Author's response to reviews

Title: Genotype-independent association between profound vitamin D deficiency and delayed sputum smear conversion in pulmonary tuberculosis

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Version: 5 Date: 23 May 2015

Author's response to reviews: see over
23rd May, 2015

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BMC Infectious Diseases
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Dear Dr. Hilary Logan,

Re: Genotype-independent association between profound vitamin D deficiency and delayed sputum smear conversion in pulmonary tuberculosis

Thank you for your email of 28th April 2015, inviting submission of a revised version of this manuscript to BMC Infectious Diseases. Our response to reviewers’ comments is as follows:

Reviewer 1

Comment 1: Were sputum specimens from all patients who should a positive result at 2 weeks cultured with DST? This is not clear from the write up – in total how many sputum specimens were subjected to culture DST? Of these, who many patients were diagnosed as having MDR-TB

Response: We thank the reviewer for the opportunity to clarify this point. Drug sensitivity testing was performed for a subset of patients who were sputum-smear positive after 2 weeks of intensive-phase anti-TB treatment, namely a) those who had been previously treated for tuberculosis or b) those who were known to have contact with patients with confirmed MDR-TB. Of the 72 patients fulfilling these criteria, 16 were found to have MDR TB. The Methods and Results sections of the revised manuscript have been modified to clarify this point (page 6, 8).

Comment 2: Line 246. How was MDR-TB managed? Was the patient put on MDR-therapy? If there was a treatment change then how was the time to sputum smear conversion calculated?

Response: MDR-TB was managed according to the WHO guidelines for MDR TB treatment (1). Sputum smear conversion was calculated from the day of start of any antimicrobial treatment, and not from the date of change from one regimen to another.

Comment 3: Introduction. Some sentences are unnecessarily long and should be rewritten in a simpler manner such as those in ‘line 106-109’, ‘line 115-119’, line 298-304.
Response: We have broken these long sentences into shorter sentences as suggested (page 4).

Comment 4: Line 165, the definition for HPF with the acronym should be provided the first time it is used.

Response: We have provided the definition for HPF in line 165 as suggested (page 6).

Comment 5: Give details as to how MTB culture and DST was performed.

Response: Sputum samples from patients selected for drug sensitivity testing were initially assessed using the GeneXpert MTB/RIF platform (2). Samples which were positive on this molecular test were then cultured on Lowenstein Jensen medium, and colonies were sent for phenotypic drug sensitivity testing using the Bactec MGIT system as described (3). The Methods section of the revised manuscript has been updated with these details (page 6).

Comment 6: Figure 1 is alright but some labels on the right side of the flow chart are redundant. It is not necessary to say ‘0 died’ or ‘0 lost to follow up’ so these can be removed.

Response: Figure 1 has been modified according to the reviewer’s suggestion.

Comment 7: The data related to Table 4 regarding the multivariate analysis of the genetic polymorphisms should be placed in the Results section rather than the Discussion section.

Response: We have moved these data to the Results section as suggested.

Comment 8: The study makes the observation that reduced vitamin D levels were coincident with bilateral TB and delayed sputum conversion. However, it is not clear what was done when the vitamin D levels in these patients were found to be deficient. Was vitamin D supplementation provided? Were patients informed regarding their vitamin D status and advised to receive vitamin D supplementation?

Response: Medical staff caring for study participants were informed of their patients’ vitamin D status, and were free to manage this as they deemed appropriate after the participant had completed follow-up. This detail has been added to the Methods section of the revised manuscript (page 5 Line 130).

Comment 9: There are several reports of vitamin D levels in the Pakistan population, lowered vitamin D levels have been identified in patients with TB. Also, vitamin D levels are found to be reduced in women. If the general population already has reduced vitamin D levels then it is the trend of deficient and insufficient levels in TB patients the same as the general population or is to different? The authors should give their
hypothesis as to what they believe could be the reason for low vitamin D levels in TB in this population.

Response: We have conducted a case control study to compare vitamin D status in TB patients vs. healthy controls, which is the subject of a separate manuscript. These data show that TB patients have lower vitamin D status than healthy controls. We hypothesise that low 25(OH)D levels may have preceded the onset of active TB, and impaired containment of latent TB infection, thereby leading to the development of active TB. This hypothesis is supported by findings of longitudinal studies conducted in Pakistan (4) and elsewhere (5) reporting that vitamin D deficiency in patients at risk of active TB precedes the development of active disease. However, as this is an observational study, reverse causality or residual confounding cannot be ruled out as explanations for this association. We have modified the second paragraph of the Discussion to address the issues raised by this comment (page 11).

Reviewer 2

Comment 1: One of the limitations in the present study (which the authors themselves have mentioned in the discussion) is that drug sensitivity was not done for 72% of the isolates. Although the authors claim that sensitivity assay was done for samples which were smear positive for >2 weeks, they could identify only 16 MDR isolates. However, the fully sensitive cases / cases for which sensitivity was not done, have a median TTSC of 15 (7.5-22.5) days which suggests that they could very well include MDR cases. Again, in the hypovitaminosis group (25(OH)D <25nmol/l), the median TTSC is 22.5 (22.5-37.5) days, which also suggests the possibility of MDR cases. Although the results clearly show that TTSC is significantly more in hypovitaminosis D group, the data will be more meaningful if interpreted along with the drug sensitivity results. This is more important because, the authors have found a significant association between isolation of a multidrug-resistant organism on sputum culture with delayed sputum smear conversion.

In an earlier study conducted in Northern India, Rathored et al (Reference 9), had reported that serum 25(OH)D concentrations were significantly lowest in MDR-TB, correlating inversely with time to sputum smear conversion, stating that lower serum 25(OH)D may increase time to MDR-TB sputum smear negativity. Yet another study done in Pakistan by Raheel Iftikhar et al (Reference 23) has shown a significant Vitamin D deficiency in patients with tuberculosis which was more pronounced in MDR-TB patients. Since both these studies claim that a severe vitamin D deficiency is associated with MDR TB, this possibility needs to be verified in the present study. The interpretations in the present study have been made on the assumption that cases with undetermined sensitivity are possibly drug sensitive cases. But, the authors cannot exclude the possibility that a delayed TTSC in the group with undetermined sensitivity could be due to the presence of MDR cases. Without verifying this data, the authors cannot claim an independent association of vitamin D deficiency with delayed sputum smear conversion.

The claim that the authors make in the introduction that 'studies investigating the potential effect of interactions between vitamin D status and polymorphisms in the
vitamin D pathway on response to antituberculous therapy have not previously been performed’ is not completely true since the study by Rathor ed et al has addressed the same question with a larger number of patients, except that they have studied the polymorphisms only in vitamin D receptor and not the DBP and CYP2R1. This point needs to be clearly mentioned in the introduction and discussion part.

Response:

We thank the reviewer for these comments.

Regarding the fact that DST was performed in a sub-set of patients only: please see response to Reviewer 1, Comment 1, and note that DST was performed only in a sub-set of those who were smear-positive at 2 weeks into treatment, as per hospital protocol. We acknowledge that there was a lack of clarity regarding this point in the Methods section of our original submission, and we hope that the new wording resolves this issue.

The reviewer raises the possibility that the presence of undiagnosed MDR-TB may have confounded the relationship between low baseline vitamin D status and delayed sputum culture conversion. To investigate this hypothesis, we repeated the multivariate Cox regression analysis of determinants of time to sputum smear conversion just in the sub-group of 72 patients who had DST results, adjusting for sex, radiological extent of disease, baseline vitamin D status and drug sensitivity as before. We found that the independent association between low baseline vitamin D status and delayed sputum smear conversion remained strong after adjustment for sex, radiological extent of disease and drug sensitivity (adjusted Hazard Ratio [aHR] 4.51, 95% CI 1.91 to 10.70, P=0.001). The independent association between presence of MDR TB and delayed sputum smear conversion was also replicated in this sub-group analysis (aHR 3.59, 95% CI 1.77 to 7.30, P<0.001). These results suggest that the association between low vitamin D status and delayed sputum smear conversion seen in the study population as a whole are likely to be independent of the effect of MDR TB. The findings of this sub-group analysis have been added to the Results section of the revised manuscript (page 9).

With regard to the comments regarding primacy: these prompted us to re-read the cited paper by Rathored et al. Respectfully, the Rathored paper does not address the question of whether vitamin D status and VDR genotype interact to influence time to sputum culture conversion. The paper does report ‘main effects’, whereby BsmI genotype is shown to associate with time to sputum culture conversion; but results of interaction analyses, testing whether the influence of VDR genotype on time to sputum culture conversion is dependent on vitamin D status, are not presented. We therefore stand by our claim that ‘studies investigating the potential effect of interactions between vitamin D status and polymorphisms in the vitamin D pathway on response to antituberculous therapy have not previously been performed’. 
Comment 2: A per tables 2,3 and 4, the total number of all three genotypes in any polymorphism does not add up to 260, which means that the results are not available for all participants. The reason for this needs to be mentioned in the text.

Response: Absence of genotyping result indicates failure of the allelic discrimination assay, which may have occurred due to poor DNA quality. We have added a sentence to the Methods section of the revised manuscript to clarify this point (Page 6, line 164).

Comment 3a: The authors have shown the number of patients with vitamin D deficiency for each genotype in table2. Additionally, they can also include a graphical representation of actual vitamin D levels between different genotypes, at least for Cyp2R1 polymorphism.

Response: It is also not clear to us why the reviewer has asked for a graphical representation of baseline 25(OH)D concentration by CYP2R1 genotype, when none of the SNP in this gene associated with risk of vitamin D deficiency at baseline (P=0.81, 0.34 and 0.60 for the three SNP investigated). We are not convinced that graphical presentation of these null data will provide the reader with any additional insight into our findings. However, if the editor would like such a figure to be presented, we have no objection to providing it in a subsequent revision.

Comment 3b: The authors can add more experimental details, particularly for allelic discrimination assays, or they can give a reference for the methodology.

Response: A new reference has been added for the methodology of the allelic discrimination assays as requested (page 6).

Reviewer 3

Comment 1: It would be a good idea to perform combine analysis of 25-OH vit D3 levels and VDR/CY2R1/DBP polymorphism with disease phenotype or response to treatment.

Response: In conducting multivariate analysis to identify determinants of 25(OH)D levels and response to treatment (Tables 2 and 3), we elected only to include variables which associated with our outcome of interest with P<0.10 on univariate analysis. This approach is in keeping with widely practiced statistical convention – adjusting for every genotype investigated, irrespective of whether an association is demonstrated on univariate analysis, would have generated an unworkably complex model which likely could not have been resolved computationally.

Comment 2: Describe the power calculation in details. It's not clear whether authors performed pre/post power analysis. They may use a statistical tool for power analysis (G*3 power).
Response: The power calculation was performed prior to study conduct, using algorithms described in the paper by Lwanga and Lemeshow, 1991, now cited (6) (page 7).

Comment 3: Based on 25-OH vit D levels patients should be grouped in a) sufficient, b) insufficient and c) deficient. (Mandal et al, http://arthritis-research.com/content/16/1/R49) and re-analyse the possible associations.

Response: We thank the reviewer for this suggestion. However, the number of patients in the ‘sufficient’ group using the suggested classification of vitamin D status is only 6 – just 2% of the total number of participants in the study. Sub-group analyses with such tiny numbers in one of the sub-categories will not be powered to yield any meaningful insight.

Comment 4: The manuscript may be corrected by a native English speaker.

Response: We assure the reviewer that the senior author of the manuscript (Martineau) is a native English speaker and he has reviewed the manuscript. If there are specific errors in the English that the editor identifies, we would be glad to correct them.

Reviewer 4

Comment 1: Can the authors describe why they chose to categorise xray severity according to whether disease was unilateral or bilateral? There are better measures of radiological severity than this. Severe cavitory disease may be unilateral; mild disease with infiltrates which clear quickly may be bilateral. Robust severity scores correlate with time to sputum smear conversion but the x-ray severity categorisation here does not. Do the authors have records of cavitory status at least (still not the best severity measure on its own), or percentage of lung affected, and if so, can these measures be used in the uni- and multivariable models instead?

Response: We adopted this classification as it was simple, readily verifiable and could be consistently performed by the range of clinicians involved in the study with a high degree of inter-observer agreement. As the reviewer will be aware, the chest radiograph is insensitive for detection of cavitation as compared with CT scanning – effectively, all patients with smear-positive TB will have a degree of pulmonary cavitation, irrespective of whether or not it is visible on plain chest radiograph; it is therefore a moot point whether attempting to identify such cavitation on plain chest radiography provides biologically or clinically useful information. Moreover, inter-observer agreement in identifying cavities and assessing surface area of affected lung in patients with active tuberculosis is poor (7). Together, these factors may explain why presence / absence of cavitation is not universally found to associate with time to sputum culture conversion (8). We acknowledge that some severity scores correlate with time to sputum smear conversion, but these often incorporate clinical parameters in addition to radiographic ones. For these reasons, we elected not to collect information on cavitory status or percentage of lung affected.
Comment 2: The crux of the results is time to smear negativity. According to Figure 3A, 80% of individuals with replete vitamin D concentration >25 converted to smear neg by 2 weeks, much faster than is usually expected. The majority of people at baseline had a low sputum smear grade, from what I can deduce (two thirds had <100 per 100 HPFs which I think is grade 1 or 2+). Can the authors elaborate on the quality of smear microscopy, specifically, any cross checking, whether the lab participates in QC processes, whether the follow up specimens were graded or classified as positive/negative only, and whether staff providing sputum smear readings were blinded to vitamin D status.

Response: Sputum smear microscopy was performed by trained staff using written standard operating procedures in the microbiology laboratory at Gulab Devi Chest Hospital, which has established QC procedures. Follow-up sputum specimens were graded as being positive or negative for acid-fast bacilli on microscopy – further quantitative detail would not have influenced our calculation of time to sputum smear conversion. Staff performing sputum smear microscopy were blinded to participants’ vitamin D status. Text clarifying these points has been added to the Methods section of the revised manuscript (page 6).

Comment 3: Methods – page 5 line 133 – would be helpful to add in latitude here (mentioned in the discussion).

Response: Latitude has been added in the Methods section as suggested (see page 5).

Comment 4: Line 147 – samples for vitamin D ELISA were stored at -20 degrees (a standard domestic freezer?) – can the author comment on stability at this temperature (instead of -70), average duration of storage, and any potential impact on results? also on accuracy/repeatability of the ELISA method used?

Response: There is a large literature testifying to the stability of 25(OH)D: Hollis et al have reported stability of 25(OH)D in human serum stored at -20ºC for more than 10 years (9), and Colak et al report equivalent stability in samples stored at -20ºC vs. -70ºC (10). The average duration of storage in our study was 91.2 days – this detail has been added to the revised manuscript. We do not therefore think it likely that significant degradation of 25(OH)D will have occurred in our study samples. Regarding accuracy/repeatability of the ELISA method used: inter-assay CV was 11.5%. This detail has been added to the Methods section of the revised manuscript (page 6).

Comment 5: Participants were followed up for 8 weeks only yet figure 3 shows some were followed out to day 70. Please explain.

Response: Data from the small number of patients who attended their final follow-up visit late were included up to 10 weeks from initiation of antimicrobial treatment. A sentence to this effect has been added to the Methods section of the revised manuscript (page 5).
Comment 6: The comments in line 258 to 262 do not appear to relate to Table 4 (there is no mention of FF, ff or Ff in Table 4).

Response: Thank you for spotting this disconnect; we have amended the text to express genotypes in terms of G/A alleles as opposed to F/f alleles, so that it is consistent with data presented in Table 4 (page 10).

Comment 7: Line 275 – the authors express surprise that so many (54%) patients had vitamin D level <25, yet they had estimated (in the sample size calculation) that 79% would have vitamin D level <25. Can the authors address if there are social reasons for the low vitamin D levels, especially in women?

Response: Women participating in the study may have had lower vitamin D status than men due to experiencing less sun exposure, and possibly having lower dietary vitamin D intake. We have added a sentence to this effect to the Discussion of the revised manuscript (page 11).

References
