

A proof-of-concept study in small and large animal models for coupling liver normothermic machine perfusion with mesenchymal stromal cell bioreactors

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Review Report

The authors have skillfully design a promising platform to study the effect of mesenchymal stem cells (MSC) during ex vivo perfusion of rat livers. This is a very interesting approach to overcome some issues observed in direct MSC infusion in organ-in-a-pump models, like microvasulature clogging. The main successful points are the modification of a rat liver normothermic machine perfusion (NMP) system to include a bioreactor for MSC cell therapy and the maintenance of hepatocyte viability during MSC-NMP. However, there are some concerns that need to be addressed:

Introduction

Authors claim that MSC only activate their secretory potential after exposure to inflammatory stimuli. However, this is not true as demonstrated earlier (i.e. Park et al., Int J Stem Cells., 2009) but MSC secretory profile is modulated by the microenvironment (i.e. Burja et al., Curr Res Transl Med., 2020).

Methods

MSC in vitro culture. Authors describe "MSCs were cultured at a concentration of 2×10^5 in 14 mL in α MEM medium". What vessel was used to seed the cells and what is the final seeding density in this vessel?

Characterization of MSC after NMP. Cells were retrieved from the bioreactor. Were the cells directly analysed (cytometry). For how long were the MSC re-cultured for the morphology tests and what were the conditions of that culture? (cell seeding density, culture time, etc.). Also, did the authors count the total number of cells retrieved from the bioreactor and compared with the original number? Exposure to perfusion fluid may have altered their metabolism and survival, acting as a licensing agent itself.

Flow cytometry. For reproducibility purposes, please add the reference numbers of antibodies used for flow cytometry and other relevant reagent.

Liver NMP. Perfusion took place in two phases: a warming phase and a stable normothermic phase. Was the bioreactor connected to the circuit during the entire perfusion (warming+stable phases)? Similar to the question related to shipment, rewarming time might affect MSC survival and metabolism during this time.

The authors seeded 20×10^7 MSC in a bioreactor. Is this number constant throughout the perfusion experiment? Do MSC die and are replaced by proliferating MSC? These dead cells might be responsible for the uncontrolled release of cytokines. In addition, average rat liver weight is 4-5 grams which results in a MSC dose of 4×10^9 MSC/kg, an extremely high dose which has a poor translation towards a clinically relevant setup.

In addition, authors sent MSC in a bioreactor from one laboratory to another at room temperature. Culture temperature is key for MSC survival and temperatures lower to 37°C are deleterious (Pratiwi et al., Cytoherapy, 2019). Did the authors study the status of MSC after the shipment?

Results

The authors describe an increase in certain metabolites such as lactate, IL-6, IL-1ra, among other, during liverless NMP+bioreactor. However, these are inflammatory cytokines and lactate production has been related to increased ROS production (i.e. Li et al., Signal Transduct Target Ther., 2022). Though the authors address the increase in inflammatory

cytokines as a positive results, they should discuss the inflammatory perfusate profile and elaborate on this in the discussion section as it appears to be a setback of their NMP circuit.

Immune cells outflow. Authors describe outflow of an increased number of immune cells when the bioreactor is connected during NMP. However, they do not show data for cell characterization in the outflow perfusate.

EV origin. The authors hint that observed differences in EV size are related to their hepatocyte or MSC origin. This is a difficult assumption to make based only on EV size.

pH stabilization. The authors claim that MSC in the bioreactor regulate the pH of the perfusate. However, exposure to MSC medium present in the bioreactor, which contains a buffered solution to maintain pH, might be the responsible factor of pH stabilization.

Reviewer #2

(Remarks to the Author)

This study reports the effects of the addition of an MSC-bioreactor to the NMP circuit in an isolated rat liver model. The study mainly shows the safety and feasibility of this approach and some beneficial effects of using an MSC bioreactor approach rather than thawed MSCs. The MSC-bioreactor effects included decreased injury markers, increased bile production; increased acute phase proteins; more effective washout of blood; reduced ATP depletion; absence of succinate accumulation.

Please address the following:

1. You state that the 'translational value is high'. This needs justification when using a rodent model as many such models have been shown not to have reproducible effects in large animals and humans.
2. Although you acknowledge that the cold ischaemic time is very short, this is a major limitation with little clinical relevance.
3. Please discuss the potential effects of raised TNF and IL-6 in the bioreactor group. These acute phase proteins can have pro-inflammatory as well as protective effects.
4. It is difficult to make conclusions about ATP depletion when the ATP:ADSP ratio has not been measured.
5. In the discussion you state that the ATP recovery mechanism is likely due to a transfer of viable mitochondria to injured cells but you do not present any data to support such a contention.
6. In the discussion you conclude that Bioreactor-MSCs had an impact on different aspects of immune modulation and inflammation. Please justify this conclusion further.
7. There are several typographical and syntax errors.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have made a revision of their manuscript replying to the comments made by the reviewers. Additionally, the authors have included new results from a different study describing one case of successful 17-day human liver NMP perfusion with a custom setup based on commercially available equipment to promote direct translation. It is interesting the development of an strategy to increase the pool of organs for research purposes. These two studies are not correlated, as each of them uses a different setup and has different objectives. In this new context, authors should address the following: Liver NMP case.

Some damage markers such as AST, are reduced until day 11, remaining stable until day 14. From there it rises, indicating a possible injury in liver. Authors may elaborate on the discussion section about fine tuning of the proposed setup.

Images in supplementary Figure 11 have not an appropriate resolution. Authors should provide better quality images.

Rat model

-Authors state that after shipment, 5×10^4 MSC are found in the supernatant, accounting for 2.5% of the total number of MSC. If 20×10^7 were seeded in the bioreactor, this ratio is not correct. Please review this data.

-There is a pronounced difference between the number of MSC seeded in the bioreactor (20×10^7) and the retrieved cells after perfusion (106). Although authors show no increase in CK18 during liverless perfusion, this 95% decrease in the number of cells raises a concern about the impact of shipment and perfusion conditions on MSC, including cell death, and the effects observed in the present study. Authors must explain the possible causes.

- In line with the previous comment, authors could measure viable MSC cell numbers in the bioreactor after shipping and after the recovery time prior to NMP to identify the actual amount of MSC at the beginning of NMP, in case they die or proliferate.

-Cell dosing is still a limitation authors should include. Extrapolation of their data indicates the need of 12×10^{10} cells for an 80 Kg person. Generating these numbers and developing/dedicating bioreactors for this purpose are holding back the translation of this setup, which should be reflected in the discussion.

-AGP and A2M increase during perfusion, common markers for liver damage. Authors could mention how they will solve this issue.

- Authors describe immune cell leakage from the liver based on particle size found in the perfusate outflow. However, more cell types, such as endothelial cell or MSC could, flush through the system. Moreover, identification of EV origin according to size is not correct, as all cells can secrete vesicles of different sizes. It is not possible to sustain this claim with the presented data.

General comments

Translation from preclinical work to human liver NMP should be proposed carefully. These studies are different at several levels such as perfusion fluid composition, organ preservation, oxygen carriers, equipment used (bioreactor, blood hemodialysis unit, etc.)

Overall, the translational value of the preclinical study should be assumed carefully. Authors themselves are aware of the differences between their preclinical model and human studies in the responses to the reviewer's comments. The authors may consider mentioning that translation in the future after confirming result in large animal models could be possible.

Reviewer #3

(Remarks to the Author)

Study A: The authors report on a single liver graft perfused using a modified commercial system. This system is very similar to the one described by Lau et al.(1)., with the primary difference being the use of continuous venovenous hemodiafiltration (CVVHDF) instead of a less expensive dialysis system. The authors reported graft survival for up to 17 days. However is unclear what viability criteria were used. Only bile production and lactate levels are included. Study B reports on the addition of an MSC-bioreactor to an NMP circuit in an isolated rat model, noting decreased injury markers. However, the rat liver was perfused for only 4 hours, and no transplant was performed.

Please address the following points:

Study A

1. The authors describe a system very similar to that of Lau et al., based on a commercially available perfusion machine, the Liver Assist. Similar to the Lau et al.'s system(1), the authors' system includes a long-term oxygenator, gas mixer, and a blood purification system for dialysis. Instead of a standard dialysis system, the authors used a continuous hemodiafiltration (CVVHDF) technique. The authors claim that CVVHDF offers significant advantages over dialysis by allowing the removal of larger molecules. However, this assertion is not supported by any evidence. Both techniques have their pros and cons and mainly filter water-soluble molecules. CVVHDF provides limited clearance of protein-bound solutes. Besides clearing water-soluble toxins, dialysis and CVVHDF also eliminate nutrients and hormones. The authors should moderate their claim of longer survival because of the use of CVVHDF instead of dialysis. They should also discuss that CVVHDF requires a dedicated machine, while dialysis is more available, cheaper and easier to integrate. Furthermore, the authors should acknowledge that their model is very similar to the one already described by Lau et al.

2. Donor graft quality is a significant determinant of long-term survival, as described by Lau et al(1). Eshmuminov et al. (2) and Lau et al.(1) used organs unsuitable for transplantation, these graft had usually a quite long cold ischemia time and were mainly steatotic or DCD grafts. The authors used a graft from a 56-year-old patient with unresectable colorectal metastases. While using a graft with liver tumors is an interesting model, it is not comparable to the poor-quality grafts used by Lau et al.(1) and Eshmuminov et al.(2) The graft clearly was of higher quality considering the likely short cold and warm ischemia, although data about the cold ischemia time are missing. The authors should discuss that despite previously receiving chemotherapy the quality of graft used and the minimal ischemic injury has played a major role in graft survival.

3. Data about liver viability are very limited. No viability criteria are defined. A liver can continue to produce bile even in the presence of massive liver necrosis. Detailed data of histology at various time points is needed, The authors describe massive necrosis at the later stage of perfusion, but data are very limited. Data about ATP content, and oxygen consumption are also missing. Such data should be included.

4.The title is misleading. The authors report a significant worsening of liver function on day 15, yet it is unclear why the experiment was stopped on day 17. Viability criteria should be defined, and the contribution of CVVHDF in reducing lactate levels should be discussed.

5. Important details of the protocol used are missing. What type of Total Parenteral Nutrition (TPN) and concentration was used? Were biliary acids used? Compared to Lau et al. and Eshmuminov et al., RBCs were added during perfusion, which may suggest increased hemodialysis by the CVVHDF. This should be discussed.

6.Was any culture of the perfusate performed? Lau et al. (3) demonstrated an increased risk of infection with prolonged duration of perfusion. This should be discussed.

7. Only a single experiment is reported. The limitations of such a single experiment should be more extensively discussed.

Study B:

1.The main innovation is the modification of a rat liver normothermic machine perfusion system to incorporate a bioreactor for mesenchymal stem cell (MSC) therapy. However, I have reservations regarding the translational value of this model. Rodent models have limited reproducible effects in humans. The sole advantage of a rat model is the potential for transplanting perfused organs, yet no transplant was performed. Additionally, the ex vivo perfusion was limited to just 280 minutes, and the cold ischemia time was notably short. Considering that the authors aim to support the possibility of manipulating organs during long-term perfusion, the animal model used seems to have limited translational value. The authors should better acknowledge the limitations of their model and explain why they did not proceed with transplantation.

2. Considering the limited number of animals per group, the authors should discuss the technical challenges of perfusing small liver grafts which may have affected the results. Also how sample size was determined is not very clear and require better justification.

3. The model used included a very short cold ischemia time and a short duration of perfusion, only 4 hours. The only conclusion that can be made is that the ischemia reperfusion injury was reduced. A model with prolonged perfusion and large animal model is needed to demonstrate "organ manipulation".

4. The title of the paper, "From 17-day human liver machine perfusion to a preclinical mesenchymal cell bioreactor towards a long-term ex-situ platform for organ manipulation," is quite misleading. Study A included only one long-term perfusion, and Study B comprised only short-term perfusion of just 4 hours.

5. The authors reported the induction of factors involved in resolution/regeneration. However the authors have very limited data suggesting regeneration. Most of their results could be explained with a reduction of IR injury. Furthermore the short duration of perfusion (4 hrs) limits any conclusion. Please justify this conclusion further.

REFERENCES

- 1) Lau, N. S. et al. Long-term ex situ normothermic perfusion of human split livers for more than 1 week. *Nat. Commun.* 14, (2023).
- 2) Eshmuminov, D. et al. An integrated perfusion machine preserves injured human livers for 1 week. *Nat. Biotechnol.* 38, 189–198 (2020).
- 3) Lau NS, Ly M, Dennis C, Toomath S, Huang JL, Huang J, Ly H, Chanda S, Marinelli T, Davis R, Liu K, McCaughan G, Crawford M, Pulitano C. Microbial Contamination During Long-term Ex Vivo Normothermic Machine Perfusion of Human Livers. *Transplantation*. 2024 Jan 1;108(1):198-203.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have reviewed their manuscript according to suggestions and concerns made by the reviewers. Additionally, they have included some pilot results in a large animal NMP model to improve the translational impact of their work. Here are some issues that need to be addressed.

1) According to the international society for cell and gene therapy, the correct term for MSC is now "mesenchymal stromal cells" (Viswanathan et al., *Cytherapy*, 2019). Authors should update their manuscript.

2) Although the addition to the NMP circuit might be novel, culture of MSC in hollow fiber bioreactors is already known. Additionally, commercial distribution of MSC has been offering the possibility of shipping cultured MSC in culture flasks or other formats, keeping their properties intact after transportation, which is quite similar to the logistic procedure described here. Novelty of this fact is scarce.

3) Authors need to update figure 4 and supplemental data including all cell types susceptible to be measured in "wash-outs", (i.e. MSC, endothelial cells, etc.) Even in low numbers, MSC can still detach and flush through the system.

4) Authors mention the section supplementary methods 1.12 which is not included in the supplementary material.

5) Supplementary figure 12. Regardless of measured parameters used to evaluate success in NMP, visual evaluation is still key during perfusion. In the images, there is a clear difference in perfusion between both organs, with the one from the MSC group having a paler color. This unequivocally show bad perfusion of the organ.

6) There is a discrepancy regarding the number of cells used for the pig experiments described in the results and methods section. 37×10^7 cells noted in the results section does not equal 37 million cells (20 ml of a 1.85×10^6 cells/ml suspension). Please correct accordingly.

7) Authors have used 2×10^7 MSC for the rat NMP setup and average liver weight was 15 grams, resulting in a dose of 1.3 million MSC per gram of tissue. However, only 28-280 thousand cells (depending on correction made based on the previous point) per gram of liver tissue was reached in the pig experiments. Comparison of results is therefore very limited between both models.

8) Additionally, cell density in bioreactors is extremely different: 18500 MSC per cm² for pig bioreactor vs. 40000 MSC/cm² in rat bioreactors. Cell density is known to alter MSC functionality, secretory profile and immunomodulatory potential. Authors should justify these differences and elaborate about possible consequences.

9) The large animal model used by the authors lacks potential for translation. Clinical strategies used in human liver NMP trials include perfusion times ranging from 9-20+ hours. Although authors are presenting preliminary results in a large animal preclinical model coupled to an MSC bioreactor, clinical translation assumptions should be made carefully, as the authors do not present sufficient data supporting their claims.

10) Even though these are preliminary results, only n = 3 animals were used for the large animal study. This small sample size needs justification and sample size calculation need to be explained.

Reviewer #2

(Remarks to the Author)

The incorporation of an MSC-bioreactor in a normothermic machine perfusion (NMP) circuit is a novel concept although other groups are working in this area. There is a body of work demonstrating that MSCs can ameliorate ischaemia reperfusion injury and it is known that this is due to the release of EVs and paracrine factors from the stem cells and that MSCs themselves have a number of potentially adverse effects. The isolated organ preservation offered by NMP removes problems with targeting stem cells to a specific organ and bypassing the reticuloendothelial system. NMP also reduces the risk of off-target adverse effects. The addition of a bioreactor to the NMP circuit is a logical development. The authors have demonstrated the feasibility of this new approach and in the rat model evidence that the addition of a bioreactor can counter some of the changes associated with I/R injury.

There are a number of issues that the authors should consider:

1. The relevance of the rat model is questionable as studies using rodent models of ischaemia reperfusion injury have a very poor record of translation into clinical practice.
2. The porcine model demonstrated the potential for upscaling but there was much less analysis of outcome compared to the rat component and it is not clear why. The porcine liver is a better model than the rat but still has important differences to the human liver. Porcine livers have a histological structure based on very distinct lobules whereas the basic unit of the human liver is acinar. In addition, porcine livers are more resistant to warm ischaemic injury than the human organ.
3. The group size for the porcine experiments is small at n=3 and the period of NMP was only 4 hours. More prolonged NMP (at least 24h and even up to several days) have been proved feasible and would have allowed more detailed study of the influence of the bioreactor on liver injury (e.g. A2M and Ipha-1 glycoprotein increases). More prolonged perfusion would also help define whether the MSCs survive in the bioreactor for longer than 4h.
4. The best model would be to use human livers that were declined for transplantation. These can even be split into left and right lobes to allow a comparison of intervention vs control NMP.

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have replied to most of the reviewers comments, with a very elaborated explanation in some cases.

Some questions still arise:

- The authors missed one of the previous comments: Authors have used 2×10^7 MSC for the rat NMP setup and average liver weight was 15 grams, resulting in a dose of 1.3 million MSC per gram of tissue. However, only 28-280 thousand cells (depending on correction made based on the previous point) per gram of liver tissue was reached in the pig experiments. Comparison of results is therefore very limited between both models.

- Regarding MSC detaching from the bioreactor. MSC do not flush through during liver-less perfusion. However, as fluid composition changes due to the liver secreted molecules and cytokines, there is no data confirming this subject as conditions change. The authors do not provide any direct proof of the cell types leaving the NMP system. Therefore, all cells present in the NMP system should be taken into account, including MSC.

- The authors have provided preliminary data on the technical feasibility of coupling an MSC bioreactor in a porcine NMP setup. The objective was to set a translatable setup for human use in the clinic. With this in mind, the very small population size (n = 3) do not allow to obtain any conclusion and larger studies need to be carried out.

(Remarks to the Author)

1. The relevance of the rat model is questionable as studies using rodent models of ischaemia reperfusion injury have a very poor record of translation into clinical practice.

The authors address the use of rodent models on the basis that this is common practice and that rodent models are an important step in the evolution of new technologies. These are reasonable points but still don't answer the issue of translatability. There is not much the authors can do about this limitation.

2. The porcine model demonstrated the potential for upscaling but there was much less analysis of outcome compared to the rat component and it is not clear why. The porcine liver is a better model than the rat but still has important differences to the human liver. Porcine livers have a histological structure based on very distinct lobules whereas the basic unit of the human liver is acinar. In addition, porcine livers are more resistant to warm ischaemic injury than the human organ.

The authors have answered my concerns to some extent. The use of a porcine model provides useful feasibility evidence that the technology can be upscaled to a large animal set-up that resembles the clinical situation.

3. The group size for the porcine experiments is small at n=3 and the period of NMP was only 4 hours. More prolonged NMP (at least 24h and even up to several days) have been proved feasible and would have allowed more detailed study of the influence of the bioreactor on liver injury (e.g. A2M a& lpha-1 glycoprotein increases). More prolonged perfusion would also help define whether the MSCs survive in the bioreactor for longer than 4h.

3. My comments relating to the small group size in the small group size in the porcine experiments have been acknowledged by revision of the supplementary information. In relation to the short duration of the perfusion experiments, the authors are planning experiments with more relevant prolonged perfusion. It would have strengthened the work to wait for these results.

4. The best model would be to use human livers that were declined for transplantation. These can even be split into left and right lobes to allow a comparison of intervention vs control NMP.

The authors agree that declined human livers that have been declined for transplantation will be useful in the future.

Overall, the authors have explored a potentially important development in normothermic organ perfusion and they present a useful dataset. Nonetheless, their findings are preliminary and the further studies suggested by reviewers and planned by the authors would lend much more weight to

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RESPONSE TO REVIEWERS

MANUSCRIPT NUMBER: NCOMMS-23-38868

We thank the reviewers for their helpful feedback. Below, we present our responses to the points raised and a description of the changes made accordingly. Reviewers' comments are typed in bold and our corresponding responses in regular text.

Reviewer #1

The authors have skillfully design a promising platform to study the effect of mesenchymal stem cells (MSC) during ex vivo perfusion of rat livers. This is a very interesting approach to overcome some issues observed in direct MSC infusion in organ-in-a-pump models, like microvasculature clogging. The main successful points are the modification of a rat liver normothermic machine perfusion (NMP) system to include a bioreactor for MSC cell therapy and the maintenance of hepatocyte viability during MSC-NMP. However, there are some concerns that need to be addressed:

Introduction

1. **Authors claim that MSC only activate their secretory potential after exposure to inflammatory stimuli. However, this is not true as demonstrated earlier (i.e. Park et al., Int J Stem Cells., 2009) but MSC secretory profile is modulated by the microenvironment (i.e. Burja et al., Curr Res Transl Med., 2020).**

We thank the Reviewer for stressing this concept and for providing an additional reference demonstrating the plasticity of the immunoregulation mediated by adipose tissue (AT)-MSCs, which can mount distinct responses to different inflammatory stimuli. These observations reinforce the rationale behind the design of our platform, where stem cells' secretory activity can be specifically activated by the inflammatory mediators release by each individual liver. The sentence "...MSCs secretory activity is induced only after cell exposure to an inflammatory microenvironment, ..." was re-formulated as follows: "...MSCs secretory activity is boosted after exposure to inflammatory microenvironment and different inflammatory stimuli can elicit distinct responses, ..." (lines 214-215, page 9 of the tracked version of the revised manuscript).

Methods

2. **MSC in vitro culture. Authors describe "MSCs were cultured at a concentration of 2×10^5 in 14 mL in α MEM medium". What vessel was used to seed the cells and what is the final seeding density in this vessel?**

Two hundred thousand human AT-MSCs were cultured at a concentration of 14.3×10^4 cells/ml in 75cm² culture flasks (Sarstedt AG & Co. KG, Numbrecht, Germany), in 14 mL of complete medium. This information was added to the Supplementary Methods 1.1.1.

3. **Characterization of MSC after NMP. Cells were retrieved from the bioreactor. Were the cells directly analysed (cytometry). For how long were the MSC re-cultured for the morphology tests and what were the conditions of that culture? (cell seeding density, culture time, etc.). Also, did the authors count the total number of cells retrieved from the bioreactor and compared with the original number?**

We thank the Reviewer for noting that relevant information was missing. The methods for cell detachment from the bioreactor and processing before flow cytometry have been added to Supplementary Methods 1.1.3.

4. Exposure to perfusion fluid may have altered their metabolism and survival, acting as a licensing agent itself.

The Reviewer's comment is pertinent. As previously demonstrated by Sierra Parraga and coworkers, the conditions required to perform the NMP procedure influence MSCs viability and function. In particular, exposure of thawed MSCs to the perfusion fluid and the suspension condition are associated with reduced cell viability, slower mitochondrial metabolism, and generation of reactive oxygen species (ROS) ^{1,2}. Conversely, the authors reported no negative influences of the perfusion fluid on survival when the cells are kept under adherent conditions ¹.

The innovative configuration of our platform allows keeping the cells in their optimal culture environment during NMP, i.e. adherent to the bioreactor fibers. Moreover, the use of a bioreactor provides the cells with a resting period after thawing, during which they can recover from structural damage and oxidative stress induced by cryopreservation. Consistently, we did not reveal massive cell death during liverless-NMP experiments. In fact, no circulating cells, detached from the bioreactor, were detected in the perfusate samples, while the release of the apoptotic marker caspase-cleaved keratin 18 (CK18) remained stable throughout the procedure (Figure 3).

Therefore, the particular setting of the present model offers MSCs the opportunity to recover from cryopreservation, while ensuring optimal culture conditions during NMP. These points were further clarified in the Introduction (lines 153-155, page 7 of the tracked version of the revised manuscript) and in the Discussion (lines 373-375, page 14 of the tracked version of the revised manuscript).

5. Flow cytometry. For reproducibility purposes, please add the reference numbers of antibodies used for flow cytometry and other relevant reagent.

These details have been added to Supplementary Methods 1.1.3

6. Liver NMP. Perfusion took place in two phases: a warming phase and a stable normothermic phase. Was the bioreactor connected to the circuit during the entire perfusion (warming+stable phases)? Similar to the question related to shipment, rewarming time might affect MSC survival and metabolism during this time.

The bioreactor was attached to the circuit immediately before liver connection and was therefore exposed to a temperature ranging from 24-26°C, which was increased to 37°C over 40 min. As suggested by the Reviewer, we investigated whether MSCs underwent cell death during rewarming, by assessing the release of human CK18 into the perfusate. The data show that the degree of apoptosis activation during liver-NMP is similar to that observed during liverless-NMP, indicating that the cells did not experience excessive distress during the different phases of the liver-NMP procedure. These results were added to the Supplementary Results 1.2.10

7. The authors seeded 20×10^7 MSC in a bioreactor. Is this number constant throughout the perfusion experiment? Do MSC die and are replaced by proliferating MSC? These dead cells might be responsible for the uncontrolled release of cytokines.

As discussed at points 3 and 5 of the present document, no massive cell death was observed during the experiments.

- 8. In addition, average rat liver weight is 4-5 grams which results in a MSC dose of 4×10^9 MSC/kg, an extremely high dose which has a poor translation towards a clinically relevant setup.**

In our experiments, the average rat liver weight was 13-15 g; as a result, 1.5×10^6 cells were used per gram of liver, which, as highlighted by the Reviewer, is about 1000-times higher than the dosage currently adopted in clinical trials. However, we believe that this is a strength, rather than a limitation. In fact, the application of a high dosage-cell therapy appears essential for maximizing liver cell exposure to the MSCs secretome within the short time window offered by the NMP procedure. By completely eliminating the risk of embolism, the bioreactor-based setting enables to safely increase the quantity of stem cells delivered in a single administration. Additionally, the use of a bioreactor avoids cell migration to target tissues, thus preventing possible cell de-differentiation or long-term retention. Overall, this represents a significant clinical advantage, considering that for certain patients and diseases, multiple administrations of standard-dose MSCs are needed to achieve the desired therapeutic effect ³.

In support of the practical applicability of our platform, we demonstrated in a pilot study the feasibility of integrating the MSCs-bioreactor into a circuit currently used in the clinical setting (Abstract N° 1307, “Set up of a DCD pig model of normothermic machine perfusion associated to a mesenchymal stromal cell coated bioreactor to improve organ preservation”, presented at ESOT congress 2021, Milan). More specifically, we performed NMP of porcine livers procured from cardiac death donors, using the Liver Assist® machine (XVIVO Perfusion AB, Sweden) coupled with a plasma filter (Aferetica srl, Bologna, Italy), seeded with mesenchymal stem cells.

These preliminary data, together with the growing availability of novel technologies for cell upscaling and manufacturing ^{3,4}, reinforce the translational value of our advanced circuit.

- 9. In addition, authors sent MSC in a bioreactor from one laboratory to another at room temperature. Culture temperature is key for MSC survival and temperatures lower to 37° C are deleterious (Pratiwi et al., Cytoherapy, 2019). Did the authors study the status of MSC after the shipment?**

Bioreactor supernatants were collected upon devices arrival to the Milan Lab and were then analyzed using an automated cell counter, gas-analysis, and ELISA. Results of this analysis are reported in the session Assessment of MSCs viability after bioreactor shipment pg 9-10 of the original version of the manuscript and in the Supplementary Table 2.

Results

- 1. The authors describe an increase in certain metabolites such as lactate, IL-6, IL-1ra, among other, during liverless NMP+bioreactor. However, these are inflammatory cytokines and lactate production has been related to increased ROS production (i.e. Li et al., Signal Transduct Target Ther., 2022). Though the authors address the increase in inflammatory cytokines as a positive results, they should discuss the inflammatory perfusate profile and elaborate on this in the discussion section as it appears to be a setback of their NMP circuit.**

The production of lactate, along with glucose consumption, indicates that the cells were metabolically active and initiated anaerobic glycolysis during Liverless-NMP.

With regard to the increased cytokine perfusate concentration, a mild inflammatory activation of the cells seeded in the bioreactor was expected, since both shipment and the NMP procedure itself represent stress-inducing factors.

Accepting the Reviewer's suggestion, we investigated whether MSCs were exposed to oxidative stress, by assessing the release of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the perfusates collected during Liverless-NMP. We found increasing concentrations of 8-OHdG over the procedure (from 15630 ± 2211 pg at 1 h to 19965 ± 3532 pg at 4 h). However, higher amounts (28211 ± 1489 pg) were detected in the supernatant collected from the bioreactor immediately after device arrival to the Milan lab. This interesting observation indicates that MSCs experience an oxidative burst during shipment and highlight the need for a technical improvement of this stage, for example by supplementing the culture medium with antioxidants (vitamin C, glutathione). We thank the Reviewer for pointing out the need to refine these important aspects, in view of a potential translational use of our platform.

2. Immune cells outflow. Authors describe outflow of an increased number of immune cells when the bioreactor is connected during NMP. However, they do not show data for cell characterization in the outflow perfusate.

Accepting the Reviewer's suggestion, we performed a qualitative assessment of individual donor cell populations removed during the initial wash-out. Briefly, cell pellets obtained from wash-out samples were originally counted using the Scepter™ cell counter (Merck KGaA) equipped with 40 μ m sensors, according to the Coulter principle. The acquired data were further analyzed based on particle size and volume using the software Scepter™ 2.0 software pro (Merck KGaA)⁵. The obtained results are presented in Figure 5, while methods are detailed in the Supplementary Information 1.1.8. Note that, since the bioreactors used in this research have membranes with pores that are 150 nm in size, the particles found in the perfusate originate solely from the liver.

We thank the Reviewer for his comment which lead to a significant improvement in the description of the wash-out samples.

3. EV origin. The authors hint that observed differences in EV size are related to their hepatocyte or MSC origin. This is a difficult assumption to make based only on EV size.

We agree with the Reviewer and acknowledge that the assessment of EV size alone is not entirely adequate to attribute their origin to either stem cells or the liver.

However, due to the inherent technical difficulties in analyzing Oxyglobin-enriched samples, a more thorough evaluation of the EVs released in the perfusate was unfeasible. Based on this, the results of EVs analysis were excluded from the main findings and relocated in the Supplementary Results 1.2.4.

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electrolytes which can directly influence perfusate pH, as reported in Supplementary Figure 6 - Gas analysis of perfusate samples collected during liver-NMP.

Reviewer #2

This study reports the effects of the addition of an MSC-bioreactor to the NMP circuit in an isolated rat liver model. The study mainly shows the safety and feasibility of this approach and some beneficial effects of using an MSC bioreactor approach rather than thawed MSCs. The MSC-bioreactor effects included decreased injury markers, increased bile production; increased acute phase proteins; more effective washout of blood; reduced ATP depletion; absence of succinate accumulation. Please address the following:

- 1. You state that the 'translational value is high'. This needs justification when using a rodent model as many such models have been shown not to have reproducible effects in large animals and humans.**

Although we acknowledge the difficulties encountered when transferring the findings from preclinical studies to the clinical scenario, we deem that starting from a rodent model was an essential step in the development of the novel circuit for liver NMP, where the livers can be treated with a MSCs-based therapy before transplantation. In fact, establishment of such an innovative platform required the consideration and implementation of various technical aspects related to both MSCs and the NMP procedure, including cell type selection, seeding density, design of the bioreactor circuit, and oxygen supply to the liver. The use of rodent models shows several advantages compared to large animals, including a more rapid recovery of hepatic homeostasis and function, less intra-experimental group variability, and greater selection of specific laboratory reagents. Thus, small animal models emerge as the optimal choice at this early stage of the research.

The knowledge gained with the present study was used to guide the translational application of the novel platform to porcine liver NMP (Abstract N° 1307, “Set up of a DCD pig model of normothermic machine perfusion associated to a mesenchymal stromal cell coated bioreactor to improve organ preservation”, presented at ESOT congress 2021, Milan). More specifically, porcine livers procured from cardiac death donors were ex situ-perfused using the Liver Assist® machine (XVIVO Perfusion AB, Sweden) coupled with a plasma filter (Aferetica srl, Bologna, Italy), seeded with mesenchymal stem cells. This pilot study demonstrates the practical applicability of our novel configuration when the clinical devices/consumables are used.

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responses to stress stimuli between the livers of the various species. Therefore, a direct comparison of human and experimental procedures may not be entirely adequate when time intervals are considered.

Consistently, the present research shows that a different duration of ischemia enabled to obtain the same outcome. In fact, the IR challenge applied in this study not only induced the release of cytolysis markers, but it likewise promoted many of the detrimental biological events typically triggered by IR, including intracellular ATP depletion (Figure 6), succinate accumulation (Figure 6), and production of inflammatory mediators (Figure 7).

Thus, although the ischemic time is shorter than that observed in the clinical setting, the experimental findings support the reliability of our model in reproducing the key pathophysiological features of liver IR injury and support its translational use as a useful starting point for future studies in porcine and human livers.

More prolonged or extreme stimuli can be applied to our model. However, here we describe the set-up phase of NMP+bioreactor and we believe that smaller but significant injuries could lead to more informative results in this research phase.

3. Please discuss the potential effects of raised TNF and IL-6 in the bioreactor group. These acute phase proteins can have pro-inflammatory as well as protective effects.

As highlighted by the Reviewer, IL-6 and TNF- α can exert either protective or harmful effects depending on various factors, including the amount released, the duration of their expression, and the underlying liver condition.

In the early stages of IRI, TNF- α and IL-6 are secreted as part of the pathophysiological immune response to the ischemic damage^{7,8}. Their role involves recruiting immune cells to the injured site and activating pathways related to tissue repair and removal of damaged cells⁹. In addition, TNF- α and IL-6 are established key factors in the onset of liver cell regeneration¹⁰.

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underlying mechanism based on previous evidence ^{14,15}. Further research is required to verify whether this process plays a role in the context of hepatic IRI.

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RESPONSE TO REVIEWERS

MANUSCRIPT NUMBER: NCOMMS-23-38868

We thank the reviewers for their helpful feedback. Below, we present our responses to the points raised and a description of the changes made accordingly. Reviewers' comments are typed in bold and our corresponding responses in regular text.

Reviewer #1

The authors have skillfully design a promising platform to study the effect of mesenchymal stem cells (MSC) during ex vivo perfusion of rat livers. This is a very interesting approach to overcome some issues observed in direct MSC infusion in organ-in-a-pump models, like microvasculature clogging. The main successful points are the modification of a rat liver normothermic machine perfusion (NMP) system to include a bioreactor for MSC cell therapy and the maintenance of hepatocyte viability during MSC-NMP. However, there are some concerns that need to be addressed:

Introduction

1. **Authors claim that MSC only activate their secretory potential after exposure to inflammatory stimuli. However, this is not true as demonstrated earlier (i.e. Park et al., Int J Stem Cells., 2009) but MSC secretory profile is modulated by the microenvironment (i.e. Burja et al., Curr Res Transl Med., 2020).**

We thank the Reviewer for stressing this concept and for providing an additional reference demonstrating the plasticity of the immunoregulation mediated by adipose tissue (AT)-MSCs, which can mount distinct responses to different inflammatory stimuli. These observations reinforce the rationale behind the design of our platform, where stem cells' secretory activity can be specifically activated by the inflammatory mediators release by each individual liver. The sentence "...MSCs secretory activity is induced only after cell exposure to an inflammatory microenvironment, ..." was re-formulated as follows: "...MSCs secretory activity is boosted after exposure to inflammatory microenvironment and different inflammatory stimuli can elicit distinct responses, ..." (lines 214-215, page 9 of the tracked version of the revised manuscript).

Methods

2. **MSC in vitro culture. Authors describe "MSCs were cultured at a concentration of 2×10^5 in 14 mL in α MEM medium". What vessel was used to seed the cells and what is the final seeding density in this vessel?**

Two hundred thousand human AT-MSCs were cultured at a concentration of 14.3×10^4 cells/ml in 75cm² culture flasks (Sarstedt AG & Co. KG, Numbrecht, Germany), in 14 mL of complete medium. This information was added to the Supplementary Methods 1.1.1.

3. **Characterization of MSC after NMP. Cells were retrieved from the bioreactor. Were the cells directly analysed (cytometry). For how long were the MSC re-cultured for the morphology tests and what were the conditions of that culture? (cell seeding density, culture time, etc.). Also, did the authors count the total number of cells retrieved from the bioreactor and compared with the original number?**

We thank the Reviewer for noting that relevant information was missing. The methods for cell detachment from the bioreactor and processing before flow cytometry have been added to Supplementary Methods 1.1.3.

4. Exposure to perfusion fluid may have altered their metabolism and survival, acting as a licensing agent itself.

The Reviewer's comment is pertinent. As previously demonstrated by Sierra Parraga and coworkers, the conditions required to perform the NMP procedure influence MSCs viability and function. In particular, exposure of thawed MSCs to the perfusion fluid and the suspension condition are associated with reduced cell viability, slower mitochondrial metabolism, and generation of reactive oxygen species (ROS) ^{1,2}. Conversely, the authors reported no negative influences of the perfusion fluid on survival when the cells are kept under adherent conditions ¹.

The innovative configuration of our platform allows keeping the cells in their optimal culture environment during NMP, i.e. adherent to the bioreactor fibers. Moreover, the use of a bioreactor provides the cells with a resting period after thawing, during which they can recover from structural damage and oxidative stress induced by cryopreservation. Consistently, we did not reveal massive cell death during liverless-NMP experiments. In fact, no circulating cells, detached from the bioreactor, were detected in the perfusate samples, while the release of the apoptotic marker caspase-cleaved keratin 18 (CK18) remained stable throughout the procedure (Figure 3).

Therefore, the particular setting of the present model offers MSCs the opportunity to recover from cryopreservation, while ensuring optimal culture conditions during NMP. These points were further clarified in the Introduction (lines 153-155, page 7 of the tracked version of the revised manuscript) and in the Discussion (lines 373-375, page 14 of the tracked version of the revised manuscript).

5. Flow cytometry. For reproducibility purposes, please add the reference numbers of antibodies used for flow cytometry and other relevant reagent.

These details have been added to Supplementary Methods 1.1.3

6. Liver NMP. Perfusion took place in two phases: a warming phase and a stable normothermic phase. Was the bioreactor connected to the circuit during the entire perfusion (warming+stable phases)? Similar to the question related to shipment, rewarming time might affect MSC survival and metabolism during this time.

The bioreactor was attached to the circuit immediately before liver connection and was therefore exposed to a temperature ranging from 24-26°C, which was increased to 37°C over 40 min. As suggested by the Reviewer, we investigated whether MSCs underwent cell death during rewarming, by assessing the release of human CK18 into the perfusate. The data show that the degree of apoptosis activation during liver-NMP is similar to that observed during liverless-NMP, indicating that the cells did not experience excessive distress during the different phases of the liver-NMP procedure. These results were added to the Supplementary Results 1.2.10

7. The authors seeded 20×10^7 MSC in a bioreactor. Is this number constant throughout the perfusion experiment? Do MSC die and are replaced by proliferating MSC? These dead cells might be responsible for the uncontrolled release of cytokines.

As discussed at points 3 and 5 of the present document, no massive cell death was observed during the experiments.

- 8. In addition, average rat liver weight is 4-5 grams which results in a MSC dose of 4×10^9 MSC/kg, an extremely high dose which has a poor translation towards a clinically relevant setup.**

In our experiments, the average rat liver weight was 13-15 g; as a result, 1.5×10^6 cells were used per gram of liver, which, as highlighted by the Reviewer, is about 1000-times higher than the dosage currently adopted in clinical trials. However, we believe that this is a strength, rather than a limitation. In fact, the application of a high dosage-cell therapy appears essential for maximizing liver cell exposure to the MSCs secretome within the short time window offered by the NMP procedure. By completely eliminating the risk of embolism, the bioreactor-based setting enables to safely increase the quantity of stem cells delivered in a single administration. Additionally, the use of a bioreactor avoids cell migration to target tissues, thus preventing possible cell de-differentiation or long-term retention. Overall, this represents a significant clinical advantage, considering that for certain patients and diseases, multiple administrations of standard-dose MSCs are needed to achieve the desired therapeutic effect ³.

In support of the practical applicability of our platform, we demonstrated in a pilot study the feasibility of integrating the MSCs-bioreactor into a circuit currently used in the clinical setting (Abstract N° 1307, “Set up of a DCD pig model of normothermic machine perfusion associated to a mesenchymal stromal cell coated bioreactor to improve organ preservation”, presented at ESOT congress 2021, Milan). More specifically, we performed NMP of porcine livers procured from cardiac death donors, using the Liver Assist® machine (XVIVO Perfusion AB, Sweden) coupled with a plasma filter (Aferetica srl, Bologna, Italy), seeded with mesenchymal stem cells.

These preliminary data, together with the growing availability of novel technologies for cell upscaling and manufacturing ^{3,4}, reinforce the translational value of our advanced circuit.

- 9. In addition, authors sent MSC in a bioreactor from one laboratory to another at room temperature. Culture temperature is key for MSC survival and temperatures lower to 37° C are deleterious (Pratiwi et al., Cytoherapy, 2019). Did the authors study the status of MSC after the shipment?**

Bioreactor supernatants were collected upon devices arrival to the Milan Lab and were then analyzed using an automated cell counter, gas-analysis, and ELISA. Results of this analysis are reported in the session Assessment of MSCs viability after bioreactor shipment pg 9-10 of the original version of the manuscript and in the Supplementary Table 2.

Results

- 1. The authors describe an increase in certain metabolites such as lactate, IL-6, IL-1ra, among other, during liverless NMP+bioreactor. However, these are inflammatory cytokines and lactate production has been related to increased ROS production (i.e. Li et al., Signal Transduct Target Ther., 2022). Though the authors address the increase in inflammatory cytokines as a positive results, they should discuss the inflammatory perfusate profile and elaborate on this in the discussion section as it appears to be a setback of their NMP circuit.**

The production of lactate, along with glucose consumption, indicates that the cells were metabolically active and initiated anaerobic glycolysis during Liverless-NMP.

With regard to the increased cytokine perfusate concentration, a mild inflammatory activation of the cells seeded in the bioreactor was expected, since both shipment and the NMP procedure itself represent stress-inducing factors.

Accepting the Reviewer's suggestion, we investigated whether MSCs were exposed to oxidative stress, by assessing the release of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the perfusates collected during Liverless-NMP. We found increasing concentrations of 8-OHdG over the procedure (from 15630 ± 2211 pg at 1 h to 19965 ± 3532 pg at 4 h). However, higher amounts (28211 ± 1489 pg) were detected in the supernatant collected from the bioreactor immediately after device arrival to the Milan lab. This interesting observation indicates that MSCs experience an oxidative burst during shipment and highlight the need for a technical improvement of this stage, for example by supplementing the culture medium with antioxidants (vitamin C, glutathione). We thank the Reviewer for pointing out the need to refine these important aspects, in view of a potential translational use of our platform.

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RESPONSE TO REVIEWERS

MANUSCRIPT NUMBER: NCOMMS-23-38868

We thank the reviewers for their helpful feedback. Below, we present our responses to the points raised and a description of the changes made accordingly. Reviewers' comments are typed in bold and our corresponding responses in regular text.

Reviewer #1

The authors have reviewed their manuscript according to suggestions and concerns made by the reviewers. Additionally, they have included some pilot results in a large animal NMP model to improve the translational impact of their work. Here are some issues that need to be addressed.

1) According to the international society for cell and gene therapy, the correct term for MSC is now "mesenchymal stromal cells" (Viswanathan et al., Cytotherapy, 2019). Authors should update their manuscript.

We thank the Reviewer for this important suggestion. The manuscript has been changed accordingly.

2) Although the addition to the NMP circuit might be novel, culture of MSC in hollow fiber bioreactors is already known. Additionally, commercial distribution of MSC has been offering the possibility of shipping cultured MSC in culture flasks or other formats, keeping their properties intact after transportation, which is quite similar to the logistic procedure described here. Novelty of this fact is scarce

As noted by the Reviewer, the novelty of our study resides in the connection of a MSCs-bioreactor to a NMP platform. This innovative experimental setup has not been explored in previous researches, nor the core properties of MSCs in a bioreactor have been investigated after exposure to a perfusion procedure. A recent analysis performed by Fu and coworkers highlights the huge therapeutic potential of coupling MSCs-bioreactors to ex situ perfusion, but does not provide experimental clues supporting its applicability, and claim the need to perform targeted experiments aimed at addressing this point³.

Our research fills this knowledge gap, providing for the first time evidence showing both feasibility and efficacy of this modern MP platform. This important information is essential to proceed with the clinical implementation of this new technology. In this regard, the possibility of shipping pre-seeded bioreactors from a specialized cell facility to any Transplant Centers, could contribute to accelerating the translational process.

3) Authors need to update figure 4 and supplemental data including all cell types susceptible to be measured in "wash-outs", (i.e. MSC, endothelial cells, etc.) Even in low numbers, MSC can still detach and flush through the system.

Figure 4 was updated to include “endothelial cells” among cells detected within the following cell diameter range: 9 -18 μm ⁴. In line with the Reviewer’s comment, the supplementary material has been likewise revised (line 184, page 10).

With regard to the Reviewer’s suggestion to include also MSCs among the cells detached during the NMP procedure, we respectfully hold a different viewpoint and would appreciate the opportunity to offer an explanation justifying our decision. The wash-out samples drained from livers subjected to the bioreactor-based perfusion showed a higher cell count and a greater concentration of nitric oxide (NO) metabolites compared to those collected during standard NMP. The increased availability of the vasodilator NO, together with increased cell concentration, support a more effective wash-out of donor blood in livers exposed to the MSCs-derived secretome compared to controls. Moreover, as described in the previous “Response to Reviewers” (Reviewer #1, point 6), the results obtained from liverless-NMP experiments clearly indicate no MSCs detachment during the perfusion procedure (lines 152-153, page 8 of the revised version of the manuscript). These evidence collectively suggest that the cells found in the perfusates derive solely from the liver.

4) Authors mention the section supplementary methods 1.12 which is not included in the supplementary material.

Authors thank the Reviewer for pointing this out. All the references to section 1.12 were removed from the main text.

5) Supplementary figure 12. Regardless of measured parameters used to evaluate success in NMP, visual evaluation is still key during perfusion. In the images, there is a clear difference in perfusion between both organs, with the one from the MSC group having a paler color. This unequivocally show bad perfusion of the organ. Supplementary figure 12 does not compare livers from the control and the NMP+b-MSCs group, rather the pictures represent a random liver before and after undergoing the bioreactor-based perfusion procedure.

As suggested by the Reviewer, the macroscopic appearance of the liver provides a first general impression of the liver during perfusion, but its clinical value is still debated. Indeed, at a recent international guideline meeting on liver viability during in-situ/ex-situ machine perfusion organized by ELITA/ESOT (Madrid, March 18-20, 2024), a dedicated discussion was devoted to this topic. While the macroscopic appearance of the liver was considered a possible parameter in in-situ perfusion (normothermic regional perfusion, NRP), it was not included among the viability parameters in ex-situ perfusion (NMP). In fact, the macroscopic appearance of the liver during NMP was hardly related to perfusion quality and viability. During the session, a patchy and/or pale appearance of the NMP liver, rather than possible differences in the light exposure of the camera, was related to non-physiological perfusion conditions (e.g., outside the human body) and to the position of the liver during NMP, but not to the overall perfusion quality when NMP fluidodynamics and metabolism were preserved. In our study, all clinically derived parameters were met, fluidodynamics parameters were preserved throughout the perfusion time and, lastly, histology was preserved. At the above mentioned meeting, three of the authors of the present manuscript participated as "experts" and here we report a preliminary and confidential extract of the statement resulting from the meeting to further support the above statements:

“Comment to Q11: What parameters measured during in-situ normothermic regional perfusion may be used to assess DCD liver viability?

[...] The parameter “gross appearance of the liver” is very subjective and often based on the perception and experience of the procurement surgeon and team involved in NRP. [...]”

“Statement Q13: What parameters measured during ex situ normothermic machine perfusion may be used to assess deceased donor liver viability?

*1) Expert Panel Statement: Perfusion quality parameters, perfusate parameters (pH, lactate, transaminases, Glucose) and bile parameters (bile flow, pH, Glucose, Bicarbonate) may be used to assess deceased donor liver viability.
Recommendation grade: conditional recommendation for
Median level of evidence: 2 (range 3 to 2+)”*

Authors thank the reviewer for the opportunity to discuss this topic and anticipate some aspects that will be further explored in literature in the near future.

This being said, based on the poor predictive potential of macroscopic aspects in the context of ex situ perfusion procedures, and in line with the Reviewer's comment, we would remove the panels a and b from Supplementary figure 12.

6) There is a discrepancy regarding the number of cells used for the pig experiments described in the results and methods section. 37×10^7 cells noted in the results section does not equal 37 million cells (20 ml of a 1.85×10^6 cells/ml suspension). Please correct accordingly. We apologize for this imprecision; the total number of cells seeded in the bioreactors was 3.7×10^7 . Authors are very grateful to the Reviewer for his accurate revision.

7) Authors have used 2×10^7 MSC for the rat NMP setup and average liver weight was 15 grams, resulting in a dose of 1.3 million MSC per gram of tissue. However, only 28-280 thousand cells (depending on correction made based on the previous point) per gram of liver tissue was reached in the pig experiments. Comparison of results is therefore very limited between both models.

8) Additionally, cell density in bioreactors is extremely different: 18500 MSC per cm^2 for pig bioreactor vs. 40000 MSC/ cm^2 in rat bioreactors. Cell density is known to alter MSC functionality, secretory profile and immunomodulatory potential. Authors should justify these differences and elaborate about possible consequences.

All the calculations made by the Reviewer are correct. However, we would like to clarify that the pilot study in porcine livers was conducted to investigate whether a bioreactor can be technically integrated into a clinical device and to test whether this new configuration can sustain the protocol for human liver MP. Our intention was to provide experimental evidence in support of the clinical applicability of our new setup, addressing the concerns raised in the previous revisions on this topic. Thus, in addition to the differences pointed out by the Reviewer, the porcine study involved several other changes compared to the small animal model, which were introduced to progress towards the setting adopted in the human scenario. First, porcine livers were exposed to a period of warm ischemia. Second, the NMP was performed using leukocyte-depleted autologous blood including red blood cells and the perfusate was pumped into the liver through both hepatic artery and portal vein. Moreover, portal vein target pressure was different compared to the setting adopted in the rat NMP, and the porcine circuit was equipped with two oxygenators. These, and other, adjustments were needed to test the feasibility of the bioreactor-based perfusion in a human-like setting.

This being said, as highlighted by the Reviewer, no comparison in the MSCs effects between rat and pig livers was provided, nor were the MSCs functionality and secretory profile explored in porcine experiments, since the experimental aims of these two work packages were different.

9) The large animal model used by the authors lacks potential for translation. Clinical strategies used in human liver NMP trials include perfusion times ranging from 9-20+ hours. Although authors are presenting preliminary results in a large animal preclinical model coupled to an MSC bioreactor, clinical translation assumptions should be made carefully, as the author do not present sufficient data supporting their claims.

We agree with the reviewer on the potential drawbacks of our large animal model from a translational point of view. For this reason, we used pig experiments only to test the possible integration of an MSC bioreactor into a commercial perfusion device, as in other studies where pigs were used for technological assessment. In this setting, a NMP duration of 4 h was chosen by our group. In the clinical literature, perfusion time varies widely within the same study and between studies. In fact, while most studies did not specify a per-protocol NMP duration, it usually depends on the goal of the perfusion, how it is applied (e.g., back to base or in the donor hospital approach), and other logistical issues. While preservation and viability might require a longer NMP time, reconditioning might benefit from a shorter NMP duration when used to face organ reperfusion, as in the DCDnet trial (NCT04744389) in which some of the present authors are involved. For these reasons, we decided to use a 4-hour NMP in our WP3. In addition, since our goal was to test the integration of the bioreactor into a commercial NMP device, most of the technological issues should become apparent early after the start of the NMP. Following the Reviewer's suggestion, we revised and, in some cases, reformulated, all the claims about the clinical translational of our results. As pointed out by the Reviewer, longer preservation times will be applied in future studies to verify the biological changes secondary to the connection of the MSC bioreactor to an NMP.

10) Even though these are preliminary results, only n = 3 animals were used for the large animal study. This small sample size needs justification and sample size calculation need to be explained.

We appreciate this important note on sample size calculation in the pilot study and consistently improved the dedicated paragraph in the Supplementary material by adding the requested information (1.11.1 Sample size, page 14).

As discussed above, the work package in large animals investigated feasibility of bioreactor integration into an existing perfusion circuit for human use. The possible outcomes were, therefore, defined as categorical, namely 1) success of execution (feasible) or 2) failure of execution (unfeasible).

Due to lack of prior data and consequent unavailability of a formal verification of sample size, the number of pigs needed as liver donors was determined based on our experience in the set up and implementation of novel perfusion platforms for preclinical models ⁵⁻⁷.

Considering these assumptions, while adhering to the ethical principles in animal research ^{8,9}, we elected to use 3 animals for each experimental group.

Reviewer #2

The incorporation of an MSC-bioreactor in a normothermic machine perfusion (NMP) circuit is a novel concept although other groups are working in this area. There is a body of work demonstrating that MSCs can ameliorate ischaemia reperfusion injury and it is known that this is due to the release of EVs and paracrine factors from the stem cells and that MSCs themselves have a number of potentially adverse effects. The isolated organ preservation offered by NMP removes problems with targeting stem cells to a specific organ and bypassing the reticuloendothelial system. NMP also reduces the risk of off-target adverse effects. The addition of a bioreactor to the NMP circuit is a logical development.

The authors have demonstrated the feasibility of this new approach and in the rat model evidence that the addition of a bioreactor can counter some of the changes associated with I/R injury.

There are a number of issues that the authors should consider:

1. The relevance of the rat model is questionable as studies using rodent models of ischaemia reperfusion injury have a very poor record of translation into clinical practice.

We thank the Reviewer for the opportunity to further discuss the scientific background of our research, which provides a proof of concept to implement a new platform for liver NMP.

Based on the existing literature, rodent models of ischemia/reperfusion injury (IRI) are widely utilized to investigate novel intervention strategies or gain insights into the biological mechanisms elicited by IR. Of interest, a recent systematic review explored the use of cell-based therapies to counteract IRI in transplantable organs². The analysis indicated that out of the 97 studies examined, 45 utilized rat models of ischemic injury, while 46 researches were conducted in mice. Therefore, although we acknowledge that clinical translation could be challenging, preclinical models still represent valuable tools for scientific investigation in this field.

Moreover, it's important to consider that the typical pathway to develop a new medical setup involves the progression from initial testing in rodent models to subsequent studies in larger animals before advancing to human research. Since we aimed at establishing an unprecedented technology, the use of a small animal model was necessary to depict the various technical and biological aspects essential to successfully integrate the bioreactor into a NMP circuit, while demonstrating MSCs' efficacy in mitigating IRI.

2. The porcine model demonstrated the potential for upscaling but there was much less analysis of outcome compared to the rat component and it is not clear why. The porcine liver is a better model than the rat but still has important differences to the human liver. Porcine livers have a histological structure based on very distinct lobules whereas the basic unit of the human liver is acinar. In addition, porcine livers are more resistant to warm ischaemic injury than the human organ.

We thank the Reviewer for the comment which allows improving the description of the research aims. As highlighted, the pilot study in porcine livers was conducted to investigate the potential of the bioreactor to be upscaled and integrated into a clinical device, while providing information supporting the ability of the new configuration to sustain the protocol for human liver MP. Our intention was to provide experimental evidence to demonstrate the technical feasibility of our new setup in a scenario resembling the clinical setting, addressing the concerns raised in the previous revisions on this topic. Therefore, as noted by the Reviewer, we did not investigate the MSCs' effects on porcine liver IRI. On the other hand, this aspect was extensively studied in rat livers, taking advantage of the expertise of our Research Team as well as on availability of a variety of bio-molecular technologies previously validated for this model¹¹⁻¹⁷.

3. The group size for the porcine experiments is small at n=3 and the period of NMP was only 4 hours. More prolonged NMP (at least 24h and even up to several days) have been proved feasible and would have allowed more detailed study of the influence of the bioreactor on liver injury (e.g. A2M a& lpha-1 glycoprotein increases). More prolonged perfusion would also help define whether the MSCs survive in the bioreactor for longer than 4h.

We recognize that sample size calculation for the pilot study needed to be expanded and, as a consequence, we changed the dedicated paragraph in the Supplementary material to better justify our evaluation (1.11.1 Sample size, page 14).

We agree with the Reviewer that the investigation of the bioreactor-based perfusion effects during prolonged procedures is an intriguing research perspective. In fact, we are currently working on the setup of extended bioreactor-based NMP, with the ultimate aim to explore the long-term consequences of MSCs. In this regard, our Group recently completed the development and optimization of a stable protocol for 12 h-NMP in rodents¹⁷.

4. The best model would be to use human livers that were declined for transplantation. These can even be split into left and right lobes to allow a comparison of intervention vs control NMP.

We appreciate the Reviewer's suggestion regarding the use of human livers deemed unsuitable for transplantation. This approach could indeed provide valuable insights into the efficacy of bioreactor-based NMP and, as such, it appears as a logical next research step. The scientific community could take advantage of the seminal results provided by the present study, and consider conducting targeted experiments to investigate the benefits of bioreactor-based NMP in human livers. We added a sentence in the Discussion section (lines 360-363, page 15 of the revised version of the manuscript) to highlight this concept.

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RESPONSE TO REVIEWERS

MANUSCRIPT NUMBER: NCOMMS-23-38868

We thank the Editor for the opportunity to re-evaluate our manuscript and the Reviewers for their helpful feedback.

Below, we present our responses to the points raised and a description of the changes made accordingly. Reviewers' comments are typed in bold and our corresponding responses in regular text.

Reviewer #1

The authors have replied to most of the reviewers comments, with a very elaborated explanation in some cases.

Thank you for recognizing our efforts to improve the clarity and the relevance of our manuscript.

Some questions still arise:

1. The authors missed one of the previous comments: Authors have used 2×10^7 MSC for the rat NMP setup and average liver weight was 15 grams, resulting in a dose of 1.3 million MSC per gram of tissue. However, only 28-280 thousand cells (depending on correction made based on the previous point) per gram of liver tissue was reached in the pig experiments.

Comparison of results is therefore very limited between both models.

We apologize if we did not make this point clear. The exact number is 2.8×10^4 cells per gram of liver tissue.

While we agree with the Reviewer that comparison of results is very limited between the models, this observation open up two different discussions. The first is the aim of the porcine study and the second is the normalization.

First, we would like to emphasize that the large animal study was not conducted to compare the effects of MSCs on porcine versus rat liver. Instead, its primary goal was to test the feasibility of integrating our bioreactor into a clinical NMP platform. This crucial step must be completed before advancing with further research and required a dedicated pilot study to avoid future failures, as recommended by NC3Rs ¹.

Concerning normalization, the selection of normalization criteria and of the compounds that should be normalized is still a matter of debate in the MP field. In fact, it is either partially applied or not applied at all in MP literature, even in those experiments that use depurative strategies such as dialysis, that could deeply manipulate the concentration of some compounds ²⁻⁵. This being said, according to the number of cells, we had the same concerns of the Reviewer. Indeed, whether the effect of the MSCs is directed to the liver or the MSCs are primed by the liver-secreted factors leads to a different normalization. In the first case, the normalization would be based on MSC/g of liver, in the other on the volume and flow of the perfusate. Since we could not get a direct answer from the literature and other experiments, we applied a normalization based on the perfusate volume and flow rate into the bioreactor, due to the inherent differences in the perfusion settings of rodent and pig model. This combined factor was comparable between the rodent and porcine models.

We thank the Reviewer for the comment and have added the paragraph “1.12.2 Preparation of the bioreactor suitable for porcine livers”, in the Supplementary information, willing to open a debate on this issue. We are, indeed, aware that the type and the selection of the normalization criteria can be criticized.

2. Regarding MSC detaching from the bioreactor. MSC do not flush through during liver-less perfusion. However, as fluid composition changes due to the liver secreted molecules and cytokines, there is no data confirming this subject as conditions change. The authors do not provide any direct proof of the cell types leaving the NMP system. Therefore, all cells present in the NMP system should be taken into account, including MSC.

We agree with the Reviewer that the factors secreted by the liver could have had a chemotactic effect on the MSCs. As suggested, we cannot directly prove the absence of small MSCs or MSCs fragments in the perfusion fluid during liver NMP. Therefore, Figure 4 was accordingly updated to include “MSCs” among cells detected within the following cell diameter range: 9 -18 μm .

This being said, we would like to point out that the bioreactor fibers are made of semi-permeable membranes with a pore diameter of around 150 nm, as already reported in the Supplementary information (Supplementary figure 1 and Supplementary figure 9). Given that the average size of the MSCs cultured under standard conditions is 15 - 30 μm ^{6,7}, the likelihood for an intact stem cell to pass through the pores appears very low and must assume a considerable degree of cell deformability⁸. Moreover, it should be noted that the same fibers are used in clinical plasmafilters to trap erythrocytes, which have a smaller diameter than MSCs.

To improve the clarity on this point, we have reported the bioreactor fiber pore size even in the main text (page 17-18, lines 449-476 of the tracked version of the manuscript) and added a detailed bioreactor description to the Supplementary information 1.2 (page 5).

3. The authors have provided preliminary data on the technical feasibility of coupling an MSC biorreactor in a porcine NMP setup. The objective was to set a translatable setup for human use in the clinic. With this in mind, the very small population size (n= 3) do not allow to obtain any conclusion and larger studies need to be carried out.

By definition, pilot studies are “designed to test the feasibility of methods and procedures for later use on a large scale or to search for possible effects and associations that may be worth following up in a subsequent larger study”⁹. Since the large animal work package was performed to investigate the use of the novel bioreactor-based configuration as a part of multistep project, it fully meets the criteria set for a pilot study. Consistently, our claim was to test the possible integration of our MSCs bioreactor in a human device, rather than providing evidence on direct clinical translatability.

We fully agree with the reviewers and the editor that sample size is a critical issue. As highlighted and accepted by Reviewer #2 (point 4) and in accordance with NC3Rs¹, in our pilot study in porcine livers, the sample size was determined as detailed in the Supplementary information 1.12:

1.12.1. Sample size

This pilot study was designed to establish a novel perfusion system by combining the Liver Assist® machine to a stem cell bioreactor. Sample size was determined according to the guidance published by NC3R¹, considering the following possible outcomes: 1) success of execution (feasible) or 2) failure of execution (unfeasible). Based on our experience in the set up and implementation of unprecedented perfusion platforms for preclinical models¹⁰⁻¹², we deemed necessary using 3 animals for the NMP+bioreactor group. Therefore, work package 3 involved a total of 6 animals.

To provide further clarification on this issue, we have added a brief description in the main text (page 20, lines 551-558 of the tracked version of the manuscript). If the Reviewers and Editor deem it preferable to more extensively use the term "pilot study" to enhance clarity regarding the aim of the large animal model, and if it aligns with the journal's guidelines, we will incorporate this suggestion in subsequent manuscript versions.

Reviewer #2

1. The relevance of the rat model is questionable as studies using rodent models of ischaemia reperfusion injury have a very poor record of translation into clinical practice.

The authors address the use of rodent models on the basis that this is common practice and that rodent models are an important step in the evolution of new technologies. These are reasonable points but still don't answer the issue of translatability. There is not much the authors can do about this limitation.

As declared in previous review rounds, we are in full agreement with the assertions made by Reviewer #2. Indeed, the low translatability rate from rodent studies to clinical applications is well documented¹³, but this observation needs to be carefully interpreted to avoid simplism (e.g. futility of rodent models in biomedical research). In fact, it is established that rodent models remain an invaluable resource for investigating the biological mechanisms underlying a given phenomenon. As a consequence, the poor translatability does not preclude the use of these models in the pathway towards clinical implementation of a novel technology or treatment.

Of note, different Societies, Associations, and Institutions relevant to preclinical research, indicated a poor methodological approach and the lack of protocol standardization among the main reasons causing the low translational rate and the failure to recapitulate many human disease symptoms¹⁴⁻¹⁷. To address this important issue, we strictly adhered to the PREPARE guidelines¹⁴ during the approval phase of the protocol, and to the ARRIVE guidelines¹⁸ during article preparation, as recommended by NC3Rs, Felasa, and NORECOPA.

We have never claimed the use of rodent models as “common practice”, but we believe that it could be considered a “common science” based on international literature (see response to point 1 raised by Reviewer #2 in the previous review round) and guidelines. The disadvantages of not initiating the process from a rodent model include incomplete comprehension of a complex phenomenon and a reliance on previously published studies to develop a protocol or interpret results. In addition, it must be noted that this iter is also needed for the legislative approval of a new device/drug [https://health.ec.europa.eu/medical-devices-sector_en].

In the transplantation field, no study have ever demonstrated that a MSC-bioreactor can be coupled to liver NMP and that MSCs can be activated by the interaction with the liver. To address this intriguing research question, after an in-vitro phase (liverless perfusion), we employed a rat model and, upon successfully accomplishing this objective, in line with the recommendations of the Reviewers and the Editor, we explored the bioreactor integration into a commercial human device.

The final step of our project will be the clinical applicability of our technology, which, in our idea, will deserve a separate research project (see also paragraph 2 of points 3-4, Reviewer #3) and a separate paper. In a “translational” perspective, the present results will be the milestones needed to properly set up this future study. We do not believe that reporting a new device or drug (MSCs are considered drugs for Italian legislation) would require the inclusion of all the research steps in a single publication.

The role of small animal model was better clarified in the Discussion (page 12, lines 279-282 of the tracked version of the manuscript).

2. The porcine model demonstrated the potential for upscaling but there was much less analysis of outcome compared to the rat component and it is not clear why. The porcine liver is a better model than the rat but still has important differences to the human liver. Porcine livers have a histological structure based on very distinct lobules whereas the basic unit of the human liver is acinar. In addition, porcine livers are more resistant to warm ischaemic injury than the human organ.

The authors have answered my concerns to some extent. The use of a porcine model provides

useful feasibility evidence that the technology can be upscaled to a large animal set-up that resembles the clinical situation.

The comments provided by the Reviewer are in complete accordance with the considerations outlined in our previous correspondence and in the various versions of the main text. As the Reviewer correctly asserted, the porcine model was employed solely for the purpose of evaluating the feasibility of upscaling the bioreactor in a commercial device. Furthermore, it is important to note that this is not merely a straightforward issue of scaling up (as outlined in points 7-8 of the previous "Letter to the Reviewers" by Reviewer #1), but also entails adapting our bioreactor to a distinctly engineered circuit, compared to that for rat liver NMP. The integration of the bioreactor in a commercial human device was successful, with no failure of the circuit or machine and no macroscopic, as pointed out by the Reviewer #2 damage to the liver (Supplementary table 6 and Supplementary figure 12). Therefore, the point was not only addressed to "some extent", but any further elaboration on the analyses or the possible underlying mechanisms (as requested in point 3 by Reviewer #2) would exceed the scope of our large animal study. Indeed, we are fully aware of the differences in structure and biological mechanisms between human and porcine livers, and we agree with the Reviewer on this matter. Nevertheless, the underlying premise of our experimental design is that porcine livers, despite their heterogeneity, may still serve a role in device setup and technology testing¹⁹. This concept is supported by a substantial body of literature, as evidenced by the fact that the Pubmed search "liver normothermic machine perfusion AND pig" provides 63 articles. A systematic review on this topic was published in 2018 in Transplantation by leading experts in the fields of liver MP and stem cells²⁰. This review identifies the porcine model as a convenient method to explore novel approaches of perfusion and to study the early events of IRI. Among the presented NMP model, we selected the Groningen approach²¹, which was adapted to be used with the commercial device Liver Assist (XVIVO). Of note, pig livers have been used in recent years to develop novel MP techniques, which have been subsequently tested on non-viable livers^{3,5}. This ultimately led to the clinical transplantation of the liver²².

We recognize that our initial presentation may have lacked clarity; thus, we have expanded the "Study design" paragraph (page 7, lines 129-130 of the tracked version of the manuscript) and the discussion on the rationale behind our large animal model (page 12, lines 279-284 and page 15-16, lines 380-388 of the tracked version of the manuscript).

3. The group size for the porcine experiments is small at n=3 and the period of NMP was only 4 hours. More prolonged NMP (at least 24h and even up to several days) have been proved feasible and would have allowed more detailed study of the influence of the bioreactor on liver injury (e.g. A2M a& lpha-1 glycoprotein increases). More prolonged perfusion would also help define whether the MSCs survive in the bioreactor for longer than 4h.
4. My comments relating to the small group size in the porcine experiments have been acknowledged by revision of the supplementary information. In relation to the short duration of the perfusion experiments, the authors are planning experiments with more relevant prolonged perfusion. It would have strengthened the work to wait for these results.

We will address these two points together, as they are subject to similar criticisms. First of all, we would like to thank the Reviewer for accepting the sample size justification for our pilot study, provided in the previous review round. Sample size justification (pilot large animal model) and calculation (rodent model) have been performed according to the PREPARE guidelines¹⁴. Making this point clear and repeatable (as requested by the ARRIVE guidelines¹⁸ observed by this journal) is for us of paramount importance.

As the Reviewer correctly observed, prolonging the perfusion time in both rodent and porcine models would have offered an intriguing pathway for further investigations. A longer perfusion time would provide a time window during which organs could be not only "reconditioned", but also "repaired".

However, as declared in the previous version of the paper and review rounds, this was not the objective of our study. Instead, the rodent model was employed to establish the bioreactor-based NMP platform, to investigate the potential of MSC utilization in liver perfusion, and their impact on early IRI, while the pig model was utilized for to test feasibility of bioreactor integration into a commercial device used in the clinical practice.

Beside the fact that the effects of a prolonged perfusion felt outside our aims, at the time of experiment execution, no prolonged perfusion rat models were available in the literature. A 12 h-perfusion model was reported for the first time by our group after completing the present study²³. It is important to note that, despite meeting all clinical criteria for viability at the end of the 12-hour perfusion, perfused livers exhibited a number of metabolic changes compared to the native liver. These changes could have influenced the interpretation of the MSC effect on prolonged liver perfusion. The same non-physiological effects were showed by Guo et al. in ischemia-free human livers, indicating that ex-situ perfusion could lead to deleterious effects²⁴ that potentially concurs to liver viability failure, even at the end of a prolonged perfusion time^{3,4}. As a result, while prolonging perfusion in a human/large animal model is an intriguing perspective, extended perfusion itself modifies liver biology and would have affected the understanding of MSC effects. For these reasons, MSC survival, effect, and outcome in prolonged perfusion necessary represents a second step of the present research.

This point is discussed in the Limitations section (page 16, lines 389-392 of the tracked version of the manuscript) and in the Conclusion section (page 17, lines 418-419 of the tracked version of the manuscript).

5. The best model would be to use human livers that were declined for transplantation. These can even be split into left and right lobes to allow a comparison of intervention vs control NMP.

We recognize the potential of non-viable human livers as a tool to explore clinical utility of novel technologies and compounds. However, the Reviewer defined non-viable humans as the “best model”, yet no supporting evidence was provided or could be identified in the literature (21 original articles or commentaries on PubMed [normothermic machine perfusion AND split OR reduced liver]: 12 of them for ex-situ clinical split liver). In addition to the low level of evidence, the use of non-viable split livers introduces two fundamental confounding factors that remain poorly understood: first, the high degree of heterogeneity among cases, and second, the presence of a resection line.

In regard to the first well-known issue⁵, “by definition” non-viable livers are not utilized for a number of reasons, including prolonged warm ischemia time and high steatosis grade. As reported in a recent series published in Nature Communication⁴, despite the exceptional results achieved, this resulted in heterogeneity in fulfilling viability criteria and in unrecognized metabolic changes due to the inherent liver physiopathological conditions. This could lead to difficulties in interpreting the data if a new technology is tested.

As for the presence of a resection line, it can introduce additional confounding factors when using split livers as an internal control. Indeed, the potential influence of the resection line on resolution and repair mechanisms has already been demonstrated in-vivo models and in humans (e.g. associated liver partition to portal vein ligation in staged hepatectomy – ALPPS^{25,26}).

In addition, the precise ex vivo pump flow required for the hemi liver remains to be established²⁷. All these variables would have further complicated the investigation of a MSC-bioreactor effects during NMP.

Finally, for completeness towards Reviewers and Editors, it is important to acknowledge that the use of non-viable livers for research purposes is no longer permitted in Italy. This observation was not reported in the previous revisions because it does not represent an insurmountable limitation. Indeed, international collaborations can be sought within the scientific community (although the funds of this grant cannot be used for non-Italian collaborations).

Despite reiterating that the ultimate objective of the present research is not the full clinical application of our MSC-bioreactor, we are pursuing these avenues. Indeed, we have recently published a paper on human liver NMP using the native liver of the recipient.

Since this topic is of high interest in the field of MP, we discussed this point as a future development of our research (page 17, lines 454-456 of the tracked version of the manuscript).

We kindly request that the comment on Italian legislation not be included in the paper, as it is not crucial for the scientific community.

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