Peer Review File

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Reviewer A

• Overall

Comment 1 Overall, this is a study looking at the effect of perfusate temperature during EVLP in rats. I think the title is a bit inaccurate as the authors do not perform the necessary inhibition experiments to truly elucidate a mechanistic basis for the improved function of lung allografts on EVLP at subnormothermic temperatures. The paper would also be strengthened in the authors could provide some insight into what these differences in graft function during EVLP might indicate for graft function in vivo post-transplant. This would also help to further differentiate this work from similar previous studies.

***Reply 1*:** **Thank you for your comment. We agree that it is difficult to present strong evidence for our hypothesis owing to the lack of inhibition studies. We have modified the title as per your comment. We also mentioned these limitations in the limitation section again and will reflect on these inhibition studies in the future.**

Change in the text: **The title was changed to 'Subnormothermic ex vivo lung perfusion possibly protects against ischemia–reperfusion injury via the mTORC–HIF-1α pathway'. The limitation of the lack of inhibition tests was mentioned at lines 357-362.**

• Title page + Abstract

Comment 2 The title is overstated since you just know that subnormothermic EVLP is associated with reduced gene expression of mTORC and HIF1a, you have not performed genetic or pharmacologic inhibition of these pathways to validate that their involvement in IRI following EVLP in your model.

***Reply 2*:** **Thank you for your comment. We agree that it is difficult to present strong evidence for our hypothesis owing to the lack of inhibition studies. We mentioned these limitations in the limitation section again and will reflect on these inhibition studies in the**

future.

Change in the text: **The limitation of the lack of inhibition tests was mentioned at lines 357-362.**

Comment 3 For the methods, would clarify that the lung function analyses were performed during EVLP and specify at what time post-EVLP the other assays were performed.

Reply 3: **Thank you for your comment. We have clarified that lung function was analyzed every hour during EVLP and we have accurately described when LIS, metabolic analysis, and qRT-PCR were performed.**

Change in the text: **We have changed the text as follows (lines 45-50).**
Lines 45-50: Lung function analyses, including oxygen capacity, compliance, and pulmonary vascular resistance, were performed hourly during EVLP. Further, after 4 h of EVLP, histological evaluation of the right lobe was performed using the lung injury severity scale. The expression levels of inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-18 were evaluated. Metabolomic analysis of left lung tissues was conducted using capillary electrophoresis time-of-flight mass spectrometry after 4 h of EVLP in the EVLP groups and after 1 h of cold preservation in the sham group.

Comment 4 You should also specify which cytokines you looked at in the abstract and either include that information where you discuss it on line 17 or line 22.

Reply 4: **Thank you for your comment. We described the observations of TNF-α, IL-6, IL-18, IL-1β, and caspase-3 and revised the abstract to reflect this.**

Change in the text: **We have changed the text as follows (lines 45-48, 52-53).**
Lines 45-48: The expression levels of inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-18 were evaluated.
Lines 52-53: ~ expression levels of inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-18 were significantly lower~

Comment 5 Your conclusion isn't supported by the data you present. Based on the current data, you can fairly conclude that lung graft function during EVLP is improved by subnormothermic perfusion and is associated with reduction in pro-inflammatory cytokine expression and glycolytic activity.

Reply 5: **We agree with your opinion and have revised the abstract conclusion section.**

Change in the text: **We have changed the text as follows (lines 63-65).**
Lines 58-60: EVLP with subnormothermic perfusion improves lung graft function by reducing the expression of pro-inflammatory cytokines and glycolytic activity during EVLP. Additionally, EVLP can be a useful target for the improvement of graft function after transplantation.

• Introduction
Comment 6 The sentence starting with "Owing to.." on line 48 is a bit cumbersome and confusing, you could cut out "Owing to the shortage of organ donors" and improve the clarity.

Reply 6: **Thank you for your comment. In response to your comment, the above sentences were deleted and modified, and the entire manuscript has been proofread for English language accuracy.**

• Methods
Comment 7 For the line starting on 100 beginning with "We selected a small…" what is this based on? Did you perform multiple experiment with similar results? How many individual experiments were performed if so?

Reply 7: **We had previously conducted studies using rat EVLP[1,2] and at that time, we were able to confirm the minimum number of animals required to produce statistically significant results, which was 5 animals. Owing to the ethical constraints related to animal testing, experiments were conducted with the minimum number of animals that could produce meaningful results and minimize suffering.**

Comment 8 For the metabolic profiling and qPCR, could you specify at what timepoint

following EVLP the samples were prepared? From Figure 1, it appears like these samples were collected immediately following 4 hours of EVLP. However, it would be helpful to explicitly state this somewhere if that is indeed the case.

Reply 8: **Thank you for your comments. As you mentioned, we collected the left lung 4 h after EVLP and performed qRT-PCR and metabolomic analysis. As per your comment, we have explicitly mentioned this.**

Change in the text: **We have changed the text as follows (lines 161-163).**
Lines 161-163: RNA was extracted from the left lung after 1 h of cold preservation in the sham group and after 4 h of EVLP in the EVLP groups .

• Results
Comment 9 The difference in oxygen capacity and PVR between normoEVLP and subnormoEVLP while on EVLP is interesting. However, previous studies using rat models have shown similar results. It would be interesting to look at whether these differences in functional characteristics during EVLP result in functional differences in the graft post-transplant. The best, albeit challenging, way, to look at this would be to take these grafts, transplant them into rats and see how the rats and grafts survive. However, even without doing this you may be get some insight into this question by taking some of the subnormo lungs and perfusing them at 37 C to see if those functional differences in subnormo are still seen once the lungs are at the temperature they would be in vivo post-transplant.

Reply 9: **We agree with your comments. In fact, we conducted this experiment when designing the methodology. After 4 h of EVLP, lung function was assessed by performing a longer perfusion at 37°C for both subnormoEVLP and normoEVLP groups. In this experiment, more than half of the lungs in both groups was affected by tissue edema and damage, making it impossible to continue the experiment. This was probably due to the prolonged exposure of very small rat lungs to EVLP.**

Comment 10 The title for 3.3 is a bit confusing since you're only looking at gene expression. The only non-cytokine you mention in this section is caspase-3. You could either move the data for caspase-3 elsewhere or perhaps omit it. Total Csp3 mRNA is not really a great marker of

apoptosis (whereas the cleaved caspase-3 protein is), so including it doesn't add much. However, if you want to use your caspase-3 gene expression data to suggest there may be reduced apoptotic cell death in lungs with subnormoEVLP compared to normoEVLP you should substantiate this by staining lung sections for cleaved caspase-3 protein.

Reply 10: **Thank you for your valuable comment. As you mentioned, measuring the level of cleaved caspase 3 can accurately describe the actual amount of apoptosis. We agree that the study could have been improved if western blot had been performed for this. The title of section 3.3 was modified and the data for caspase 3 was omitted.**

Change in the text: **We deleted caspase 3 data and its contents from the main text.**

Comment 11 The data for figures 5 and 6 are swapped.

Reply 11: **Thank you for your comments. Even though we carefully reviewed and edited the manuscript, we made a mistake during the upload process. This was revised and carefully reviewed once again.**

Comment 12 Use of "inclined" on 220 is a bit odd, perhaps "trended" would be more clear.

Reply 12: **Thank you for your comments. We changed line 248-252 according to your advice, and once again submitted the article for English proofreading. The English proofreading certificate is attached.**

Change in the text: **We have changed the text as follows (lines 248-252).**
Lines 248-252: The levels of metabolites from early glycolysis steps, such as glucose-6-phosphate (normoEVLP vs. subnormoEVLP, 0.001765 ± 0.000654 vs. 0.004330 ± 0.000839; P = 0.1338), fructose-6-phosphate (0.0003834 ± 0.000128 vs. 0.0009974 ± 0.000226; P = 0.1073), and fructose-1,6-bisphosphate (0.001849 ± 0.00061 vs. 0.002430 ± 0.00030; P = 0.6732), indicated a tendency toward lower values in the normoEVLP group than in the subnormoEVLP group

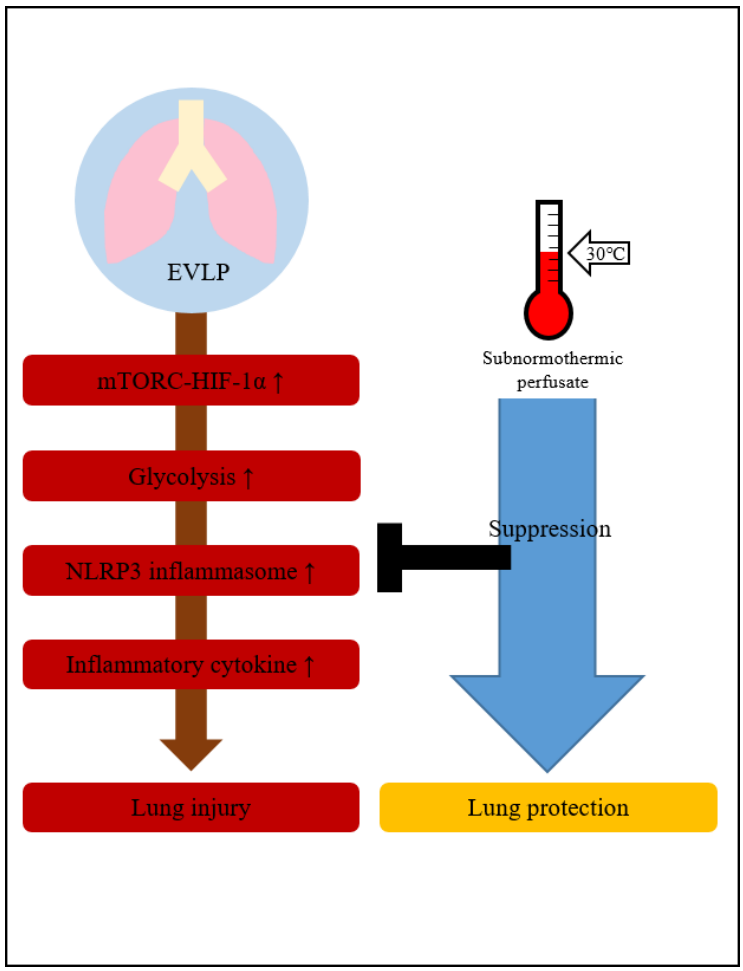
• Discussion

Comment 13 In reference to line 252, is there any data for how functional perimeters during EVLP correlate with graft function post-transplant? Further, in terms of using EVLP as a way to screen marginal grafts for the potential for transplant, is there any evidence to suggest that perfusing them at subnormothermic temperatures may mask grafts will function poorly at physiologic temperatures in vivo?

Reply 13: **Since we did not perform transplantation, we could not determine whether graft function after transplantation matches the function during EVLP. However, in previous studies, improvements during EVLP were observed at 28°C, including a decrease in pro-inflammatory cytokines and myeloperoxidase activity, improved compliance, and reduced PVR[3]. Another study conducting EVLP at 25°C demonstrated a decrease in inflammatory mediators during EVLP and confirmed a reduction in histologic graft injury after transplantation[4]. Based on this, we predict that our study, if extended to evaluate transplantation in the future, will yield similar results.**

Comment 14 I think the discussion and description of limitations is fair. It sounds like in terms of mechanism you are suggesting that ischemia during EVLP results glycolysis in an HIF-1a- and mTORC1-dependent manner and that this shift to glycolysis results in NLRP3 inflammasome activation in some cell populations within the graft and that use of subnormothermic perfusate reduces this effect. Although without genetic or pharmacologic inhibition of components of these pathways you cannot specifically say this is the case, it would still be helpful to include a figure with a graphical summary of the hypothesized pathway you are proposing to aid in clarity.

Reply 14: **Thank you for your comment. Below, we have added a graphical summary of the hypothesized pathway.**



Reviewer B

Thank you for providing me with the opportunity to review the manuscript detailing the protective effects of hypothermic ex vivo lung perfusion against ischemia-reperfusion injury. Please find below my comments and suggestions for revising this manuscript.

Comment 15 One of the biggest concerns in this manuscript is the number of data points in the figures. The authors indicated that there were eight cases in each group, but Figures 3 to 7 show only five data points in each group. Could the authors clarify this discrepancy?

Reply 15: **We apologize for the confusion. This study was conducted with a total of 15 rats (5 per group), and the data were analyzed using these. The relevant content in the section has been corrected.**

Change in the text: **We have changed the text in lines 44-45, 127-129.**

Comment 16 This comment is somewhat related to comment #1. Figure 2 displayed mean and standard error, but what is the case number for each group? Is it n=5 or n=8? Please provide clarification.

Reply 16: **We apologize for the confusion. This study was conducted with a total of 15 rats (5 per group), and the data were analyzed using these. The relevant content in the section has been corrected. We have mentioned case numbers in all the figures and not only in Figure 2; further, we have included standard errors for all continuous variables in the results.**

Change in the text: **We have changed the text at lines 44-45, lines 127-129, and Figure 2).**

Comment 17 Lines 228 to 230: I recommend removing this sentence since the statement is speculative and is more appropriate for the discussion section. Instead, consider adding several sentences summarizing the results. The first sentence of the paragraph and the Figure 6 legend are too simplistic and lack information.

Reply 17: **We agree with your comments. We have reflected your comments and modified section 3.6 to describe the results of the experiment in more detail and have also reflected this in the Figure 6 legend. Additionally, the content of lines 228-230 has been moved to the discussion (line 340).**

Change in the text: **We have changed the text as follows (lines 258-268, 340-343)**

Lines 258-268: The TCA cycle metabolites pyruvate, cis-aconitic acid, and α-KG were not significantly different between the normoEVLP and subnormoEVLP groups (Figure 6). The levels of isocitric acid (sham 0.000767 ± 0.0005, normoEVLP 0.000085 ± 0.000085, subnormoEVLP 0.000299 ± 0.00013; sham vs. normoEVLP, P = 0.0008; sham vs. subnormoEVLP, P = 0.0123; normoEVLP vs. subnormoEVLP, P = 0.293), furamic acid

(sham 0.0019 ± 0.00013, normoEVLP 0.00096 ± 0.00021, subnormoEVLP 0.0012 ± 0.000080; sham vs. normoEVLP, P = 0.002; sham vs. subnormoEVLP, P = 0.0129; normoEVLP vs. subnormoEVLP, P = 0.5604), and malic acid (sham 0.0182 ± 0.0011, normoEVLP 0.0099 ± 0.0012, subnormoEVLP 0.1125 ± 0.00035; sham vs. normoEVLP, P = 0.0002; sham vs. subnormoEVLP, P = 0.0008; normoEVLP vs. subnormoEVLP, P = 0.5968) were significantly decreased in both EVLP groups compared to the sham group. There were no significant differences between the normoEVLP and subnormoEVLP groups, and they showed similar trends in both groups . Line 340-343: However, the TCA cycle for aerobic glycolysis showed similar trends in both groups in this study. This result is considered to show similar energy conversions in both groups due to the inflow of acetyl-CoA and succinyl-CoA through branched-chain amino acids and β-oxidation in addition to pyruvate.

Comment 18 Although the authors acknowledged the limitations of this study, a major concern is the study design. The comparison between groups was conducted under significantly different conditions. Ideally, in vivo perfusion is needed for comparison. At a minimum, the subnormoEVLP group should have a normothermic phase for comparison.

Reply 18: **We agree with your comments. In fact, we conducted this experiment when designing the methodology. After 4 h of EVLP, lung function was assessed by performing a longer perfusion at 37°C for both subnormoEVLP and normoEVLP groups. In this experiment, more than half of the lungs in both groups was affected by tissue edema and damage, making it impossible to continue the experiment. This was probably due to the prolonged exposure of very small rat lungs to EVLP.**

Comment 19 Why does the normoEVLP group exhibit a low P/F ratio (falling below 300 at 4 hours) and an increase in PVR with 60 minutes of CIT? This seems unusual. In my experience, normoEVLP with limited cold ischemic time results in an unchanged PVR during EVLP.

Additionally, the authors stated that the oxygen capacity in normoEVLP was 271.25, but Figure 2 does not represent that. Please verify your dataset and make the necessary corrections.

Reply 19: **Thank you for your comments. There was a mistake in our data. As such, the entire document was verified and corrected once again. Regarding the increase in PVR and decrease in OC during normothermic EVLP 60 min after CIT, which you mentioned, we considered this to be a limitation of EVLP. Owing to perfusion using a circuit, there was inflammation or damage, resulting in a decrease in OC and an increase in PVR; however, the quality of the donor lung remained that of a standard donor.**

Change in the text: **We have changed the text as follows (lines 206-208).**
Lines 206-208: Functional parameters during EVLP significantly differed between the normoEVLP and subnormoEVLP groups regarding OC (1-h normoEVLP vs. subnormoEVLP, 302.2 ± 17.51 vs. 419.4 ± 18.76; 4-h normoEVLP vs. subnormoEVLP, 306.6 ± 16.09 vs. 368.6 ± 30.51, P < 0.0001)

Minor Comments:
Comment 20 Line 266: "normothermia" should be replaced with "subnormothermia" or "hypothermia."
Reply 20: **Thank you for your comments. We have changed "normothermia" to "subnormothermia".**

Change in the text: **We have changed "normothermia" to "subnormothermia" in line 311.**

Comment 21 Some numerical values in the results section do not have units following them.

Reply 21: **Thank you for your comments. We have presented units of numerical data, excluding relative values, in the results section.**

Change in the text: **We have changed lines 209-213.**

Comment 22 Many continuous values lack standard error in the results section. Please correct this.

Reply 22: **Thank you for your comments. We did not present standard errors for relative values, but we have modified the results section to include standard errors as per your comments.**

Comment 23 The y-axis label in Figure 2 should be reviewed. Is it "LA PaO2/FiO2 – PA PaO2/FiO2" as the authors stated in the Methods and Materials section? Confirm and correct if needed.

Reply 23: **As you mentioned, $P_{LAO2} – P_{PAO2}/FiO_2$ is correct. We have changed the y-axis in Figure 2.**

Reviewer C

Interesting work, however I do have some concerns listed below that needs attention:
Major comments:

Comment 24 The decision to use 30°C instead of 25°C warrants justification, especially given the reference (https://doi.org/10.1016/j.jtcvs.2021.01.066) cited by the authors. The referenced publication highlights the advantages of 25°C EVLP, I suggest to the authors to justify the choice of 30°C in their study.

Reply 24: **The studies we cited also presented different views on perfusate temperature during EVLP. Burki et al.[5] reported that 25°C was more advantageous than 30°C. Arni et al. reported that 28°C was better than 21°C, 24°C, 28°C, 32°C, and 37°C; although 28°C showed the best results, lower temperatures such as 21°C were unfavorable in terms of lung weight increase[6]. Gloria et al.[4] showed that histological grading at 2 h after transplantation was significantly less impaired in the 25°C group, while other indices during and after EVLP showed that the lungs in both 30°C and 25°C groups were significantly less damagedand had better function than the 37°C group. Therefore, subnormothermia during EVLP is advantageous and the ideal temperature among these subnormothermic conditions has not been accurately determined. Additionally, this study targeted the possibility of long-term readjustment or reassessment with other additional**

interventions (e.g. drugs or inhibitors) during EVLP. Therefore, we chose a temperature of 30°C in order to select a temperature below body temperature. If possible, this temperature should not be too low such that sufficient metabolism for viability assessment, organ repair, and organ reconditioning can be maintained.

Comment 25 Hexokinase (HK) activities are known to be affected by glucose 6-phosphate (G6P) levels, where increased G6P can inhibit HK. However, the authors' findings reveal a lower expression of HK in subnormal conditions compared to normal conditions. Since, authors did not look the activation, this observed change in expression warrants further discussion and justification. Similarly, examining the expressions of PFK alongside the product between the normal and subnormal groups, presents an intriguing contrast. While PK expression and pyruvate levels remain consistent, the noticeable differences in PFK and HK expression, along with their product amounts, merit in-depth discussion.

Reply 25: **Thank you for your valuable comments. We also spent a lot of time considering the glycolysis pathway metabolites in Figure 5. There is no significant difference in the early steps of glycolysis, such as G6P, F6P, and F1,6P, but subnormoEVLP tends to have a higher relative value of metabolites than normoEVLP. This is not the case for the metabolites of the remaining late steps except pyruvate. In metabolite analysis, these intermediate metabolites can have two meanings. First, this metabolic pathway may have been more active; therefore, the metabolites were depleted and presented at lower levels. Second, because this metabolic pathway has high activity, intermediate metabolites may have accumulated and increased. Both interpretations are possible, and to make this clearer, we checked the enzyme level to help clarify whether the accumulation or depletion of metabolites means an increase or decrease in the pathway, respectively. In conclusion, we confirmed that HK and PFK enzyme levels were significantly increased in normoEVLP and decreased to sham level in subnormoEVLP. Based on this, it was determined that glycolysis activity increases in normoEVLP and decreases again in subnormoEVLP, and intermediate metabolites tend to accumulate due to a decrease in pathway activity in subnormoEVLP. In the case of PK, about 90% of lung PK is PKM2. Because the dimer form of PKM2 mediates anaerobic glycolysis and the tetramer form mediates conversion to the TCA cycle, it is difficult to indicate the degree of glycolysis simply by PK expression. As a result, we believe that overall glycolysis activity decreases**

in subnormoEVLP as the levels of HK and PFK decrease.

Change in the text: **We have changed the text as follows (lines 331-335).**

Lines 331-335: In our study, changes in the glycolysis pathway were particularly significant. HK and PFK, important enzymes involved in glycolysis, were significantly downregulated in the subnormoEVLP group compared with those in the normoEVLP group, whereas the levels of glycolysis intermediate metabolites tended to be increased in the subnormoEVLP group. This means that intermediate metabolites accumulate due to decreased activity in the glycolysis pathway in subnormoEVLP.

Comment 26 In the introductory section (Lines 66-69), the authors introduced the discussion on HMP. To enhance clarity, I recommend relocating this discussion to the "Discussion" section. Furthermore, as this is the sole mention of HMP, elaborating on why subnormothermic EVLP was chosen over HMP would provide valuable insight.

Reply 26: **Thank you for your comments. We have changed our manuscript reflecting your comments.**

Change in the text: **We have changed the text as follows (lines 285-296).**

Lines 285-296: Previous studies on the ideal temperature for organ preservation using machine perfusion have been conducted, particularly subnormothermic or hypothermic machine perfusion (HMP) in kidney and liver transplants. Based on better nutrient supply and endothelial protection (27), HMP is the gold standard for circulatory death kidney transplantation (28). In liver transplantation, HMP stimulates aerobic metabolism while limiting organ metabolism and oxygen demand (27). HMP settings are already in clinical use for kidney (27, 29) and liver (30) transplants.

Meanwhile, several recent studies have been conducted on the perfusate temperature in lung transplantation, demonstrating that subnormothermic EVLP can effectively lower the metabolic rate, decrease oxygen demand, and facilitate the removal of metabolic waste products while restoring adenosine triphosphate (ATP) levels (27,29,30). Extreme subnormothermic perfusate temperatures, such as 21°C, have disadvantages in terms of PVR and edema. Therefore, moderate subnormothermia is more advantageous than low subnormothermia (8).

Comment 27 Line 75 introduces "Metabolomic analysis, measuring many metabolites." This description lacks specificity and technical depth. I suggest the authors expand on the scope of the metabolites being measured to offer a more detailed and technical explanation through adding such as instead of ‘’many’’.

***Reply 27*:** **Thank you for your comments. We have slightly modified the order and content of these sentences in this paragraph for clarity.**

***Change in the text*:** **We have changed the text as follows (lines 102-104).**
Lines 102-104: Metabolomic analysis may provide important information about the extent of tissue damage or repair (17). This analysis quantitatively assesses dynamic responses to physiological and pathological changes (18).

Comment 28 Regarding the histology results the cited article (https://doi.org/10.1016/j.jtcvs.2021.01.066) results appear significantly more greater. I recommend discussing this disparity.

***Reply 28*:** **Thank you for your comments. As you mentioned, the LIS of normoEVLP in our study was about twice as high, and the actual histology also confirmed that the lungs in our study were more damaged. It is difficult to determine the exact cause, but the two main differences between the studies are as follows. First, our study started EVLP 1 h after cold static preservation, but the reference study started EVLP immediately after harvesting. Second, our study had histologic evaluation at 4 h after EVLP, while the reference had an additional 2 h of in vivo perfusion.**

Comment 29 To improve the structural coherence of the methodology section, I suggest reordering the explanation. Specifically, placing the RNA extraction and qRT-PCR processes (Section 2.6) before detailing the Metabolic profiling by capillary electrophoresis time-of-flight mass spectrometry (Section 2.5) would enhance the logical flow of the methodology. (Beacuse first ‘’RNA isolation’’ is displayed under Metabolic profiling by capillary electrophoresis time-of-flight mass spectrometry-Line 129)

Reply 29: **We agree with your comment. As you mentioned, since metabolic profiling was performed after RNA extraction, it is reasonable to modify this by considering the actual sequence of events. We modified the manuscript to place the RNA extraction section first, and then describe the qRT-PCR and metabolic profiling.**

Change in the text: **We have changed lines 158-192.**

Comment 30 It's crucial to clarify the basis for relative values in Figure 6, particularly concerning HK, PK, and PFK calculations. Is GAPDH utilized as the reference?

Reply 30: **Thank you for your comment. The transcript levels of HK, PFK, and PK were normalized to those of GAPDH and analyzed using the standard ΔΔCT method. We marked this on Figure 5 to make it clear.**

Comment 31 The labeling of figure legends for Figure 5 and Figure 6 appears mixed.

Reply 31: **Even though we carefully reviewed and edited the manuscript, we made a mistake during the upload process. We apologize for the confusion; this has been revised and carefully reviewed once again.**

Comment 32 The authors focused on examining caspase 3 levels, known as an executioner caspase. It may be more advantageous to assess active caspase levels instead of expressions, providing a more justified understanding of apoptosis in both subnormal and normal conditions.

Reply 32: **Thank you for your valuable comment. As you mentioned, measuring the level of cleaved caspase 3 can accurately describe the actual amount of apoptosis. We agree that the study could have been improved if western blot had been performed for this. The title of section 3.3 was modified and the data for caspase 3 was omitted.**

Comment 33 In line 291, the article introduces the phrase ‘’Aerobic glycolytic activity…’’. However, the cited work (doi: 10.3390/cells10040748) does not explicitly discuss decreased glycolytic activity. In the cited work authors imply: ‘’Since there are no compensatory mechanisms in this EVLP setting, the decreased lactate levels or reduced glucose consumption

levels may indicate respectively, aerobic glycolysis in the 28 °C group and increased anaerobic glycolysis in the 37 °C group'' So in this paper authors would justify the ''decreased aerobic activity'' since the lactate levels (known for anaerobic activity is decreased).

Reply 33: **Thank you for your comments. We read and checked the reference article again and revised the content based on your comments.**

Change in the text **We have changed the text as follows (lines 338-340).**
Lines 338-340: Decreased lactate levels may indicate that aerobic glycolysis decreased in subnormothermic (28℃) EVLP and anaerobic glycolysis increased in normothermic (37℃) EVLP (7).

Reviewer D

Thank you for the opportunity to review this manuscript. The study's focus on the potential benefits of reducing the temperature to 30°C during ex vivo lung perfusion (EVLP) for lung transplants is intriguing. The approach of subnormothermic perfusion, extensively researched in kidney and liver transplants, is novel in the context of lung grafts. This approach of subnormothermic perfusion, while extensively studied in kidney and liver transplants, has not been thoroughly investigated in the context of lung grafts. The study's experimental design is robust and methodical. By incorporating comprehensive evaluations, such as lung function tests, histological assessments, and metabolomic analyses, the study provides a thorough understanding of the effects under investigation. The findings reveal that subnormothermic EVLP, especially through its influence on the mTORC-HIF-1α pathway, offers protective advantages, contributing to the improvement of lung graft outcomes.

However, I have several concerns and suggestions that I believe could enhance the manuscript:

Comment 34 The introduction, while providing a basic overview, could benefit from added depth and breadth in background information. It would be advantageous to more clearly articulate the study's objectives and aims and emphasize the importance and novelty of this research.

***Reply 34*:** **Thank you for your comments. Because our study contained results from various fields, we had difficulty clearly explaining the background or methodology of the study. With the help of your comments and those of other reviewers, we have revised the introduction and discussion sections to make it more readable and justified.**

results:

Comment 35 Figure 2: Why is the oxygen capacity of subnormoEVLP already very high in the first hour and decreased in the following hours, while in the normoEVLP group it is stable?

***Reply 35*:** **In subnormoEVLP, OC shows a tendency to decrease over time, but there was no significant difference in OC according to EVLP time (repeated measures general linear model, p=0.138). Although it was not statistically significant, it is necessary to consider the reason why OC tended to decrease in subnormoEVLP compared to OC remaining stable without change in normoEVLP.**

EVLP duration	*OC in subnormo EVLP*
1 h	*419.4* ± 18.76
2 h	*406.6* ± 8.21
3 h	*402.2* ± 20.93
4 h	368.6 ± 30.51

Comment 36 Figure 3: The author used acellular perfusate. why there is still a lot of inflammatory cell infiltration in the normoEVLP group, and why the alveolar seems to have a dilation in the subnormoEVLP?

Reply 36: **Although we used acellular perfusate, inflammatory cells such as resident lung cells – lymphocytes, macrophages, eosinophils, and neutrophils[7] – exist in the lung parenchyma. For this reason, there may be inflammatory-cell infiltration observed in the histological results even when acellular perfusate is used. In addition, in normoEVLP, the alveolar space is narrowed due to alveolar wall thickening. In comparison, subnormoEVLP may show dilation of the alveolar space; however, this was not seen in this study.**

Comment 37 Figures 5–6: The presentation and interpretation of these results are unclear.
In most metabolites, no comparisons were made between the normoEVLP group and subnormoEVLP; does that mean its not significant or that the author didn't do it?

Reply 37: **Thank you for your comments. Even though we carefully reviewed and edited the manuscript, we made a mistake during the upload process. This was revised and carefully reviewed once again. We compared whether there were significant differences in metabolites between all the groups. In addition, significance was indicated with a p-value only when it was statistically significant. There were no significant differences in the metabolites in Figures 5 and 6 between the normoEVLP and subnormoEVLP groups. A description of these results has been added to sections 3.5–3.6 and the figure legend.**

Change in the text: **We have changed sections 3.5 and 3.6 and the legends of Figures 5-6.**

Comment 38 Discussion: Lines 268–292 could be enhanced by reestablishing the connection between previous studies and the current findings, particularly regarding the significance of the results in HK and PFK.

Reply 38: **Thank you for your comments. As per your comment, theses paragraphs were mixtures of various contents, making them difficult to read and understand. We have revised these paragraphs.**

Change in the text: **We have changed lines 313-340.**

Comment 39 It would be valuable to expand upon why this research is significant and how it contributes to the field. Highlighting gaps in the current literature that this study addresses or detailing the potential implications of the findings would provide greater context and relevance.

Reply 39: **I agree with your opinion. Although our study showed somewhat broad and general insight into the perfusate temperature of EVLP, the differences observed in glycolysis activity and the expression levels of mTORC, HIF-1a, and NLRP3 during EVLP suggest that these molecules act as potential target candidates to induce improvement in lung function. Therefore, I believe that through further experiments**

utilizing the inhibition or activation of these molecules, we can verify the enhancement of lung function using EVLP.

Comment 40 References: No. 8 and No. 29, No. 7 and No. 30, are actually the same project; No. 7-8 are just the abstracts of conferences. No. 23 is also an abstract of conferences; the real paper can be found at https://pubmed.ncbi.nlm.nih.gov/36326834/

Reply 40: **Thank you for your comment. We apologize for not properly reviewing the same two studies in the reference. Additionally, as the experiments and manuscript writing progressed, the abstract reference had already been published in separate articles. We have modified the references accordingly.**

While the manuscript presents a compelling and well-structured study, addressing these points could significantly strengthen its impact and clarity. I look forward to seeing the advancements in the revised manuscript.

Reviewer E

In the present manuscript, the authors identified subnormothermic ex vivo lung perfusion to protect against ischemia-reperfusion injury via the mTORC–HIF-1α pathway using a rat model. Their conclusions are based on the assessment of pulmonary function parameters, as well as histopathological evaluation, metabolic profiling using CE-TOFMS and qRT-PCR of lung tissue samples. Hence, the authors applied different methods. The manuscript is well written; the data is presented in a comprehensive way. Please find my comments and suggestions below.

Comment 41 Highlights:

- the statements chosen as highlight are not optimal as they – in my opinion – do not properly reflect the key aspects, i.e. highlight °2 has no content (what was the idea behind? to state that inflammatory cytokines were reduced in the subnormothermic EVLP group? or to list experimental methods?; description should focus on the subnormothermic EVLP group (highlight °3 and "this energy metabolism switch" (highlight °4))

Reply 41: **Thank you for your comments. We have modified the highlights based on the results of our study and focused on subnormoEVLP.**

Change in the text **We have changed the text as follows (Highlights).**

~~**• Studies examining the effects of perfusate temperature on graft function are limited.**~~

~~**• Inflammatory cytokine expression levels were evaluated in male Sprague Dawley rats.**~~

• Subnormothermic ex vivo lung perfusion (EVLP) exhibited favorable outcomes in terms of oxygen capacity, pulmonary vascular resistance, and diminished lung injury in comparison to normothermic EVLP.

• Subnormothermic EVLP was associated with a significantly lower expression of inflammatory cytokines and mTORC, HIF-1α, and NLRP3 compared to normothermic EVLP.

• Compared to the subnormothermic EVLP group, the normothermic EVLP group predominantly used glycolysis for energy metabolism.

• Subnormothermic perfusion improves lung graft function during EVLP through this energy metabolism switch.

Comment 42 Introduction:

- the rationale for subnormothermic EVLP at precisely 30°C should be given in more detail (i.e. why not 28°C?)

Reply 42: **The studies we cited also presented different views on perfusate temperature during EVLP. Burki et al.[5] reported that 25°C was more advantageous than 30°C. Arni et al. reported that 28°C was better than 21°C, 24°C, 28°C, 32°C, and 37°C; although 28°C showed the best results, lower temperatures such as 21°C were unfavorable in terms of lung weight increase[6]. Gloria et al.[4] showed that histological grading at 2 h after transplantation was significantly less impaired in the 25°C group, while other indices during and after EVLP showed that the lungs in both 30°C and 25°C groups were significantly less damaged and had better function than the 37°C group. Therefore, subnormothermia during EVLP is advantageous and the ideal temperature among these subnormothermic conditions has not been accurately determined. Additionally, this study targeted the possibility of long-term readjustment or reassessment with other additional interventions (e.g. drugs or inhibitors) during EVLP. Therefore, we chose a temperature of 30°C in order to select a temperature below body temperature. If possible, this temperature should not be too low such that sufficient metabolism for viability assessment, organ repair, and organ reconditioning can be maintained.**

Methods:

Comment 43 - how were dynamic lung compliance and peak airway pressure measured (including calculation, units)? Where are the results of the peak airway pressure measurements shown?

Reply 43: **We measured airway pressure (cmH_2O) and tidal volume (L/s) during EVLP using Labchart (ADInstruments, Sydney, Australia) software. Using these measurement values, compliance was calculated as follows:**

$$\textbf{compliance} = \frac{\textbf{Tidal volume}}{\textbf{airway pressure (measured peak airway pressure} - \textbf{PEEP)}}$$

Change in the text **We have changed the text as follows (lines 143-147).**

Lines 143-147: **Functional parameters were measured, including oxygen capacity (OC, calculated as [left atrial (LA) perfusate PO2 - pulmonary arterial (PA) perfusate PO2]/FiO2 through arterial blood gas analysis), pulmonary vascular resistance (PVR, calculated as [PA pressure - LA pressure] × 80 / PA flow), and dynamic lung compliance (Tidal volume (TV) / [Peak airway pressure – Peak end expiratory pressure (PEEP)]).**

Comment 44 - an explanation should be given for the gradual perfusate temperature increase of 10 vs. 20 min for the subnormo- vs. normoEVLP groups (i.e. reference)

Reply 44: **When initiating EVLP, the perfusate temperature was started at 20°C and gradually increased to 37°C over 30 min. Ventilation was controlled by adjusting pressure to allow lung expansion and achieve a consistent tidal volume, requiring approximately 3–4 min. The EVLP flow was increased slowly, and the desired target flow was initiated 1 h after the start of EVLP; strictly speaking, both groups underwent complete EVLP from 1 h onward. According to Figure 1, owing to differences in target temperatures, the subnormoEVLP group appeared to start EVLP first, causing confusion. However, adjustments were made during the initiation of EVLP, and both groups actually started EVLP with the desired flow and settings from the first hour.**

Comment 45 - why were only 5 out of 8 lungs per group analyzed for the metabolic profiling? how were they selected?

Reply 45: **We apologize for the confusion. This study was conducted with a total of 15 rats (5 per group), and the data were analyzed using these. The relevant content**

in the section has been corrected. We have mentioned case numbers in all figures.

Change in the text: **We have changed the text in lines 44-45, 127-129.**

Comment 46 - how much reverse transcribed RNA was applied per qRT-PCR analysis? please add this information to the methods section.

Reply 46: **Thank you for your comments. qRT-PCR was performed using cDNA synthesized from 1 µg of isolated RNA.**

Change in the text: **We have changed the following text (lines 165-166).**
lines 165-166: For qRT-PCR, 1 µg RNA was reverse transcribed using AMV Reverse Transcriptase (#M0277L, New England Biolabs, Ipswich, MA, USA).

Results:
Comment 47 - overall, extremely short description of results; introductory sentences as well as a final sentence summarizing the key finding in each section may be helpful for the reader

***Reply 47*:** **Thank you for your comment. As per your suggestion, I have provided a detailed explanation in the results section and adjusted the manuscript's focus to align with subnormoEVLP.**

Comment 48 - the focus of the manuscript is the subnormothermic EVLP group, yet the wording in many instances emphasizes the description of the normothermic EVLP group (i.e. in results section) – in my understanding, this should be the other way round

Reply 48: **Thank you for your comments. Following your suggestion, we have described the results more clearly in the results and discussion sections.**

Additionally, we have focused our discussion on subnormothermic EVLP.

Comment 49 Figure 2: lung function parameters

- include 0 h baseline measurement of OC, PVR and compliance for normoEVLP and subnormoEVLP groups
- statistics in square brackets = over 4 hrs of EVLP?!
- figure legend: include number of animals analyzed per group (n=8), and statistical test applied

Reply 49: **The 0-h timepoint represents the immediate instance when the donor lung is connected to EVLP, before the initiation of ventilation or flow. At this stage, the lung has not yet undergone ventilation, making it a state where compliance, PVR, or OC cannot be discussed. This absence of measurements at 0 h aligns with similar practices in other existing studies, given the inherent limitations of assessing lung parameters in the absence of ventilation at the outset[7]. We tested statistical significance using two-way ANOVA. The value displayed in the large square bracket is the column factor p-value, which indicates that there is a difference between the two groups regardless of the time (row) factor. As you suggested, statistical methods and the numbers for each group are described in the legends of all figures.**

Comment 50 Figure 3: LIS scores and histological findings

- why was sham group not analyzed here?
- why different color code for experimental groups from here on as compared to Figs. 1 + 2? I suggest to stay with the white/red/blue color code.
- 3B: scale in µm is missing
- figure legend: include number of animals analyzed per group (n=8?), and statistical test applied

Reply 50: **I have unified the color codes within each group by modifying the color codes in the figures. The lens ratio in Figure 3B was not indicated separately as it lacks a specific unit. Statistical methods and the number of subjects per group have been included in the figure.**

Comment 51 Figure 4: Pro-inflammatory cytokines and Caspase-3 mRNA levels

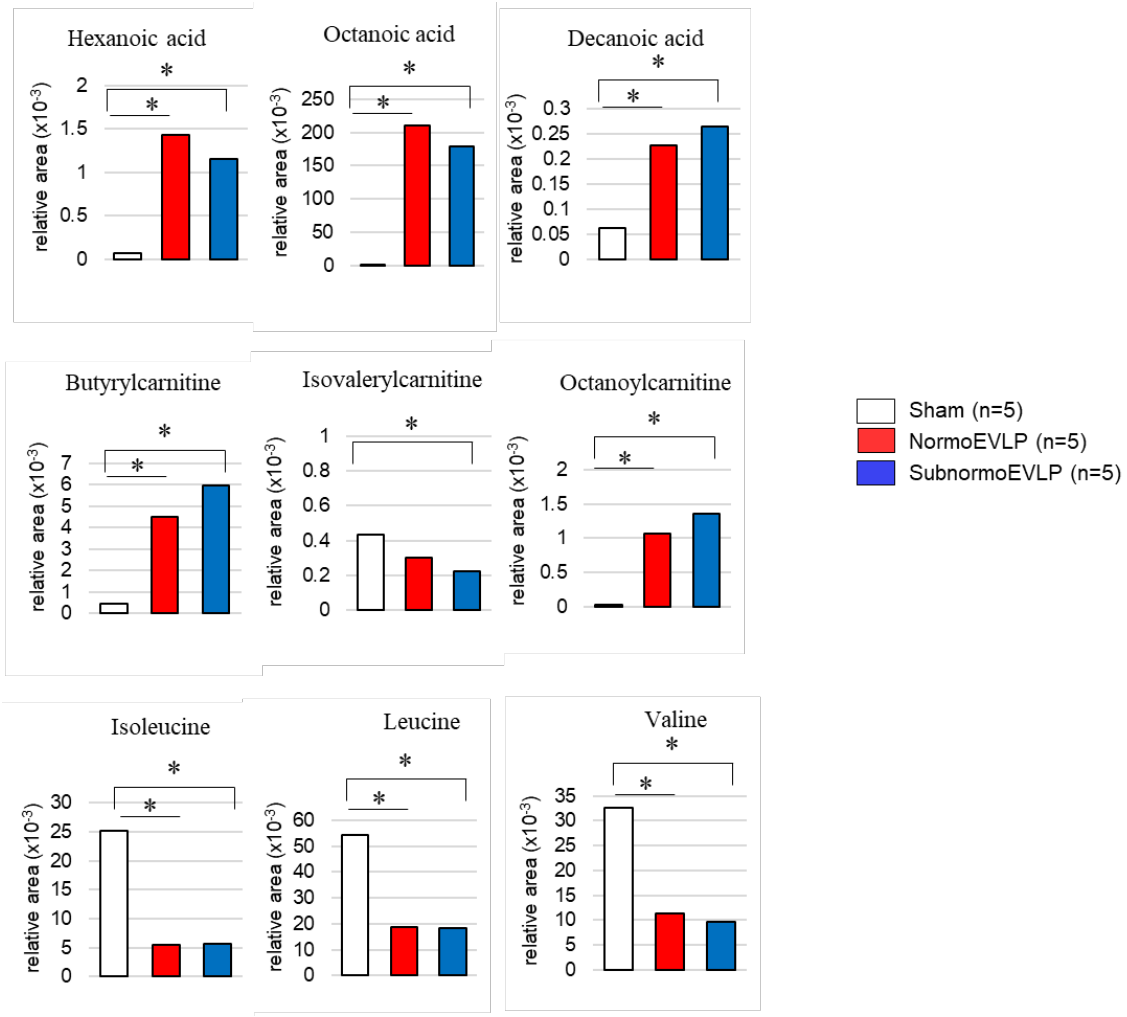
- why different color code for experimental groups from here on as compared to Figs. 1 + 2?
- figure legend: include number of animals analyzed per group (n=5), and statistical test applied
- analyzing mRNA levels from lung tissue provides information at the endpoint of the experiment; analyzing protein levels from perfusates (i.e. by Luminex-based multiplex assays) would allow a kinetic starting at 0, 1, 2, 3, and 4 hours
- how about additional inflammatory molecules, i.e. IFN-g, anti-inflammatory IL-10 and endothelial factors?

Reply 51: **Thank you for your valuable suggestion. I have unified the color codes within each group by modifying the color codes in the figures. Statistical methods and the number of subjects per group have been included in the figure. I agree with your perspective, and as you mentioned, it is not possible to analyze mRNA from lung tissue at different timepoints beyond 4 h post-EVLP. Performing Luminex assays on perfusates collected over time would indeed have been a useful experiment, allowing for the examination of diverse protein expression with minimal sample amount. I appreciate your insightful feedback, and in future EVLP experiments, I will consider incorporating such studies to capture temporal changes. Moreover, revisiting other inflammatory markers, including IFN-γ, IL-10, and endothelial factors, is a crucial suggestion. I will incorporate these diverse factors into future experiments, as they play significant roles in the overall context. Thank you once again for your valuable insights.**

Comment 52 Suppl. Fig. 1:

- which dataset was used for the PCA analysis? I assume glucose metabolism, TCA cycle, and mTORC, HIF-1a, NLRP3 expression levels?!
- were additional metabolic pathways analyzed/tested (i.e. OXPHOS, FAS etc.)? If so, which were they, and what was the result?

Reply 52: **A total of 292 metabolites were identified in metabolome profiling, and principal component analysis (PCA) loading was performed on the entire set of these metabolites. These metabolites were predominantly associated with carbohydrate metabolism, the TCA cycle, glutamine metabolism, urea cycle, choline metabolism, methionine salvage pathway, aromatic amino-acid metabolism, purine metabolism, and pyrimidine metabolism. Despite investigating other energy-related pathways such as lipid metabolism and amino-acid metabolism, no significant differences between subnormoEVLP and normoEVLP were observed, except for glucose metabolism. To avoid potential confusion caused by excessive data, results for these pathways were not presented separately. Attached are the outcomes for branched-chain amino acids and lipid metabolites for your reference.**



Comment 53 Figure 5: Metabolite levels in glycolysis pathway

- files provided for Figs. 5 and 6 appear to be mixed up, as data displayed does not match figure legend and description in results section

- figure legend: include number of animals analyzed per group (n=5), and statistical test applied

- subnormo group is comparable to sham group for metabolite levels of early glycolysis steps; yet, metabolite levels from later glycolysis steps are not discussed -- please comment on this as they either are comparable (i.e. 3-PG, pyruvate, lactic acid) between normo- and subnormoEVLP groups or are opposite (i.e. PEP)

***Reply 53*:** **Thank you for your valuable comments. Figures 5 and 6 were inadvertently mixed up, and I apologize for the confusion. This issue has been**

rectified, and I have provided descriptions of the number of subjects per group and the statistical methods used for each. We also spent a lot of time considering the glycolysis pathway metabolites in Figure 5. There were no significant differences in the early steps of glycolysis, such as G6P, F6P, and F1,6P, but subnormoEVLP tends to have a higher relative value of metabolites than normoEVLP. This is not the case for the metabolites of the remaining late steps except pyruvate. In the metabolite analysis, these intermediate metabolites of the pathway can have two meanings. First, this metabolic pathway may have been more active; therefore, the metabolites were depleted and presented at lower levels. Second, because this metabolic pathway has high activity, intermediate metabolites may have accumulated and increased. Both interpretations are possible, and to make this clearer, we checked the enzyme level to help clarify whether the accumulation or depletion of metabolites means an increase or decrease in the pathway, respectively. In conclusion, we confirmed that HK and PFK enzyme levels were significantly increased in normoEVLP and decreased to sham level in subnormoEVLP. Based on this, it was determined that glycolysis activity increases in normoEVLP and decreases again in subnormoEVLP, and intermediate metabolites tend to accumulate due to a decrease in pathway activity in subnormoEVLP. In the case of PK, about 90% of lung PK is PKM2. Because the dimer form of PKM2 mediates anaerobic glycolysis and the tetramer form mediates conversion to the TCA cycle, it is difficult to indicate the degree of glycolysis simply by PK expression. As a result, we believe that overall glycolysis activity decreases in subnormoEVLP as the levels of HK and PFK decrease.

Comment 54 Figure 6: Metabolites levels in TCA cycle

- files provided for Figs. 5 and 6 appear to be mixed up, as data displayed does not match figure legend and description in results section

- figure legend: include number of animals analyzed per group (n=5), and statistical test applied

Reply 54: **Thank you for your valuable comments. Figures 5 and 6 were inadvertently mixed up, and I apologize for the confusion. This issue has been rectified, and I have provided descriptions of the number of subjects per group and the statistical methods used for each.**

Comment 55 Figure 7: expression levels of mTORC, HIF-1a, NLRP3 and caspase-1

- figure legend: include number of animals analyzed per group (n=5), and statistical test applied
- was mTORC1 or mTORC2 amplified by qRT-PCR? please specify
- correlation of metabolite levels as well as HIF-1α expression levels to lung function parameters to support the author's hypothesis

Reply 55: **Thank you for your valuable comments. I have provided descriptions of the number of subjects per group and the statistical methods used for each.**

Comment 56 Discussion:

- the novelty of the present study should be clearly stated as well as its translation to the clinics (i.e. how could this new finding be used in the clinics)
- an outlook with next experimental steps should be given

Reply 56: **I agree with your opinion. Although our study showed somewhat broad and general insight into the perfusate temperature of EVLP, the differences observed in glycolysis activity and the expression levels of mTORC, HIF-1a, and NLRP3 during EVLP suggest that these molecules act as potential target candidates to induce improvement in lung function. Therefore, I believe that through further experiments utilizing the inhibition or activation of these molecules, we can verify the enhancement of lung function using EVLP.**

Comment 57 General comments/suggestions:

- provide figure headings and corresponding results sections with a content statement
- expand the description of the results: include introductory sentences as well as a final sentence summarizing the key finding in each section as this may be helpful for the reader
- in each figure legend include number of animals that were analyzed in the respective experiment as well as statistical test applied
- files provided for Figs. 5 and 6 appear to be mixed up

Reply 57: **Thank you for your comment. As per your suggestion, I have provided a more detailed explanation in the results section, and I have also made modifications to the figure legends. Additionally, I have included the statistical methodology and the number of subjects in the figure legends. I have reviewed the figure files, including Figures 5 and 6.**