Author's response to reviews

Title: CCL3L1 copy number, CCR5 genotype and susceptibility to Tuberculosis

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Author's response to reviews: see over
Dear Editor

Many thanks for your message communicating the responses of reviewers to our manuscript. We appreciate the well-informed and insightful comments from the reviewers, and we have been happy to incorporate all their suggestions in our revised manuscript. In the detailed documentation below, we have reproduced the reviewers' comments, and our own comments in response are highlighted in red.

We hope that these revisions respond adequately to the comments and suggestions made by these reviewers, and that you will now consider our manuscript suitable for publication in *BMC Medical Genetics*.

**Reviewer's 1 report:**
Carpenter et al. presents a study examining the relationship between genetic variation in the gene encoding the chemokine receptor CCR5 as well as its copy number variants (CNV) with clinically diagnosed tuberculosis. The study comes from an experienced lab, was well written, used appropriate methodology and was more than sufficiently powered to test their hypothesis that CCR5 genotype and CNV numbers would associate with tuberculosis. Although the authors were able to report differences in CCR5 CNV between the Peruvian and African cohorts examined, no associations with CCR5 genotype or CNV and tuberculosis were established. Given the thorough approach taken and the need to disseminate genetic association studies regardless of the significance of the reported observations, I would deem this article suitable for publication. However, I feel it would be more appropriate as a short communication. My comments are below.

**Discretionary revisions**
Results (general) – Although no significant associations were identified, listing the p-values in text, or in the tables may be helpful to the reader.

**Minor essential revisions**
Pg 9-10- The CNV v TB analysis results are very repetitive given that no associations were found. Please be more concise.

These paragraphs have been condensed.

Pg 11- The authors suggest type I error in the association between TB and CCR5 genotype following logistic regression analysis. Please follow this statement with a multiple testing procedure to confirm.

A Bonferroni correction has been performed and the p-value has been added with a comment to the text in the results section, pg11

**Major compulsory revisions**
Pg 8- Student's t-test was used to compare CNV. Would it not be more appropriate to use a non-parametric test such as the Mann-Whitney U test or Wilcoxon test, or even a chi-square test? Please rationalize the use of the t-test or employ a test that is more suitable.

We have taken advice from an independent statistician on this point. As our CNV data closely follows a normal distribution (see QQ plot below for control samples from Peru), then a parametric t-test is an appropriate test. We have added in a comment to justify the use of the t-test in the methods section (p8). We could supply QQ plots for all the data and place these in a supplementary information if required.
Reviewers 2 comments
The author's objective was to investigate the influence of genetic variation in the genes coding for MIP-1a and CCR5 in susceptibility to TB infection in three different populations, Peruvian, Xhosa and South African Coloured. Furthermore, copy number variation of CCL3L1 and the functional promoter polymorphism in CCR5 -2459A>G (rs1799987) were investigated. The study is appropriately designed and the manuscript is well written, however, I have some concerns in this submitted manuscript, mainly regarding the following issues.

1. The selection of controls is incomplete and needs to be provided in greater detail. I wonder whether there is epidemiologic bias in the recruitment of controls. The lack of evidence for an influence of variation in genes coding for MIP-1a or CCR5 individually or together in TB susceptibility in the three populations could be due to a bad selection of controls because these populations have high rates of both, active tuberculosis and latent tuberculosis, and people selected as healthy control could be latently infected with Mycobacterium tuberculosis and this could represent an important bias that would lead to erroneous results. Thus, I think that the authors should try to get a PPD or Interferon-gamma release assays (IGRA) assay would help in a better selection of control group.
Furthermore, ESAT-6 and CFP-10, two antigens found in these tests, only cross-react with a few environmental Mycobacterium strains (M. Kansasii, M.
marinum and M. szulgai). Authors should explain this point.

This is a study of susceptibility to clinically active TB in Peru and South Africa. The Peruvian control samples and the South African paediatric control samples are all age-matched at initial testing and followed up and have not developed clinically active TB, thus appear to be controlling infection. The description of the study and the aim of the study have been amended to “susceptibility to clinically active TB” throughout the manuscript to avoid confusion and more details have been added to the description of the controls in the materials section. This is a retrospective study and the relevant material has unfortunately not been preserved so it is not possible to perform these assays.

2- Authors state in Study population that “The adult !Xhosa and Coloured samples (n=493) consisted entirely of control samples and were more stringent controls as there was no history of previous TB disease and all had a positive Mantoux test to confirm exposure to M. tuberculosis, which is also suggestive of HIV negativity.”
I think it is a mistake. Control subjects were really latently infected?. The control group should be negative in the Mantoux test. What was the purpose of the authors, comparing individuals with active TB against healthy subjects or with latently infected individuals?

The Mantoux test has received much discussion, and the interpretation of the test is subject to debate. However in this case it was used to ensure that all control samples were exposed to TB but had not developed clinically active TB. All subjects in the South African !Xhosa and Coloured study lived in townships in Cape Town which are very crowded and over two thirds of the controls had been in contact with someone with TB, often a household member. We were using these exposed adults to control for paediatric cases, and it is always possible that a control could become a case in future; however these adult individuals had controlled the infection throughout childhood and never developed clinically active TB.

Reviewer 3 report:
The manuscript CCL3L1 copy number, CCR5 genotype and susceptibility to tuberculosis is well written and not associated with evidence of such genetic markers and tuberculosis in three distinct populations. The presented results it is important to identify future genetic markers that may help in identifying new therapeutic and vaccine.

No comments to be addressed

Reviewer 4 report:
Even if the authors question is interesting and well defined, there are not sufficient results to be accepted for publication.

This is the first study to investigate \textit{CCL3L1/CCL4L1} copy number in TB in African populations and the largest dataset to ask these questions in Peru. These datasets have sufficient power and have been published with reference to other genes. There are plenty of results however we are not reporting positive associations.

Here below some suggestion to be taken in account by the authors
- In "Material and Methods" "Study population" : it may be better to tell also here that Xhosa and South African Coloured are from South Africa and to briefly described how they are ethnically different, if they are mixed or isolated populations. The description of these 2 populations has been expanded in the materials and methods section. The origin of samples to South Africa has been made clearer and details of the ethnicity have been added.

- In "Material and Methods" "Study population" : why not all individuals were tested for HIV-1? Mantoux test is suggestive for HIV negativity?

This is a study of susceptibility to clinically active TB and not for the identification of HIV-1 positive individuals in the cohort. A positive Mantoux test would not be seen in clinical AIDS, but we have decided to remove the sentence “Mantoux test is suggestive of HIV negativity” as it is causing confusion. Due to the obvious effect on an individual if the test is positive and the implications for their future health, the ethical consent does not cover HIV-1 testing.

- in "Material and Methods" "Copy number measurement": why Peruvian population is compared with Caucasian population?

To calibrate the copy number of an unknown sample, samples of established copy number are included in all experiments to use as calibrator samples. As the Peruvian population varied between 0-6 then calibrator samples were used that represented copy numbers of 1, 2, 3 and 4. These calibrator samples were confirmed in their copy number by consistency of numerous repeated measurements using different methods. The fact that these samples are of European origin has no impact on their ability to be used as a calibration of the experiments with Peruvian samples.

- Maybe an ANOVA test is more appropriate than t-test because authors are comparing more than 2 groups.

An ANOVA would be appropriate as an initial test of difference in \( CCL3L1/CCL4L1 \) copy number between the 3 populations, but then a t-test is required to examine the differences between pairs of populations to identify which particular populations are significantly different from each other. The use of the ANOVA has been added to the methods section.