Author’s response to reviews

Title: Serum IL-10 as a marker of severe dengue infection

Authors:

Gathsaurie Neelika Malavige (gathsaurie.malavige@ndm.ox.ac.uk)
Laksiri Gomes (laksiri79@yahoo.com)
Lukmall Alles (lakmaalallace@gmail.com)
Thashi Chang (thashichang@gmail.com)
Maryam Salimi (maryam.salimi@wolfson.ox.ac.uk)
Sachie Fernando (sachieuf@yahoo.com)
Kushan Nanayakkara (kushannanayakkara@ymail.com)
S D Jayaratne (laljayaratne@gmail.com)
Graham S Ogg (graham.ogg@ndm.ox.ac.uk)

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Author’s response to reviews: see over
Prof. Philippa Harris,
Executive Editor,
BMC Infectious Diseases.

Dear Prof. Harris,

Thank you for your consideration of our manuscript for publication with BMC Infectious Diseases. We wish to thank the reviewers for reviewing our manuscript and for the positive comments. We have addressed each of the comments raised by the reviewer below. Revisions made to the manuscript have been tracked.

Reviewer 2:
The authors measure blood cytokines in 259 patients during fever and in 65 of those a subset of cytokines is also measured at defervescence. The aim of the study is to assess whether IL-10 levels can be used as a predictive marker for severe dengue, comparing levels in the first and the second sample.
The introduction is well written and some of the numerous studies measuring the same cytokines in dengue patients are cited. The methods used are accurate.
The conclusion is that IL-10 is not useful as a predictive biomarker, and the authors mention the large variability within the patient groups, representing the complexity of dengue pathology and its underlying mechanisms.
The conclusion that dengue is an extraordinarily complex disease has been reached in many other studies, and there is a consensus that one biomarker alone will not be sufficient to predict severity with a reasonable sensitivity. It is therefore somehow surprising that the authors try to assess the predictive value of IL-10 alone.

Response: We wish to thank the reviewer for this comment and we certainly agree with her. Dengue infections are a very complex and dynamic disease and it is clear especially from our work and others a single biomarker cannot be used to predict severe dengue. However, from our previous work we found that IL-10 was associated with apoptosis of T cells and lymphopenia and therefore, we were interested in IL-10 levels in dengue. IL-10 is an immunosuppressive and immunomodulatory cytokine and we felt it was important to highlight the fact that apart from proinflammatory cytokines, immunosuppressive cytokines
too are likely to be implicated in dengue disease severity. Although, not surprisingly, IL-10 was found to be a poor predictive marker of severe dengue, levels were significantly higher in patients with shock and levels also correlated with markers of liver injury. Therefore, our attempts to find out if IL-10 can be used as a biomarker were not very successful, we believe that this data is likely to highlight the fact that pathogenesis of dengue infections is more than merely due to proinflammatory cytokines, but that immunosuppressive cytokines too are likely to play a role.

**Major compulsory revisions:**

1. The authors mention that the value of this study lies in the fact that a “large cohort” of patients was analyzed. Even though the number 259 is certainly large, the critical analysis including the second time point was only done in a subset of patients, and it is not clear from the tables and figures how many patients were included for those calculations. N=… should be added to all tables and figures.

Response: Apologies for the lack of clarity. We have added the number of patients included in the analysis in all figures. N= had already been included in the relevant tables.

2. Primary and secondary patients were mixed for the analysis. It could be more informative to split these two groups and calculate correlations separately. However it might be difficult to assess the status if the first sample is drawn only at day 7 (IgG is already present even for primary). How did the authors do that?

Response: we wish to thank the reviewer for this suggestion. We repeated our analysis with after splitting these groups separately. We did not observe any difference in IL-10 levels in primary dengue in patients with severe and non severe dengue. However, the IL-10 values were higher in those with secondary severe dengue. These results are included in the revised manuscript. We agree that in primary dengue infection, IgG antibodies start to appear around day 7. However, the IgG antibody titres on day 7-8 are very low. The commercial ELISA that was used in determining IgG antibodies states that the IgG cut offs are set at a higher level to detect secondary dengue infection. This ELISA assay has been validated as both sensitive and specific for primary and secondary dengue virus infections (Vaughn et al, 1999; Sang CT et al, 1998).

3. The figure numbers in the text do not tally with the figures. Figure 1 and 2 do not seem to be mentioned at all in the text and figure 4 as mentioned in the text is missing.
Response: We are sorry about the mix up regarding the figures. We have amended these in the revised manuscript.

4. Why does IL10 have to rise or fall after the febrile phase to be suitable as a predictive biomarker? (discussion). The important value is the febrile phase IL-10, and that value does not necessarily seem to correlate with eventual severe disease, hence is not a suitable predictive biomarker.
Response: Although we did collect the first sample in the so called febrile phase, some of the patients may have already been in the critical phase (fluid leakage phase). As the only means to detect fluid leakage was by clinical assessment of fluid leakage (detection of pleural effusions or ascities) and by the rise of the haematocrit, mild degrees of fluid leakage would not have been detected. So in fact although we assume that patients were in the febrile phase the day of admission (day 4 of illness), there is a possibility that some may have been in the critical phase at this time. We believe this could be one reason why its difficult to find a biomarker. We have discussed this in the revised manuscript.

5. The comparison with MCMV is very hypothetical, the pathology of the two viruses is rather different.
Response: We wish to thank the reviewer for this comment. We have deleted this in the revised manuscript.

Reviewer 3
Major compulsory revision
(1) Most of their patients presented late at days 4-5 rather than the first 3 days, thus limiting comparability of their data with other studies. This should be discussed.
Response: We thank the reviewer for giving us an opportunity to discuss this. As the patients recruited in our study are adults, it is quite unusual for them to seek medical care until at least 3 days of fever. This is especially so in countries such as Sri Lanka due to poor over crowded hospital wards. Patients with dengue infection are cared for in general medical wards, which also admit patients with other infectious diseases, myocardial infarctions, stroke etc… Due to the overcrowding many are not provided a bed or have to share a bed with another patient. Therefore, most patients delay admission to the wards until day 4-5 of illness.
(2) Specify what laboratory investigations were done several times a day as the following sentence stated that there was resource limitation.
Response: Full blood counts were done several times a day in all patients. However, in patients who had more severe dengue, AST/ALT were done daily along with serum electrolytes, urine full report, APPT, PT, etc…Due to reasons specified in answer to question (1) it was not possible to do all laboratory tests daily in all patients.

(3) WHO 2009 defined severe dengue as severe plasma leakage, bleeding and organ impairment. The authors have selectively defined SD as "shock" or AST/ALT >1000. Please specify in Results per WHO 2009 criteria.
Response: we thank the reviewer for giving us the opportunity to clarify this. Since none of our patients had severe bleeding, this was not taken as criteria to define severe dengue in our study cohort. The 2009 WHO criteria define organ impairment as Liver: AST or ALT >=1000, CNS: Impaired consciousness, heart and other organs. None of our patients had impaired consciousness or features of myocarditis. Therefore, the only type of organ impairment they had was liver impairment. However, we do realize that what we have mentioned in the manuscript is misleading and therefore, we have rephrased this in the revised version.

(4) Results should be in the right section: 15.4% had SD and 19.3% had primary dengue. This can be incorporated into a paragraph providing some information about the study: age, gender, % with any co-morbidity, warning signs, categories of SD, primary vs. secondary, bleeding.
Response: We agree that it is very important to include these data. They have been included in the revised manuscript.

(5) Under cytokine assay in Methods, they mentioned IFN was measured in 78 but the abstract mentioned 65. Please check. Additionally they mentioned viraemic phase but no PCR or NS1 was apparently done, so please delete or change to " samples collected at presentation on median of day x".
Response: We thank the reviewer for pointing this mistake. We have corrected the numbers and the other relevant information in the revised manuscript.

(6) Results, paragraph 2, line 5, do the authors mean 48 rather than 8?
Response: Yes we do mean 48 and not 8. Thank you for pointing this out.

(7) Discussion, paragraph 1, lines 6-7, on influenza and rotavirus, the information is not pertinent, please delete.
Response: We have deleted this as requested by the reviewer.
(8) Discussion, paragraph 5, lines 2-6, on HIV, the information is not pertinent, please delete.
Response: we have deleted this as requested by the reviewer.

(9) Please incorporate all data in figures into Results with median values and ranges, and CI and P values of statistical comparison.
Response: We agree this is important and we have included all this data in the revised manuscript.

(10) Table 1, please delete column 4 on ROC value.
Response: we have deleted this in the revised manuscript as requested by the reviewer.

Minor essential revision

(1) Spell all acronyms in full at first mention: WHO, IL, IFN, GM-CSF, MIF, MIP, VEGF
Response: We have spelt all the acronyms in full in the revised manuscript.

(2) Harmonise US or UK spelling but not both: "haemorrhagic" (UK), "minimise" (US)
Response: we apologise for the different forms of spelling which we have corrected in the revised manuscript.

(3) Background, paragraph 1, line 5, do the authors mean to say "several" rather than "severe"?
Response: yes we did mean several, which we have corrected.

Reviewer 4

Major Compulsory Revisions

1. Abstract: The authors need to be cautious in the use of terms ‘severe dengue’ and ‘acute dengue’ and clarify what they mean by acute here. In general I suggest using the terms defined in the WHO 2009 guidelines (used by the authors) namely- DF and DHF or DSS.
Response: We wish to thank the reviewer for raising the classification issues of dengue disease severity. As mentioned we have used the 2009 WHO classification criteria, which classifies dengue was dengue with warning signs and severe dengue (see more details in response to question 3).
2. Abstract: Providing p values in the results section does not have any benefit and only serves to clutter the paragraph. I recommend removing this and sticking to a summary description of results.

Response: We thank the reviewer for this comment and would like to delete all the p values from the abstract as requested by him. However, reviewer number 3 actually wanted more data listed such as the median, range and p values wherever possible. Therefore, we have left the p values as they are.

3. Methods: Paragraph-1: The authors should clarify how they have calculating day of infection (e.g. ay-4 day-5 etc). Is this days since onset of fever (fever days) or is it days since admittance to hospital. This is a very important distinction. Also the authors should provide a clearer definition of severe dengue- The 2009 guidelines call for labeling dengue as DF and DHF or DSS. Why are the authors using the label ‘severe dengue’ which is very confusing to the reader.

Response: We wish to thank the reviewer for his comments regarding timing of sample collection. We have clarified this in the revised manuscript. The 2009 WHO criteria classifies dengue disease severity as dengue with warning signs and severe dengue (see below). In the 2009 WHO disease classification the following statement is mentioned regarding disease classification:

Changes in the epidemiology of dengue, as described in the previous sections, lead to problems with the use of the existing WHO classification. Symptomatic dengue virus infections were grouped into three categories: undifferentiated fever, dengue fever (DF) and dengue haemorrhagic fever (DHF). DHF was further classified into four severity grades, with grades III and IV being defined as dengue shock syndrome (DSS) [29]. There have been many reports of difficulties in the use of this classification [30–32], which were summarized in a systematic literature review [33]. Difficulties in applying the criteria for DHF in the clinical situation, together with the increase in clinically severe dengue cases which did not fulfill the strict criteria of DHF, led to the request for the classification to be reconsidered. Currently, the classification into DF/DHF/DSS continues to be widely used. [29]

Therefore, in the 2009 WHO guidelines the disease severity is classified as follows:
Although the 2011 WHO guidelines again classify dengue disease severity as DF and DHF, in our study we used the 2009 WHO guidelines as this was used to classify patients. We do agree that this is somewhat confusing, but we have strictly followed the 2009 WHO guidelines.

Reviewer: Also the criteria for classifying their patients as severe dengue (DHF I am guessing) are inadequately described. Overall, a separate table detailing the features of the cohort, exact numbers, age group, primary vs secondary; severe vs non severe will provide clarity.

Response: We fully agree with this comment. Therefore, as requested by reviewer 2 we have included a paragraph at the start of the results section describing these features.

4. Methods: Paragraph-3: What precise method was used to measure cytokine levels. Is this an ELISA kit? if so what was the serum amounts used and the dilution for the ELISA.

Response: As described in the methods section, quantitative cytokine assays were done in duplicate on serum according to manufacturer’s instructions. The dilutions for the ELISA and the volume of serum used were according to what the manufacturer had recommended.
5. Methods: There is no description of how transaminase levels/activity was measured. Response: We thank the reviewer for giving us the opportunity to explain this. The transaminase levels are routinely performed in the hospital in all patients with dengue infection. They use an autoanalyser for this purpose. We did not perform the transaminases or the other routine investigations that are carried out in the hospital such as the full blood counts, serum electrolytes etc… in our laboratory. We recorded the laboratory findings of these patients from their clinical records.

6. Results: Para-1: A lot of information here belongs to the figures and tables (and appears to be repeated here). The results indicate platelet counts and white cell counts etc. but in the actual table (table-1) the phrase ‘lowest platelet count’ etc is seen. What is this measurement? (lowest compared to what?). I would be more useful to present this data in the two different time points as potentially bar charts.

Response: We again wish to thank the reviewer for giving us the opportunity to explain this. The full blood counts were done in all patients at least twice a day since admission to hospital and we have recorded all this information. The phrase lowest platelet count refers to the lowest platelet count seen throughout the illness. We have amended this in the revised manuscript.

7. Results: Para-2: Overall measurements in both febrile and critical phase samples were only done in 65 patients. Why was this and how were these patients chosen? Only 7 of them had severe dengue. Why not chose more from the 40 severe patients to have a balanced set for their analysis. As it is it is impossible to conclude with any degree of confidence that these differences are statistically meaningful as far as severe and non-severe are concerned.

Response: we wish to thank the reviewer for this comment. We did not chose the 65 patients for paired serum samples. Although we wished to collect paired serum samples from all, since the majority of patients were bled at least twice a day (some four or five times a day), many were reluctant to provide another sample for research. Again those with more severe forms of dengue were bled more frequently and were less likely to provide another blood sample. We do agree more patients with severe dengue would have been better for statistical analysis but this was not unfortunately possible.

8. Results: Para2: The authors state “Serum IL-10 levels rose in 17/65 of these patients, while serum IL-10 levels fell in the other 8 patients (Fig 1B)”. Do they mean other ‘48’ patients?
Response: thank you for highlighting this. We do mean 48, which we have corrected in the revised manuscript.

9. Results: Para-3: The authors mention they measured interferon levels in only 78 patients. How many of these were severe dengue and why were these subset of patients chosen? It is unclear why the authors chose to examine interferons and why this is not a part of their overall conclusions. Did the levels of interferons differ between severe and non severe dengue patients?
Response: Serum IFNγ and IFNα were only done in the first 78 patients recruited for the study. IFNα levels were similar in patients with severe and non severe dengue, and the changes in IFNγ were not very distinct. As there was a limited budget, based on the results of the 78 initial patients, we did not measure these 2 cytokines in the rest of the patients.

10. Results Para4: Although MIF was measured in 65 paired samples it is not mentioned whether the levels reported are from the febrile or critical samples.
Response: We apologise for the lack of clarity. We have amended this sentence in the revised manuscript.

11. Results Para5: The authors have mentioned that 50 patients in their cohort had primary infections. In reporting differences in cytokine production between primary and secondary did the authors combine all primary in one grp and secondary in another irrespective if disease severity? Did any of the primary infections result in severe disease?
Response: We wish to thank the reviewer for this very important question. 9 (18%) of those with primary dengue infection developed severe dengue. We have included more data regarding the levels of IL-10 in those with severe primary dengue infection and non severe disease

12. Results Para6: Have the authors tried to combine multiple cytokines and test if this can improve classification of severe patients? A multiple regression approach can provide greater insight into this.
Response: This indeed is a good suggestion. However, since all the mentioned cytokines were not measured in all the patients, we did not perform this analysis.

13. Discussion: Para 3: In the paired samples, number of severe patients is too small to make a significant conclusion.
Response: we agree that comparing data of 7 patients with 57 patients in not very significant. However, we did see some interesting patterns and therefore, we thought it would be interesting to report our findings.
14. Discussion Para-6: The authors make an interesting suggestion that IL-10 levels maybe a better indicator of liver inflammation. However the correlation coefficients appear to be too low to inspire this confidence. The authors should be careful how they interpret the correlation values.

Response: We are sorry that the reviewer misinterpreted our sentence. We were not at all trying to imply that IL-10 was a better marker of liver inflammation. We have mentioned that it correlates with serum ALT which is a better marker of liver inflammation as it is liver specific than AST because it is produced by skeletal muscles as well.

- Minor Essential Revisions

1. The authors should ensure their figures and figure legends are compatible. As it is the numbering of the legends and figures are do not coincide.
Response: we have corrected these in our revised manuscript.

2. Figure legends should provide a concise but complete description of the figures and not just provide a title.
Response: we have provided a better description in the revised manuscript.

3. There is too much data provided within the text in the form of cytokine levels, p values, etc. The results section should provide descriptions and use figures to display all data. In the current format the reader will find it impossible to follow the authors’ rationale
Response: We thank the reviewer for this comment. However, the third reviewer wanted all the information in the figures mentioned in the text.

4. Result sections does not refer to figures adequately in the text. There is no mention of figures-3-7 in the text at all.
Response: we apolgise for this error. We have amended this in the revised manuscript.

5. Figures or legends should indicate ‘n’ (number of samples evaluated) for each plot, correlation.
Response: We have amended this in the revised manuscript.