviewers for their time taken to read and comment on our manuscript. I think the comments and suggestions have certainly improved the quality of the manuscript.

In response to the reviewers' comments, especially those of reviewer #2, we have made extensive revisions to the manuscript as follows:
1. Changed the analysis model from linear regression to non-linear ANOVA.
2. Expanded Tables 3 and 4 to include adjusted and unadjusted models.
3. Completely revised the discussion to address the findings of the model and the tables.
4. Revised the figures to accommodate comments from reviewer #2. Deleted Figure 1 and expanded Figure 3.

In addition, the manuscript underwent further internal editorial review and changes were made to correct typographical errors, omissions, and improved clarity throughout. All changes are highlighted in yellow.

In response to your questions regarding the ethical approval and consent, the project was approved by the National Ethical Review Committee, Nairobi, Kenya, and the Human Use Research Committee of the Walter Reed Army Institute of Research, Silver Spring, Maryland. This information was contained in the original version of the manuscript but we have reworded that section to enhance clarity (Page 6, second paragraph). Consent was obtained from adults and/or parents/legal guardians of children prior to any evaluations or procedures. Because we do not reveal any identifying information, consent for publication was not required. It is not routine for our consent forms to include a clause for consent of future publications.

I hope the above responses and the point-by-point responses attached satisfactorily address all the issues of concern. However, I will be happy to answer any additional questions that may arise.

Sincerely,

José A. Stoute, M.D.
Reviewer: Jørgen Kurtzhals

Minor essential revisions:
1. P. 7, l. 8-9: Please provide details on the criteria for selection of study subjects. (e.g. random selection from a census?)

R: The inclusion and exclusion criteria are outlined in page 7. In the second paragraph of page 7 we have added that “All individuals who met the entry criteria were invited to participate. On the days of recruitment individuals or parents self-reported to the clinic for enrollment in the study.”

2. P. 7, l. 13-15: The paper relies heavily on the fact that patients were parasite free. Please indicate the detection level of the microscopy (i.e. were 1000 WBC counted in thick smears?) Who did the microscopy and how were results quality controlled?

R: Negative smears were required to have a minimum of 200 high power fields scanned. The parasite densities were reported per 500 WBCs. Quality was controlled by double reading the slides and adjudicating the discrepancies by third microscopist. In addition, all our microscopists undergo rigorous periodic testing every three months using unknown slides. Satisfactory completion of the tests is required for each microscopist to continue to work reading slides. We expanded on this on page 8 under “Blood samples and smears”.

3. P. 11, l. 10-12 plus Figure 1 appear irrelevant and could simply be mentioned briefly in the introduction.

R: This seemed to be the only unanimous point of confusion. Therefore, we have elected to drop this figure and the reference to this sample of volunteers.

4. P. 13, l. 3-7: This paragraph is not easily comprehensible to me. What does it mean? I cannot see the described pattern in Figure 4b-c, and why suddenly an interpretation in the results section? Should probably just be omitted.

R: We have deleted the paragraph in question.

P. 16, first paragraph: Please refer to comment 2 above: C3b deposition could be caused by previous infection but in this area one could expect higher than
50% asymptomatic carriers (as reported in Table 1), and it is very likely that some study subjects were actually harbouring parasites below the detection limit. In fact, the treated subjects are probably more likely to be parasite free at the time of blood collection than the untreated subjects. This should be discussed.

R: The discussion has been extensively revised. We have added the sentence “It is also possible that some or many of these individuals have levels of parasitemia below the limit of detection of microscopy.” to the end of the paragraph on page 17.

P. 16, last paragraph: I agree with proposed reasons for low parasitaemia in the very young. However, it could also be due to selection bias. Thus, the very young children are non-immune and any high parasitaemia would be clinically important and either exclude the child from enrolment or lead to treatment.

R: The discussion was extensively revised. The age distribution of the parasite prevalence for all the individuals screened showed a similar distribution as that of the individuals included except higher prevalence distributed across all the groups. We have noted this in page 13 under “Red cell C3b, malaria prevalence, and parasite density”. Therefore, selection bias seems unlikely on this basis. In addition, we did not exclude potential participants on the basis of symptoms unless they had severe anemia (1 case).

Discretionary revisions:

1. There is too much use of non-standard abbreviations. E.g. p. 9, l. 1: antibody binding capacities (ABC) and l. 4 ICs (meaning immune complexes)

R: We have removed the usage of “ABC” on page 9, except in figures, but retained the use of “IC” to stand for immune complexes because this is a frequently used phrase in the manuscript and it is a standard abbreviation used by us in previous publications.

P. 12, last line-P. 13, l. 2: The authors should not overinterpret single observations on only 6 individuals.

R: The six individuals did have a low CR1 together with high C3b. The overall results of the study suggest that low CR1 is linked to high C3b. Therefore, we do not believe it is an overinterpretation. Nonetheless, the reader can judge this independently as the N is stated.
2. There are some typing errors, including missing words that sometimes disturb the reading (e.g. p. 13, 2nd line from bottom, add 'change' before the last word). However several other places, please go through this.

R: We have corrected this error and made editorial corrections in other places where appropriate.
Reviewer: Jim Todd

Reviewer's report:

This is an interesting paper, trying to establish the role of different complement receptors and immune complexes in severe anaemia or cerebral malaria in young children. The study design is good, and it has been carried out in an appropriate way. I have a few comments mostly on the explanations given in the paper.

Major Compulsory Revisions

1. Background 3rd paragraph, page 5. The second sentence could better explain the role of CD55 and CD35 in the activation and binding of C3b molecules. This seems to be a general statement about their role, and it is not clear what the question is that will be answered by this paper. Some indication of this is found in the first paragraph of the discussion, but the rationale for those objectives should be given in the background.

R: We have extensively revised the background section (page 5) to clarify the mechanism of action of CR1 and CD55.

2. How are C3b cells implicated in malarial infection along with other C3 convertases? This is further confused as the power calculation is given for differences in CR1, making the outcome for the paper unclear. This could be better explained, both in the rationale in the background and the methods where the power calculations are given.

R: We have added a sentence at the bottom of page 5 and top of page 6 to address this. C3b coated red cells are more susceptible to erythrophagocytosis. Regarding the sample size calculation, this was done using CR1 levels and not C3b levels because we had no previous data on the latter. An explanatory statement has been added to the second sentence, second paragraph, page 7.

3. Methods, 1st paragraph, page 6. The first sentence mentions 84 children from a previous study. It is by no means clear what role these children had in this work, apart from providing the basis for the age selection. I would suggest this is confusing, and that explicit reference to these children could be removed, both in methods and the first paragraph of the results.

R: We have done as suggested.
4. Methods, Stats analysis, page 10. The ANOVA says that differences were compared across age groups, using independent t-tests. Yet in the results, no t-test results are given in the text, the ANOVA results are given in the figures, and the multiple regression gives results on the age (months), which is presumably a linear effect across age. I would suspect a linear effect for this analysis would be inappropriate, as the graphs do not suggest a linear relationship between C3b and age, or between haemoglobin and age.

R: The legend for Figure 3 shows the results of independent samples t-test between malaria-treated and malaria negative individuals for different age groups. ANOVA, as opposed to linear regression, is now used to study the association between independent and dependent variables. Tables 3 and 4 were revised accordingly also in response to other comments below.

Minor Essential Revisions

1. Methods, general. Where did this study get ethical approval? The only reference to ethical review was for the 84 children in the previous study.

R: The original version contained this information but we have reworded it to make it clearer. The study was approved by the Walter Reed Army Institute of Research Human Use Research Committee and by the National Ethical Review Committee, Nairobi, Kenya (page 6, second paragraph).

2. Methods, 2nd paragraph, page 6. The power calculations are for CR1, which does not sit well with the title and research question raised. If the study really was powered for CR1 as the outcome, then it would be better to omit this from the paper. If there are relevant power calculations for haemoglobin or C3b then these should be given (even if calculated post-hoc).

R: As mentioned above, we have modified the explanation of the power calculation (page 7, 1st paragraph) to mention that this was done based on CR1 because we had no C3b data at that time. Therefore, we would prefer to leave this section as it is. Nonetheless, we will be glad to delete any further reference to it if the reviewer further objects.

3. Results, 1st paragraph. I would suggest removing the first paragraph, and figure 1, as these seem to have little relevance to the main results. Figure 1 has different age categories to those in the main analyses.

R: We have done as suggested.

4. Results, 2nd paragraph, page 12. I would suggest taking the ANOVA results away from the graphs (figures). The ANOVA results are just the crude (uncorrected) analysis of the outcome by age. The p-values in Figure 4 seriously impede the visual understanding
of the graph, and the multiple p-values are not clear. If these need to be presented then they should be included in the text. For the associations with C3b and haemoglobin, it would be best to include these as a separate row in Tables 3 & 4, showing the uncorrected effect of age. The it would be possible to see the effect of confounders by comparing the crude analysis with the adjusted analysis, adjusting for CR1, CD55 and malaria parasitemia.

R: We have moved the P values to the legends as suggested. Tables 3 and 4 now show the uncorrected (unadjusted) and corrected (adjusted) analyses.

5. Results, Multiple linear regression. A crude analysis could be given in the tables to allow the reader to see the effect of adjustment. It is extremely unlikely that the outcomes are directly and linearly related to age. It would be more useful if the multiple regression allowed for the effect of the age groups, which were defined and used in the sample selection in the first place.

R: See the previous response. Also, we have included the age groups as a categorical variable instead of the age as a continuous variable. However, we collapsed the groups from <6 to ≤36 months to decrease the number of categories and cut down on multiple comparisons.

6. The discussion is well argued, and they correctly allow the fact that increased C3b cells could be due to malaria infection, or the malaria treatment. In the last 2 sentences of the discussion TNF-alpha is introduced, which may be rational, but needs further explanation (or dropping from this paper).

R: We have expanded this last point to enhance clarity.

Discretionary Revisions

1. Methods, 2nd paragraph, page 7. Were the subjects tested for HIV, and specific bacterial infections?

R: No.

2. Methods, 3rd paragraph, page 7. Individuals were screened, and re-screened, and each time asked to come back after 2 weeks. How many times did this happen? Presumably these individuals are defined as ‘malaria treated’ in the analysis, but this is not made explicitly clear. Individuals who were repeatedly re-screened may be suffering from a complication, and might provide some bias in the analysis. Was any attempt made to re-analyse the data excluding the 10 individuals who were only included after the third screen?

R: The answers to these questions can be obtained from Table 1. There were three rounds of screening. 190 were enrolled in the first screen which means that they were smear negative. 194 were referred to the second screen, most of these due to positive smears. Of these, 151 were enrolled after the second screen. Of those enrolled, only 3 were enrolled after the third screen. This is a very small minority (0.9%) unlikely to bias
the results in any way. We have clarified the usage of “malaria-treated”, page 13.

3. Results, 3rd paragraph. What does the word ‘discordant’ mean in the second paragraph? If it means the age pattern differs, then this should be said. It may be useful to show some graphs of the association between the 2 outcomes (haemoglobin and C3b) and the potential explanatory factors (CR1 and CD55).

*R: The term discordant is used to refer to the fact that the parasite density seems to peak earlier than the parasite prevalence. This is explained under “Red cell C3b, malaria prevalence, and parasite density”, page 13.

4. The sentence explaining the “B” or coefficient, is not needed in a scientific journal, although the implications of the unit change may need to be discussed in the Discussion.

*R: We have deleted this sentence.