Author's response to reviews

Title: The association between the ratio of monocytes:lymphocytes at age 3 months and risk of tuberculosis (TB) in the first two years of life.

Authors:

Vivek Naranbhai (vivekn@well.ox.ac.uk)
Soyeon Kim (skim@sdac.harvard.edu)
Helen Fletcher (Helen.fletcher@ndm.ox.ac.uk)
Mark F Cotton (mcot@sun.ac.za)
Avy Violari (violari@mweb.co.za)
Charles Mitchell (cmitche@med.miami.edu)
Sharon Nachman (Sharon.Nachman@stonybrookmedicine.edu)
George McSherry (gmcsherry@hmc.psu.edu)
Helen McShane (Helen.mcshane@ndm.ox.ac.uk)
Adrian VS Hill (adrian.hill@ndm.ox.ac.uk)
Shabir A Madhi (madhisa@phru.co.za)

Version: 2 Date: 9 May 2014

Author's response to reviews: see over
Dear Dr D’Souza,

RE: Response to reviewer’s comments

Thank you for co-ordinating the review of our manuscript “2435299671185919 - The association between the ratio of monocytes:lymphocytes at age 3 months and risk of tuberculosis (TB) in the first two years of life” and for offering us an opportunity to respond. We have adhered to the requested editorial changes by:

• Formatting the abstract for a research article. (Lines 57-90)
• Confirming that the study had approvals from the relevant ethical review committees and that we obtained informed consent from parents/guardians. (Lines 157-162)
• Amending the author contribution section (Line 358)

We thank Professors Mayanja, Maeurer and Dickman for their careful and constructive review of our manuscript and the opportunity to enhance our manuscript. Based on their comments we have made the following overall changes to enhance the manuscript:

• Revised the introduction to expand on the statements we made in the original version to enhance readability
• Removed figure 1B as, based on the reviews it appears to be misleading and the original analysis was conducted allowing for non-linear relationship between ML ratio and TB disease by using fractional polynomial models in any case.
• Finally, we have revised the discussion to enhance readability and have indicated our interpretation of the clinical meaning of the findings in the context of existing tools to stratify TB risk

Below, we provide a detailed response to each review comment. The attached manuscript file contains the revisions highlighted. We trust that our responses have addressed the comments from the reviewers.

Sincerely,

Vivek Naranbhai on behalf of the authors
Point-by-point response:

Reviewer #1: The manuscript is overall well written with a relevant message where the authors show that baseline Monocyte Lymphocyte ratio is predictive of progression to active TB in children. The paper follows onto other work in animals and adults published by other authors. There is adequate reference to the primary study which is already published.

Reviewer comment #1.1. Background - authors need to check references 1 and 2 - they do not seem to be related to the text - lines 74 to 77

Author response #1.1
We offer sincere apologies for this electronic referencing error. The references have been corrected. The correct reference in lines 97 and 99 respectively are:
And
and,

Reviewer comment #1.2. Reference is made to Fletcher paper on 2 to 3 occasions; this unpublished paper was sent as an addition to the submitted paper. However the readers will not have access to this paper, unless it is published before current manuscript.

Author response #1.2
We acknowledge the fact that this work links to, as yet, unpublished work from Fletcher et al. We have therefore referenced a presentation of this work in a peer-reviewed conference (line 118). Thus work is informed largely by work prior to Fletcher et al., as we describe in the revised introduction, and does not rely on the findings of Fletcher et al.

Reviewer comment #1.3. The subheading "176 Baseline ML ratio is associated with TB disease free survival" does not seem to tally well with the following text, such as the lines "Specifically, the ML ratio was 199 significantly associated with TB disease alone or in combination with all-cause 200 death and latent TB infection." Since this section of results most strongly reflects the title and abstract, the text part of the results needs more clarification and explanation of the statistics in text form.

Author response #1.3
Thank you for pointing out this discrepancy. We have amended this section as follows. We have added a new section heading:
"Baseline ML ratio is associated with probable or definite TB rather than all-cause mortality or latent MTB infection" (line 242)

We have expanded the paragraph reporting the findings in full explaining the statistics in the text:
"To explore whether the ML ratio was specifically associated with TB disease or with other outcomes, we tested whether the association between the ML ratio and TB disease was robust to
alternative endpoint definitions (Table 1). The ML ratio was significantly associated with elevated hazards of TB disease alone (HR 1.23, 95%CI 1.04-1.45, p=0.02) and with elevated hazards of TB disease plus all-cause death (HR 1.18, 95%CI 1.02-1.37, p=0.02) or TB disease plus latent MTB infection (HR 1.21, 95%CI 1.04-1.41, p=0.02). Increasing the stringency of the case definition by excluding individuals with possible TB and including only definite and probable TB diagnoses strengthened the association between ML ratio and TB disease (HR 1.50, 1.19-1.89, p=0.006). The ML ratio was not significantly associated with all-cause death (HR 1.25, 95% CI 0.99-1.57, p=0.06). Amongst HEU children, with TST at 96 weeks, 33 of 725 (4.5%) had latent MTB infection. There was no association between baseline ML ratio and latent MTB infection (HR 1.00, 95% CI 0.66-1.5, p=0.99) neither was there evidence of interaction between ML ratio and INH prophylaxis. No INH effect was observable in a model of INH effect across the ML ratio spectrum. Lines 244-260.

Reviewer comment #1.4. Figure 1A labelling could be in sync with the text use of HEU which in the table are labelled as HIV negative.
Author response #1.4
We have amended Figure 1A label by adding HEU as the label. Line 453.

Reviewer comment #1.5. Cut off for TST should be included in text rather than being assumed
Author response #1.5
The criteria for diagnosing latent MTB infection has now been reported in full: "Latent MTB infection was defined as a positive TST (induration ≥5 mm in horizontal diameter in HIV-infected children and ≥10 mm in HEU children) in the absence of evidence of active disease as per contemporary guidelines [15]. The criteria used for categorizing TB as either possible, probable or microbiologic confirmed (definite) have been previously detailed[14]." Lines 180-185.

Reviewer comment #1.6. Spacing before a bracket needs to be reviewed in a number of areas
Author response #1.6
This typographical error is corrected in the revised manuscript.

Reviewer #2: The authors analyze the association of monocyte / lymphocyte ratio in the risk to develop clinical TB. This is a very interesting study that takes up earlier observations concerning the role of monocytes and (potential) markers for immune protection / risk for TB.

Reviewer comment #2.1. Please rephrase the statements in the manuscript, they are unclear and difficult to read.
2.1a. Lines 81-84: Please brake down the sentences in individual statements and comment on the outcome of each study cited, since this is the key issue of the report.
2.1b. Lines 224-230: The reader gets lost with the statements here. Please rephrase if and cut it in digestable pieces, i.e. what has been shown and what is the biological / clinical meaning for the interpretation of your data.

Author response #2.1
The relevant sections have been expanded and reported in a more clear way as follows:
> Lines 81-84 of the original manuscript introduction have been replaced by a section that describes the findings and implications of each cited study in full:
> "Recent and historic studies suggest that the peripheral blood monocytes:lymphocytes ratio may be associated with subsequent mycobacterial disease outcomes. Fletcher et al. used whole-
transcriptome microarrays to examine leucocyte gene expression in 10 week-old BCG-vaccinated, HIV-uninfected infants for clues why some developed TB by aged 2 years whilst others did not[6]. They found that opposing levels of myeloid and lymphoid specific gene transcripts were more frequent amongst infants who later developed culture-confirmed TB disease, than amongst matched controls. Since the quantity of myeloid and lymphoid transcripts in peripheral blood are a marker of the frequency of leucocyte subsets, the transcript ratio may reflect leucocyte subset frequencies. However, these were not available in their study. Nevertheless, in support of the specific role of relative proportions of monocytes and lymphocytes in TB pathogenesis, studies performed between 1921 and 1930 by Florence Sabin and colleagues demonstrated in rabbit models of TB that “monocyte-lymphocyte ratio in the peripheral blood might be taken as an index of the progress and extent of the disease” [7]. Sabin and colleagues then demonstrated that experimentally altering the ratio by depleting or increasing monocyte frequency resulted in commensurate changes in rabbit survival following challenge with Mycobacterium bovis[7-9]. Recent results in cattle show in vitro that the ratio of monocyte-derived macrophages to lymphocytes is associated with inhibition of mycobacterial growth [10, 11]. Therefore, we recently reported that the ratio of monocytes:lymphocytes (ML) in HIV infected South African adults prior to combination antiretroviral therapy initiation (cART), was predictive of TB disease during the subsequent 5 years on cART [12]. In what appears to be the first and only study of the role of this ratio in childhood mycobacterial disease in 1928, Rogers studied the ML ratio in 41 children with either a negative tuberculin skin test, latent TB infection or active TB disease. He observed that the ML ratio correlated with the extent and severity of disease, but called for “the work [to] be checked up on a larger series”[13].” Lines 113-142.

→Lines 224-230 of the original manuscript discussion have been replaced by a section that reports how each of our findings contribute towards a specific element of causal inference: “Several factors support ML ratio being on the causal pathway for TB development. Firstly, monocytes are target cells for mycobacterial growth and lymphocytes are the major effectors for mycobacterial clearance. Second, there appears to be a dose or gradient effect with higher ratio’s being more predictive than lower ratios across the ML ratio gradient. Third, as we demonstrated, altered ML ratios precede active disease hence reverse causality is unlikely. Fourth, our data in children is consistent with in vitro findings. Fifth, there is overall coherence of this finding with experimental animal and observational adult studies. Finally, the association between ML ratio and subsequent TB disease has partial specificity. The association is stronger for ML ratio rather than monocyte counts and definite/probable TB rather than possible TB. We have also recently also reported that the ML ratio may be associated with childhood malaria incidence[19], hence the ML ratio may actually have pleiotropic associations with some childhood infectious diseases. However, the effect size seems modest.” Lines 280-294.

Reviewer comment #2.2. Line 131-133: Please clarify the background here. Latent TB was diagnosed by a positive skin test - Is that considered indeed to be indicative of latent TB in children? what is the clinical meaning of a positive skin test in the area where the study has been conducted? Could it be that individuals have been exposed, the skin test is positive and yet there is no latent TB? Please clarify this point and discuss here studies that support the information.

Author response #2.2

We acknowledge the challenges of defining latent MTB infection and therefore relied on the clinical definition of latent MTB infection in children shared by the World Health Organisation and guidelines in the region. We have added this statement to the manuscript “Latent MTB infection was defined as a positive TST (induration ≥5 mm in horizontal diameter in HIV-infected children and ≥10 mm in HEU children) in the absence of evidence of active disease as per contemporary guidelines [15]. The criteria used for categorizing TB as either possible, probable or microbiologic confirmed (definite) have been previously detailed[14].” Lines 180-185.
Reviewer comment #2.3. The report is based on statistical analysis and modeling outlined in the materials and methods section. This may sound clear for a statistician, yet not for the reader unfamiliar with the field. Please explain step by step each method; use supplementary data sets if necessary for more explanations if needed. Please make clear what you like to achieve / address with each methods.

Author response #2.3
We have revised the materials and methods section to make it more understandable (lines 190-206) and have tried to describe the results in sufficient detail to explain what we did and why. We hope this enhances the clarity of the manuscript.

Reviewer comment #2.4. Monocytes can also be infected by HIV and this is associated with the HIV variants. Do you have information on the HIV population in each patient and the level of HIV+ monocytes (associated with the tropism of the HIV isolate) ? This is part of the core of this report. Please discuss also the interaction of HIV with monocytes and their potential functional impairment.

Author response #2.4
Regrettably we do not have information on HIV tropism of the virus isolates from the infants in this study. Although we agree this could be an interesting avenue for future research, the association between ML ratio and TB incidence is present in both HIV infected and HEU infants in this cohort. We have tried to raise this possibility in the discussion by adding “A third possible explanation is that an altered ML ratio is due to a specific monocyte subset defect. Since HIV infects and alters monocyte function in a subset specific manner[22], it is possible that HIV exposure alters monocyte functions and ratios. The similarity in effect between HIV infected and HEU infants however mitigates the likelihood of the association being driven by HIV infection.” Lines 305-310.

And by recognizing that “Thirdly, although we observed association between ML ratio and incident TB disease in all HIV exposed infants regardless HIV infection status, further studies are required to establish whether the association is present in infants not exposed to HIV,” Lines 320-324.

Reviewer #3: I was asked to review the statistics methods, in particular the methods underlying Figure 1B. Tuberculosis is not within my realm of expertise, so I will try to focus on general statistical issues.
The manner in which the statistical methods are described and the results reported and interpreted instills me with a sense of confidence that the statistical analysis has been conducted by highly competent scientists.

Reviewer comment #3.1
I am concerned, however, at the decision to report the hazard ratios for a 1 unit increase in ML ratio given that 0.4 units covers around 95% of the distribution. Note that I am not questioning the decision to assume linearity (not at this point anyway) just the decision to report the HR for a 1 unit difference. For a 0.2 unit difference (which would be close to a comparison of the 75th percentile to the 25th percentile) the HR would be 1.17^0.2 = 1.03.
The HR of 1.17 is reported in the abstract without mention of the units. Readers would be entitled to interpret this as "infants with a high ML ratio have a 17% higher risk of the outcome than infants with a low/normal ML ratio". This is not my field, but it would seem that a difference of 0.2 units in ML ratio is quite large, yet
a difference in ML ration of that magnitude is associated with only a 3% higher risk of the outcome. I would encourage the authors to the results in terms of a clinically meaningful difference in ML ratio (whatever that may be).

**Author response #3.1**

We concur with Professor Dickman’s assertion that the unit increase we used is not very clinically useful. We have therefore presented more interpretable HR’s in the abstract and in the discussion per 0.1 unit increase. We also retain the main findings reported per 1 unit increase so as to allow comparison with previous studies in this area:

“Per 0.1 unit increase in ML ratio at 3 months of age, the hazard of probable or definite TB disease before 2 years increased by roughly 4% (~1.50.1). While significant, the modest effect size suggests that the ML ratio plays a modest role in predicting TB disease-free survival; its utility may therefore be limited to combination with other tools to stratify TB risk, or to study pathophysiologic determinants of TB disease.” Lines 274-279 and in abstract

In addition we have tried to contextualize the findings insofar as how the ML ratio may compare with other clinically used methods to stratify TB risk:

“Children with MTB infection are at risk for tuberculosis (TB) disease, therefore identification of these children is important. The current tools to identify children with MTB infection are tuberculin skin testing (TST) or interferon-gamma release assay (IGRA). Although isoniazid (INH) preventive therapy (IPT) is more effective in HIV-infected individuals with a positive TST result [4] neither TST nor IGRA are sufficiently good at predicting TB disease. In a recent meta-analysis, a positive TST or IGRA result in children or adults was associated with an increase in incidence of about 1.4-2 fold with wide confidence limits due to study heterogeneity [3]. Current World Health Organisation (WHO) guidelines do not support routine TST or IGRA testing before IPT provision in children [5].” Lines 97-107.

Our conclusion has therefore also been amended as follows “This association may be of value in further stratifying risk beyond or in combination with current tools such as TST or IGRA and in suggesting novel pathophysiological mechanisms of TB disease susceptibility.” Lines 329 and abstract.

**Reviewer comment #3.2**

I fear that you must add me to the list of reviewers who do not understand figure 1B. From the context, I understand that this analysis has been performed to assess the functional form of the association between ML ratio and the outcome. This assessment has resulted in the conclusion that an assumption of linearity is appropriate. Assuming this is the case (and I accept the possibility that I may have completely misunderstood), I don't understand the need to bootstrap and I don't necessarily support the conclusion.

The functional form of the association between ML ratio and the outcome can be assessed by modelling ML ratio using, for example, a fractional polynomial. Why the need to bootstrap? I could get a curve similar to the solid line in Figure 1B without bootstrapping. I would need to center ML ratio at some sensible value (e.g., the median) and the HRs in the figure would be the hazard ratio comparing each value of ML ratio to the median. What exactly do the values of the HR in figure 1B represent? This component of the analysis needs to be better described; I suggest both a conceptual description for non-statisticians (why is this analysis performed and what do we conclude) along with a technical description (possibly for a web appendix).

If I am correct that the purpose of the bootstrap analysis is to assess the functional form of the association between ML ratio and the outcome, then I
question the conclusion that a linear association is appropriate. Compared to infants with an ML ratio of 0.15, those with a 0.1 unit lower value have an increased risk that is of similar magnitude to those with a 0.1 unit higher value. This range of values (0.05 to 0.25) contains a significant proportion of the data. Is it possible that influential observations (infants with extremely high ML ratio) are driving the linear association? It's possible that I have completely misunderstood what has been done and my comments are nonsense, in which case please ignore my comments other than as motivation for providing a better description of what was done and how it should be interpreted.

**Author response #3.2**

We apologise for the confusion introduced by Figure 1B. Our a priori statistical analysis plan was that we would use fractional polynomials to model the ML ratio association with TB free survival, and Table 1 presents the result of this primary analysis. In all cases, a linear model was the most parsimonious and selected in the fractional polynomial models. We also attempted to independently evaluate the shape of the association but realize that the method we used is neither as robust not as interpretable as a standard fractional polynomial approach. Our original Figure 1B did not have 95% CI plotted, a major flaw of this figure. Moreover, it presented instantaneous HR, that is the ratio of hazards of TB or death at that specific ML ratio vs. all other ML ratios. Consequently we have decided to remove this figure entirely from the paper. We have retained the statement beneath table 1 reporting that the ML was tested as a fractional polynomial. We have also added the following to the methods section: “We explored non-linear fits with fractional polynomials, in all cases the best statistical fit was linear.” Lines 199-200.

**Reviewer comment #3.3**

page 7: “In contrast, neither the monocyte count nor the lymphocyte count alone was significantly associated with the primary study endpoint.” The association between ML ratio and the primary study endpoint was barely statistically significant, so I don't think comparisons with other potential predictors should be made using statistical significance alone.

**Author response #3.3**

We have included further justification for our interpretation that relies on our previous (larger) studies in adults, the original animal studies and in vitro data rather than statistical comparisons alone.

“This finding is consistent with previous studies in adult humans[12], in rabbits[9] and in vitro (data not shown),” Lines 237-238.