Author’s response to reviews

Title: Evaluation of apoptosis and angiogenesis in ectopic and eutopic stromal cells of patients with endometriosis compared to non-endometriotic controls

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Author’s response to reviews:

Reply to Dr. Sandra Cecconi (Reviewer 1):

- Considering the fact that endometriosis is notably an inflammatory disease, a pivotal role is played by Fas/FasL and TNF activation, both activators of the extrinsic apoptotic pathway. The partial activation of this pathway has been reported in a recent paper, highly pertinent to this Ms, that is not cited in the text (PMID: 31416694). Please add and discuss differences.

We appreciate this valuable recommendation. Based on the reviewer’s comment, we added some information on potential action of Fas/FasL system in endometriosis in the discussion section. Besides, we added the mentioned reference "PMID: 31416694" in the manuscript and discussed it (Pages 8 and 9, Lines 185-208).

- It is important to add the analysis of some of angiogenic biomarkers (VEGFR2, HIF1A, HGF, and PDGFB) to the panel of angiogenic factors studied to support conclusions. It is not correct to speculate about increased angiogenic potential by investigating only VEGFA.

- Lines 219-37: As stated before, since the authors evaluated only VEGF-A gene expression as marker of angiogenesis, this result is not sufficient to support the effective increase in angiogenesis. Please, add experiments with at least one angiogenic marker other than VEGFA (See point 2). Alternatively, the strength of this paragraph and conclusions needs to be modified.

Thank you very much for this constructive comment. In this regard, we performed HGF gene expression in ESCs of the study groups as well, the results of which are now added to the Ms (Page 2, lines 40, 47 and 48 (abstract section); page 4, lines 82-84 and 87 (background section); page 7, lines 172-174 in results; and page 12, lines 277-289 in discussion section).

- The analysis of gene expression is not per se indicative of protein expression levels. Immunolocalization of proteins and/or WB analysis should better support results.

We appreciate the valuable comment of the reviewer. We totally agree with reviewer’s comment in that the level of gene expression does not necessarily reflect the expression at the protein level. However, a relatively large number of samples we studied in each group could give us a reliable picture of marker expression. Indeed, based on the limited budget we had for performing the experimental study, and
despite of our willingness, doing protein analysis by such means as Western blotting was not truly feasible.

- Lines 118: "Finally, cells from 17 eutopic and 11 ectopic endometrial tissues of endometriotic patients and 15 eutopic endometrial tissue from non-endometriotic patients were used in this study". These numbers are in contrast with the total number of patients claimed in the beginning of "Patients" section. The authors enrolled for the study 25 endometriotic women and 20 non-endometriotic women. Here, they stated that they use 28 tissues of endometriotic patients. Did the AA take more than one sample for some patients? Please, explain.

The authors apologize for this inconsistency. In fact, 25 endometriosis patients and 20 control patients were enrolled in the study. However, some samples were excluded from the study as a result of tissue contamination, inconsistent pathology report or absence of enough cell growth especially in case of EESCs. Finally, cells from 17 eutopic and 11 ectopic endometrial tissues of endometriotic patients and 15 eutopic endometrial tissue from non-endometriotic patients were used in this study. In some patients, doing experiment with paired samples of ectopic and eutopic tissues was not possible due to the limitations mentioned above. The abstract is now corrected for this inconsistency (Page 2, lines 34-36 (abstract section); page 5, line 115 (sample collection section).

- In this section the paragraph "Statistical analysis" is missing. The authors reported statistical significance without explaining anywhere which test has been used. Additionally, it is not clear how experimental data were expressed. What is the "expression ratio"? Usually, value of each gene is compared with the related housekeeping (gene expression/beta-actin).

Per reviewer comment, a section describing statistical analysis and how the data is expressed was added to the end of M&M section. Expression ratio which is analyzed in house by a Pair Wise Fixed Reallocation Randomization Test (REST) shows ratio of normalized gene expression (gene:housekeeping) between two experimental groups (Pages 6 and 7, lines 147-152).

- Line 146-48. Since the authors reported immunofluorescence staining as method for the evaluation of the purity of all ESCs groups, this reviewer suggests adding representative images for each ESCs group.

Figures showing representative results of IF staining and FC experiments are now added to the Ms (Figure 1, page 7, lines 154-160 (results section)).

- Figures: in all the legends the authors claimed that:
  * "Data are expressed as mean and error". Please clarify.

In this study relative expression values are shown as mean ± standard error (SE). This point revised in the Ms (Page 23, lines 531 and 538).

- Each bar represents levels of … gene expression in two different endometrial cells". Do you mean two cell groups? If so, please correct accordingly.

The reviewer is totally right. It was corrected (Page 23, lines 530 and 537).

- The authors reported the results of Bax as not significant without discussing this result. Please, explain which could be the reason(s) of such Bax gene expression.

Revised (Page 10, lines 239 and 240).

- Line 55: Correct "Despite being a quite common among women" with "Despite being quite common among women", or "Despite being a quite common pathology among women".

It was corrected (Page 3, lines 57 and 58).

- Line 162: Correct "as" with "is".

It was corrected (line 176).

- 437-38: Put "value" as subscripts

Corrected (In all of the Ms, "p" was mentioned so we omitted value) (Lines 534, 540 and 541).

Reply to Dr Jacqueline Donoghue (Reviewer 2):
- It was disappointing not to see the flowcytometry results or the immunofluorescence results. These data would add value to the paper as they demonstrate your capacity to perform the work and allows the reader to see what the data. When these results are simply dismissed as a reference it leads to the question of was the work actually performed. You have gone to the trouble of writing the methods for these data and then don't present them. I strongly urge the inclusion of at least one figure that shows some of this data.

In line with the reviewer’s recommendation, figures showing representative results of IF staining and FC experiments are now added to the Ms (Figure 1, page 7, lines 154-160 (results section)).

- The presentation of the RT-PCR data is not suitable. The result statement was "significantly higher gene expression levels of Bcl-2 (Figure 1a) and Bcl-xL (Figure 1b) in EESCs compared to EuESCs or CESC (p<0.01)." The statement indicates that the gene of interest will be presented with three data points and a one-way ANOVA statistical analysis has been performed. However, the statistics performed has not been described, the bar graphs describe E v Eu etc and the error bars a so large that there cannot possibly be any significance. I strongly suggest adding the statistical analysis done to the methods section and ensure the data presented is the mean of each sample analysed (EESC, EUESCs, CESC) +/- standard error of mean. If the data has been normalised to a control sample or eutopic endo or other, make sure this information is also contained in the methods section as data analysis and presentation.

Per reviewer comment, a section describing statistical analysis and how the data is expressed was added to the end of M&M section. In this study we used REST software to test the group difference for significance using the Pair Wise Fixed Reallocation Randomization Test. Expression of each gene in each individual was first normalized to the corresponding housekeeping gene and then the ratio of normalized gene expression was compared between two groups. So, each bar represents the expression ratio of a gene in two groups. Data were expressed as mean ± standard error. A p <0.05 was considered significant (Pages 6 and 7, lines 147-152).