Manuscript title: the impact of housing conditions on porcine mesenchymal stromal/stem cell populations differ between adipose tissue and skeletal muscle

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Response to reviewers

We wish to thank the Editor and the reviewers for their insightful comments to improve our manuscript. We have addressed all the queries of the reviewers. Please find below our point-by-point discussion in which we reiterate the reviewers' point and provide a rebuttal.

Reply to reviewer #1 (r*eviewed, 09 Jul 2021)*

This article describes the impact of housing conditions (good *vs* poor) on the proportion of porcine adult stem cell populations both in adipose tissue and skeletal muscle, in two lines selected for their residual feed intake (HRFI *vs* LRFI). Stem cell populations are deeply analyzed by a combination of 5 different cell markers (CD45, CD34, CD38, CD56 and CD140a) and the results presented here highlight some differences in stem cell populations after the sanitary challenge, with differences observed between adipose tissue and skeletal muscle of both RFI lines.

If the hypotheses behind the search for differences within tissues and within the sanitary challenge are well explained in the introduction, the rationale of the use of the two RFI lines is not obviously stated. What was expected in those two lines?
AU: this part of the introduction has been reviewed to show the rationale for using the two pig lines. It is now clearly indicated that "it has been shown that the impact of a challenge based on poor hygiene of housing conditions on growth performance, immune system and health was lower in pigs selected for low RFI (Chatelet et al., 2018). We hypothesized that hygiene of housing conditions will impact the proportions of hematopoietic and/or MSC cells in subcutaneous adipose tissue (SCAT) and skeletal muscle and that the response may differ between pigs with different RFI. "

What differences in the responses to the sanitary challenge between the RFI lines published by Chatelet et al, 2018 justify doing this study on both lines?
AU: This has been reformulated as indicated above.

Could also the results of the comparison of the 2 lines be more deeply discussed?

AU: This point of discussion has been completed but has not been extended significantly due to the lack of data in the literature. It would be too speculative.

The reference to "the period 1 of a larger study" (L133), is not clear. Housing conditions are *per se* sufficiently developed in the M&M.
AU: The sentence has been modified to clarify the information.

For the flow cytometry analysis, the ratio of dispensed cells/tube *vs* acquired events seems odd for SCAT (L183-192): 50,000 cells were dispensed for SCAT and a minimum of 50,000 events were acquired. In addition, the viability marker used is not provided.
AU: Following tissue dissociation, the number of recovered cells was higher for skeletal muscle (10^6 cells /g) than for adipose tissue (10^5 cells /g). For this tissue, we dispensed all recovered cells and acquired a minimum of 20,000 events. Propidium iodide (Miltenyi Biotec) was used to exclude dead cells as now indicated in the M&M section.

In Table 1: it would be appreciated if the final dilution/concentration of antibodies used could be mentioned.
AU: The final dilution has been included in Table 1.

The statistical analyses used are two-way ANOVA. The effects per group (5 to 9) would suggest to use non parametric test. Please justify the test used.
AU: We agree that ANOVA and many other statistical tests assume that we should have data from populations that follow a Gaussian bell-shaped distribution. However, biological data generally do not follow a Gaussian distribution precisely. As indicated in Graphpad recommendation's, "many kinds of biological data follow a bell-shaped distribution that is approximately Gaussian. Because ANOVA, t tests, and other statistical tests work well even if the distribution is only approximately Gaussian (especially with large samples), these tests are used routinely in many fields of science." Indeed, ANOVA is still commonly used for data related to investigation of cells in vitro (see Basini et al., Orexin B inhibits viability and differentiation of stromal cells from swine adipose tissue. Domest Anim Endocrinol. 2021 Apr;75:106594. doi: 10.1016/j.domaniend.2020.106594; Gao H et al., CD36 Is a Marker of Human Adipocyte Progenitors with Pronounced Adipogenic and Triglyceride Accumulation Potential. Stem Cells. 2017 Jul;35(7):1799-1814. doi: 10.1002/stem.2635. as examples).

An alternative approach would be to use nonparametric tests but we cannot consider the interactions between treatment.

In Tables 2, 3 and 4: the exact n per group should be clearly stated.
AU: the number of animals per group is now provided in tables.

Figure 1 is confusing and does not fit exactly with the gating strategies proposed in Figures 2 and 3. Wouldn't it be easier to clearly mention the panel of antibodies used for each tissue?
Gating strategies illustrated in Figures 2 and 3 seem not complete: in Figure 2, CD45-CD56- cells are not gated in red and the expression of CD34 within those cells not shown as it is for the CD45-CD56+ cells, but results of those cell populations are reported in Table 3. In Figure 3, also for CD45-CD56-

cells, no further gating is shown for CD34 and CD140a expressions but results for CD45-CD56-CD34+, CD45-CD56-CD34-, CD45-CD56-CD34+CD140a+, CD45-CD56-CD34-CD140a+ are reported in Table 4. In addition, the name of the gate CD45-CD56+CD34+CD140a- is a mistake, CD45-CD56+CD34-CD140a+ cells are shown.

AU: We think it is important to keep Figure 1 to make it easier for the reader to understand our analysis strategy. We have modified Figure 1 to better explain our gating strategy and match with Figures 2 and 3. We corrected the mistake in figure 3.

For skeletal muscle cells, the CD45- gate seems very odd. Do the FSC high cells are really CD45+ cells? The viability marker also appears high in those cells and the gating was adapted. Should the gate be adapted as for the viability dye? Otherwise, which hematopoietic cells could it be?

AU: Actually, cells digested from muscle have not been purified using percoll to keep both hematopoietic and mesenchymal stromal cells even though many debris remained in each sample. These debris can generate auto-fluorescence and disturb the electric signal. To set the threshold, we based ourselves on the control isotype of CD45. As indicated in the text, all flow cytometry analyses were performed with appropriate isotype matching negative and Flow Minus One (FMO) controls.

Language remarks:
L50 "compared to" instead of "compared with"
L83 "flow cytometry" instead of "flow-cytometry"
L143 "fed ad libitum with a standard" instead of "fed ad libitum a standard"
L275 "in both SCAT and muscle" instead of "in both at SCAT and muscle"

AU: the corrections has been made for L83 and but not for L143 and L275.

**Reply to reviewer #2 (r*eviewed, 23 Nov 2021)*

The introduction provides a generalized background of the topics discussed but could better explain the connection between existing knowledge and the research question. To make the introduction more substantial, the authors may wish to provide further information/clarification as to why the body composition is related MSCs in lines 70 to 74.

AU: Some additional information has been included in the introduction.

The authors may wish to provide examples of some of the applications of this research, along with appropriate references. In lines 96-97, the purpose of this research is mentioned as the need to investigate the functional signatures of muscle and adipose tissue and could be backed up by references and current data. The authors could provide a more direct link between the importance of functional signatures and the hypothesis presented. I feel that the authors could provide more context of the plan/technique to be used to address the research questions in lines 82 to 97.

AU: "Functional information" is not an appropriate term in the context of this study. Indeed, we need to demonstrate first that we can change the proportions of MSCs cells before undertaking the evaluation of the functional signature of some cells requiring a higher number of cells. This part has been reformulated.

The experimental setting selected for this specific study seems appropriate but lacks some details. The information provided between lines 175 and 195 does not include the concentrations used for the labeling of the cells. Perhaps the authors could provide more information about this. The authors may wish to add Immunofluorescence staining of cell cultures to its methodology.

AU: more information (final dilution) have been included in table 1. Unfortunately, we could not perform immunofluorescence staining because we worked on frozen cells and not at the tissue level.

These images could help present a more clear picture to readers of the different fluorescent antibody markers effect in cells of interest. It can also help validate the results of the experiments performed, since all the results are based in one assay (flow cytometry) that has in some cases a not ideal p value.

AU: The CD56 marker (using the same antibody as the one used in this study) have been previously validated by IHC in a previous paper (Perruchot et al., 2013). The CD34 (Perruchot et al., 2013) and CD140a (Perruchot et al., 2020) markers have been also validated by IHC. It is clear that further in-depth investigations of the different cell populations are needed. For us, it was important to determine whether these cells are influenced by different factors during growth before going further in the investigation. In this area, the literature is poorly documented.

There are several instances where assertions are made that are not substantiated with references, more specifically, in lines 132 to 149, 151 to 160, 162 to 173 and 175 to 195.

AU: We checked the manuscript and appropriate references are cited within the text.

The results of the flow cytometry assays in between lines 276 to 289 could be presented and explained in a more appropriate format. The big CD marker names given to each plot makes the data interpretation even more confusing, specially given that there is no table or chart explaining the nature of each marker and it's tissue association.

AU: In flow cytometry papers, cell populations are classically named with the CD marker names. Indeed, we talk about putative cell population so we can't be completely sure about their phenotypes ie satellite cells, pericytes … To give a more informative name, we need to further investigate the function of the different cell populations. It could be performed in a next study.

From lines 385 to 389, the author indeed states that the data does not support the hypothesis of a difference between the two RFI lines, which also differs from previous published data. This could be further analyzed.

AU: As indicated to the other reviewer, this point of discussion has been completed but has not been extended significantly due to the lack of data in the literature. It would be too speculative.

Something to note is that the flow cytometry plots shown in the research paper are only illustrations of the initial sorting but there are no plots of the sorting between different RFI animal lines and the hygiene conditions. This data is only given in tables that are not very clear and table 3 and 4 need to have the columns centered.

AU: Given the high number of data, it is unrealistic to show all plots in the manuscript. Data have to be placed in tables.

Figure 4 legend describes the data presented, but I am wondering if it is necessary to include the p values of the 3 conditions. Does it benefit the data interpretation? I think a more in-depth discussion of Fig. 3 and tables 3 and 5 would be helpful. I feel these are the key results for this paper, and therefore it merits more discussion. The findings properly described reiterate previously published data by the author, which is important.

AU: We think that it is important to show the p-value. The data related to tables and figures have been discussed. We could extend the discussion. However, with the limited number of studies, it would be a too speculative discussion.

The limitations of the study are not discussed. I would imagine that having such low p values in most of the cell populations are a limiting factor. The authors conclusion that their findings clearly show that the relative proportions of hematopoietic and of some MSC populations were affected by hygiene of housing conditions in a tissue dependent manner in pigs of both RFI lines references their findings. I will only observe that they further indicate that these populations can be targeted for growth modulation and body composition, but I don't find enough information to support this statement in this article at the moment.

AU: With regard to the conclusion, some slight changes have been introduced. It is clear that we need to perform further investigation in this area of research.