Author's response to reviews

Title: Immunomodulatory factors in cervicovaginal secretions from pregnant and non-pregnant women: A cross-sectional study

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Author's response to reviews: see over
Dear Dr Ojcius

RE: Manuscript: 9626089945154928
    “Immunomodulatory factors in cervicovaginal secretions from pregnant and non-pregnant women: A cross-sectional study”

We thank the reviewers for their insightful comments on our above-noted manuscript. We have incorporated all their comments as outlined point by point below. These suggestions have strengthened our manuscript and we trust you will now find it suitable for publication.

Below are the comments from the reviews and our responses are italicized.

Sincerely,

Grace Aldrovandi MD CM for the authors

Reviewer 1: Brenna Anderson

Reviewer's report:

The authors present a cross-sectional study examining the relationship between immune factors and pregnancy. The study is fairly small and performed many comparisons, which limits the ability to draw conclusions from the data presented.

We agree with the Dr Anderson that the sample size is small, but would like to point out that some effects remain significant after adjusting for multiple testing. Thus despite the small size and larger number of comparisons it is possible to draw conclusions from the data.

I have the following comments.

1. The last 2 sentences of the background do not belong in this section.
   
   We deleted these sentences.

2. Methods: Did the authors collect information regarding the time within the menstrual cycle as it relates to collection of samples. In addition to changes in immune function related to pregnancy, it has also been shown that immune
functions change across the cycle.

We agree with the possibility of an influence of the menstrual cycle on cytokine levels among non-pregnant women. Unfortunately, we did not collect data on LMP in our research subjects however none of the women were actively menstruating at the time of collection. In samples in which trace blood was detected adjusted analysis did not significantly change the results.

3. Why did the authors use PBS for their CVL rather than normal saline?

To avoid an influence of pH on the measurements, we preferred to use PBS rather than saline only.

4. The authors report that they have used a Bonferroni correction for multiple comparisons. This may be worth discussion with a statistician. My understanding is that with a large number of comparisons such as in this case, a number of corrections must be made. What are the details of the use of Bonferroni here? Was a sample size calculation performed a priori?

Bonferroni correction is very conservative, well accepted standard statistical method for adjusting for multiple comparisons. Briefly, this method requires that the threshold for the p-value to be divided by the number of comparisons. In this case, we used the standard threshold of 0.05 and divided it by the number of analyzed cytokines (n=39). Accordingly, we judged any result to be significant if the p value was below 0.0013 (0.05/39). Since this method is commonly used as well as conservative, we did not see the need to explore other methods to adjust for multiple testing.

The study was originally powered based on previous analysis that found a difference in IL-7 in breast milk among HIV-infected women who did and who did no transmit HIV to their infants (Walter et al. J Acquir Immune Defic Syndr. 2007 Oct 1;46[2]:200-7.). The primary hypothesis tested a difference for IL-7. Only as secondary analysis, the here observed comparisons were planned. There however was no power calculation conducted.

5. Results: There are more headings than necessary here.

We reduced the number of headings.

Reviewer 2: Attila Molvarec
Reviewer's report:
The authors investigated in this cross-sectional study several immunomodulatory factors in cervicovaginal secretions from pregnant and non-pregnant women in a comprehensive manner. They concluded that pregnancy is associated with reduced CCL22 concentration in cervicovaginal secretions, which could influence
the risk for HIV transmission. Overall, the manuscript is well written and could add to our knowledge in the field. The following issues need to be addressed before acceptance of the manuscript:

This comment does not require a reply.

Major compulsory revisions:
1. The authors mention in the manuscript that CCL22 expression fluctuates in the endometrium during the menstrual cycle. Did they examine the effect of menstrual cycle on CCL22 concentration in CVL? On which cycle days were their samples collected?

Unfortunately, we did not collect the date of the LMP among non-pregnant women and cannot conduct these analyses.

2. The authors found several correlations of CVL CCL22 concentration with other immunomodulatory factors. These results should be discussed. Did the correlations remain significant when non-pregnant and pregnant women were analyzed separately?

We agree with the reviewer that such correlations are interesting and hypothesis generating. However, since our sample size was modest, the number of cytokines relatively large and the relationships between these factors complex, we preferred not to speculate and discuss these in detail. We however added a comment in the discussion section mentioning the possibility of other regulatory mechanisms.

There were some differences between pregnant and non-pregnant women; however, in linear regression modeling of log-transformed CCL22 concentration none of the interaction terms between pregnancy and the listed factor were significant (See table 3b). In other words, pregnancy did not significantly modify the interaction between CCL22 and other immunomodulatory factors in CVL. We therefore did not include these data in the paper, but in the interest of full disclosure list the results below.

Table 3b: Immunomodulatory factors associated with CCL22 in CVL by pregnancy status

<table>
<thead>
<tr>
<th>Factor</th>
<th>Pregnant</th>
<th>Non pregnant</th>
<th>Interaction term (p value)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation</td>
<td>p-value</td>
<td>Correlation</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>( R_p = 0.24 )</td>
<td>0.28</td>
<td>( R_p = 0.43 )</td>
</tr>
</tbody>
</table>
All concentrations were in log_{10} pg/ml. \( R_p \) = Pearson correlation coefficient; \( R_S \) = Spearman correlation coefficient

* Results of linear regression modeling of log-transformed CCL22 concentration. The models included as independent variables the listed factor, pregnancy as well as an interaction term between pregnancy and the factor of interest.

3. In the adjusted model, the inverse association of CVL CCL22 concentration with time since last coitus remained significant. Do the authors have a hypothesis for this observation?

*We added a sentence in the discussion section on the possibility of coitus as a regulatory mechanism and included a reference on the effect of sperm on female genital tract immunology.*

4. Analysis of covariance (ANCOVA) should also be undertaken to determine whether the observed difference in CVL CCL22 concentration between pregnant
and non-pregnant women is independent from potential confounders.

*We agree with the reviewer that adjusted analysis is required and conducted multiple regression analysis (table 4). This approach is similar to ANCOVA and a standard statistical method for confounder control. We therefore did not see the need to conduct ANCOVA in addition.*

Minor essential revisions:

1. On page 8, there is a mistake regarding the p value for multiple testing. It should be less than 0.0013 (not greater than this value).

*We corrected this error.*

2. The authors should mention in the manuscript that normal pregnancy is characterized by a shift towards Th2-type immunity and away from Th1- and Th17-type immune responses at the systemic level. In this context, the following papers should be cited:

*We included these citations and a comment in the introduction.*


**Editorial Requests:**

1) Please can you move the References section to after the Acknowledgements.

*We moved them.*

2) In addition, the ‘???’ in the Acknowledgements needs filling in with a name.

*We corrected this error.*