Author’s response to reviews

Title: Colony Stimulating Factor-1 and Leukemia Inhibitor Factor expression from current-cycle cannula isolated endometrial cells are associated with increased endometrial receptivity and pregnancy

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Author’s response to reviews:

Dr. Tarek Shokeir,

We would like to thank the Editor and the Reviewers for potentially accepting our paper, “Colony Stimulating Factor-1 and Leukemia Inhibitor Factor expression from current-cycle cannula isolated endometrial cells are associated with increased endometrial receptivity and pregnancy.” (Ref.: BMWH-D-17-00047R1). We reviewed comments below and have taken the appropriate actions.

Editor Comments:

1. Many of your arguments and responses to reviewers’ comments (point by point) are not stated in the revised manuscript. Please clarify these issues in the text, notably in the methods and discussion. Any corrections should be highlighted in yellow.

Response: All major corrections were highlighted in yellow. Minor corrections to the spelling and grammar we did not highlight. However, we do know that we did not identify the location of any major correction; therefore, we added the location to the correction to the original responses, which are below.
2. Please include wording "PBS" in the abbreviation list.

Response: This was added (Abbreviation section, page 13, line 290).

Reviewer reports:

1. If improvements to the English language within your manuscript have been requested, you should have your manuscript reviewed by someone who is fluent in English. If you would like professional help in revising this manuscript, you can use any reputable English language editing service. We can recommend our affiliates Nature Research Editing Service (http://bit.ly/NRES_BS) and American Journal Experts (http://bit.ly/AJE_BS) for help with English usage. Please note that use of an editing service is neither a requirement nor a guarantee of publication. Free assistance is available from our English language tutorial (https://www.springer.com/gb/authorseditors/authorandviewertutorials/writinginenglish) and our Writing resources (http://www.biomedcentral.com/getpublished/writing-resources). These cover common mistakes that occur when writing in English.

Response: Dr. Leornardo M. Porchia, who contributed to the preparation of this manuscript, is an American, who is fluent in English. He reviewed the manuscript. However, no improvements to the manuscript were requested by any of the reviewers or the editor.

Editorial Policies:

Please read the following information and revise your manuscript as necessary. If your manuscript does not adhere to our editorial requirements, this may cause a delay while this is addressed. Failure to adhere to our policies may result in rejection of your manuscript. In accordance with BioMed Central editorial policies and formatting guidelines, all manuscript submissions to BMC Women's Health must contain a Declarations section, which includes the mandatory sub-sections listed below. Please refer to the journal's Submission Guidelines web page for information regarding the criteria for each sub-section (https://bmcwomenshealth.biomedcentral.com/). Where a mandatory Declarations section is not relevant to your study design or article type, please write "Not applicable" in these sections. For the 'Availability of data and materials' section, please provide information about where the data supporting your findings can be found. We encourage authors to deposit their datasets in publicly available repositories (where available and appropriate), or to be presented within the manuscript and/or additional supporting files. Please note that identifying/confidential patient data should not be shared. Authors who do not wish to share their data must confirm this under this sub-heading and also provide their reasons. For further guidance on how to format this section, please refer to BioMed Central's editorial policies page (see links below).
Declarations

- Ethics approval and consent to participate
- Consent to publish
- Availability of data and materials
- Competing interests
- Funding
- Authors' Contributions
- Acknowledgements

Response: To the best of our knowledge, the manuscript does adhere to the editorial policies. We prefer to make the patient data available upon request, as to adhere to the institute’s policies regarding patient data. Therefore, we selected the most appropriate suggestion from the webpage and replaced what was originally written. All declarations are on pages 14-15, lines 293-314.

Original Responses:

Reviewer 1:

1. The rationales for identifying these 2 markers have been provided and the aims were well outlined. The methods were adequate. It would be interesting to stain the endometrial cells with antibodies against LIF and CSF-1, in order to confirm their mRNA findings. These would further strengthened the overall conclusions. However, if this is not possible, then can be put under further studies for validation.

Response: We would like to thank the reviewer for the compliment. It is true that we could more thoroughly characterize the cellular composition of the samples; however, this is a challenging technical issue since there is a very small amount of cells to begin with. While we are entirely interested in further characterizing the cells’ identity and their relative contributions with future populations as well as the gene expression profile of the sample, we believe that this data will be more relevant in future studies, rather than being an essential part of this manuscript. We address this issue in the discussion (Discussion section, page 12, lines 256-276).

2. Limitations of this study should be further discussed. Were these cells only derived from the luminal epithelium, or did this include the glandular epithelium as well, in which the glands
are known to be the main source for LIF?. What about the stromal / decidual cells? Could they secrete LIF and CSF-1 too?

Response: We understand the reviewer’s concern and we expand on this limitation of the study in the discussion. Briefly, due to the nature of sample collection, we support the notion that our samples contain mainly luminal epithelial endometrial cells. As noted by Reviewer #2, LIF mRNA expression is restricted to the endometrial glands during the secretory phase (Cullinan et al., PNAS, 1996). However, it has been reported in endometrial epithelial cells (references [1-3] here) also produces LIF. As indicated in item #1, we included this information is the Discussion section, page 12, lines 256-276).

3. Can the author briefly described the IVF treatment regime received by these women? Would progesterone treatment causes increased in LIF and CSF-1? Or these women received different treatment based on their conditions?

Response: It has been reported that progesterone promotes LIF expression and this was expected. We agree with the reviewer that the procedure could affect CSF-1 and LIF levels if certain hormones are present. As for women receiving different treatments, patients in this study followed the same standardized protocol, which is now more extensively described in the methodology (Methods section, page 5, lines 97-111).

4. Authors concluded that determining the levels of LIF and CSF-1 can be used to predict endometrial receptivity development in women. Would these be too late to determine as the embryo is at the same time being transferred? Uterine flushing prior to embryo transfer can be done and endometrial cells retrieved and assessed for their LIF and CSF-1 levels, if in case they were high, then embryo can be transferred. These would be more practical rather than transferring the embryo and at the same time determining the development of endometrial receptivity.

Response: The reviewer does posit an interesting alternative. Some very preliminary data indicated a possible alternative: a test transfer with an empty cannula can be done 6-24 hours before transfer. We are still optimizing the procedure, hopefully to be able to produce the results within 2 hours. This way, we may know if the transfer may proceed. As for uterine flushing, this has not been a good alternative as have experienced high levels of variability recovered volume and RNA production was of poor-quality and highly variable. This issue is address in the Discussion section (Discussion section, pages 12-13, lines 269-276).
Reviewer 2:

1. I am not sure whether or not endometrial cells will be dislodged by simple touching the cannula with the endometrial cell surface. Are these cells attached outside the tube or getting inside the tube?

Response: The procedure utilized in this study is meant to be non-invasive. The passing of the cannula merely grazes the sample tissue, but does not produce a puncture. Numerous studies show that the endometrial cells are “sticky” and get stuck to the outside of the cannula. We collected the cells attached to the cannula, as we received the cannula without any internal liquid (because the review of absence of embryos after transfer) and we have to shake it in PBS at least twice to “detach” the cells and get all the available material. We added more information to the methods section to help clear up any confusion (Methods section, page 6, lines 117-119).

2. Please make sure that these cells are not coming from the luminal fluid.

Response: As mentioned above, no/minimal luminal fluid was present in the sample.

3. It is necessary to characterize the cell-types. Are these epithelial or stromal cells? Appropriate markers should be used to characterize cell-types. The reviewer is surprised to see that there are no contaminations of blood cells.

Response: Samples showing blood contamination were excluded from this study. Moreover, the cannula is washed with PBS before collecting the endometrial cells. This explains the reviewer’s surprise to the absence of blood cells. Blood present on the cannula is evidence of compromised IVF procedure, which leads to lower (if not diminished) pregnancy rates and therefore should not be included, minimizing the chance a confounding variable.

Due to the nature of sample collection, we support the notion that our samples contain mainly luminal epithelial endometrial cells. This is in agreement with our gene expression results, since there is evidence in literature that these cells express CSF1 and LIF. (LIF expression in endometrial epithelial cells please see references [1-3]; CSF expression in endometrial epithelial cells please see references [4-6]). We revised the manuscript to clarify these issues, notably in the methods (Methods section, page 6, lines 117-119) and discussion (Discussion section, page 12, line 256-268).

4. It is not possible to collect uterine glandular cells by simply touching the cannula with the uterine luminal surface. This point is raised since LIF mRNA expression is restricted to the endometrial glands during the secretory phase (Cullinan et al., PNAS, 1996). It is possible
that samples are contaminated with luminal immune cells. Please perform immunocytochemistry to demonstrate that these two proteins are expressed or present in luminal epithelial cells.

Response: We agree with the reviewer that it is not possible to collect the glandular cells by simple cannula-endometrium contact; therefore, the cells that could attach could be the columnar epithelial cells. While we also agree that it will be interesting to characterize the identity of the cells expressing the messenger RNAs studied in this work, we believe that this is not an essential part of this particular study. It is true that we have not thoroughly characterized the cell composition of the samples. As mentioned above, this is a challenging technical issue since there is a very small amount of cells. Therefore, we cannot exclude the possibility that an amount of CSF1 and LIF mRNA comes from immune cells or secretory cells. However, in this manuscript, we are providing evidence for the correlation between the expression of these markers with the particular type of endometrium sample and pregnancy success. While we are entirely interested in further characterizing the cells’ identities of the populations obtained and their relative contributions to the gene expression profile of the sample, we believe that this data will be more relevant in future studies, rather than being an essential part of this communication. We address this issue in the discussion (Discussion section, pages 12-13, lines 269-276).

In respect to the suggested immunocytochemical approach, we are interested in the identification of these cells (luminal epithelium, glandular epithelium, stromal, etc.) by immunocytochemistry and other methods with higher sensitivity and accuracy to show epithelial markers and detection of CSF and LIF. We prefer in situ hybridization to assess these cells. We insist that either of this techniques should be part of a subsequent communication. However, if you believe this has to be done, we respectfully request an extension to the revision deadline to accommodate for this. Hopefully two months would be sufficient.


