**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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Attention: Tarek Shokeir, M.D

BMC Women's Health

Dear Editor,

We would like to thank the Editor and the Reviewers for this review of 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-17-00047). We have considered comments and have taken the appropriate actions. For each comment, we have written a reply below and any corrections to the manuscript were highlighted in yellow.

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 strengthen the overall conclusions. However, if this is not possible, then can be put under further studies for validation.

Response: We would like to thank reviewer #1 for this assessment. While we believe it would be interesting to characterize the protein expression of CSF1 and LIF in endometrial cells, we think this would be beyond the scope of this work. The focus of this paper is to support the validity of measuring only available mRNA within this kind of sample. However, we do not discard the idea of characterizing this in future studies.

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 endometrial epithelial cells (references [1-3] here) are also capable of expressing LIF.

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 previously been reported that progesterone promotes LIF expression. Therefore, reviewer #1 query on administration of progesterone is completely valid. In this regard, it should be noted that all patients in this study followed the same standardized protocol, which is now more extensively described in methodology (lines 106-112 in the manuscript). This protocol did not include the administration of exogenous progesterone.

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 observation. Some very preliminary data indicate a possible alternative: a test transfer, with an empty cannula, can be done 6-24 hours before the transfer. We seek to optimize the procedure in order to produce results in as little as 2-3 hours. This way, we may know if the transfer may proceed. As for uterine flushing, we do not consider this a viable option, as it has seemingly produced too high a variable in terms of volume recovery and mRNA quality in our experience.

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). These cells are freed from the cannula by vigorous shaking in PBS at least twice to “detach” the cells and get all the available material.

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 (around 15%). 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 and discussion.

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. 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’ identity 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.

In respect to suggested immunocytochemical approach, we are interested in assessing 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 would prefer in situ hybridization to assess the distribution of the expression of these mRNAs in the analyzed cells. We support either techniques as part subsequent communications. However, if you believe this has to be done within this work, we respectfully request an extension to the revision deadline to accommodate for this, hopefully two months would be sufficient.

We very much hope all changes requested are sufficient for consideration of our paper for publication in BMC Women's Health.

I look forward to hearing from you at your earliest convenience. Thanks for your evaluation.
Best regards,

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References


