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A novel modified Steen solution limits inflammatory processes during ex vivo lung perfusion and improves graft function post-transplantation

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cytokines

BACKGROUND: Ex vivo lung perfusion allows donor lung preservation, assessment, and re-conditioning before transplantation, but is associated with increased inflammatory injury over time. Addition of antioxidative and anti-inflammatory agents to perfusate formulations could limit iatrogenic injury during perfusion. The effectiveness of a modified Steen solution containing acetyl salicylic acid, retinoic acid, and methylprednisolone was examined using a porcine extended criteria donor ex vivo lung perfusion and transplantation model.

METHODS: Porcine donor lungs underwent 24 hours cold storage and were then randomized to 4 hours normothermic ex vivo lung perfusion with modified Steen or original Steen, followed by single lung transplantation into a recipient pig. RNA-sequencing was used to assess tissue inflammatory changes during perfusion. Organ function was examined during perfusion and following transplantation and compared between groups.

RESULTS: Lungs perfused with modified Steen showed reduced pulmonary vascular resistance (p = 0.0391) and stable pulmonary artery pressure despite achieving higher flows (p = 0.0001) compared to Steen. Lung tissue showed negative enrichment of the tumor necrosis factor-α (TNF-α) signaling via nuclear factor-κB (NF-κB) pathway (p = 0.0040) in modified Steen compared to Steen. Recipients of lungs perfused with modified Steen also showed improved post-transplantation oxygenation (p = 0.0462).

CONCLUSIONS: This study highlights the superiority of modified Steen compared with original Steen. The modifications to Steen solution appear to limit inflammatory injury via the NF-κB signaling pathway during perfusion, leading to improved post-transplant function. Modified Steen provides the potential to improve post-transplant outcomes following ex vivo lung perfusion of extended criteria

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lungs and could also facilitate extended assessment and preservation, as well as administration of advanced therapies.

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Background

The accessibility of lung transplantation as a therapeutic option for end stage lung disease is limited by a shortage of donor lungs and compounded by low utilization rates due to current interpretation of suitable donor selection criteria.[1,2] Use of much more extended criteria donor (ECD) organs is becoming more frequent, despite their potential association with worse outcomes[3] and increased risk of primary graft dysfunction (PGD).[4]

Ex vivo lung perfusion (EVLP) allows objective assessment of ECD lungs that fail to achieve criteria for immediate use and has the potential to facilitate their acceptance for transplant. It also allows prolonged preservation times, providing logistical benefits. In addition, EVLP has shown promise as a therapeutic platform to recondition donor lungs in recent years. Thus far, clinical trials have already demonstrated the safety and feasibility of transplanting ECD organs assessed by EVLP, showing comparable survival with those implanted following traditional cold static storage.[5-7]

The success of EVLP is built on the foundational studies performed by Professor Stig Steen, who carried out the first human lung transplant following ex vivo assessment.[8] The group was instrumental in the development of Steen solution, an organ perfusion solution which is used clinically as the standard of care for EVLP. The commercial production of Steen was critical in enhancing the accessibility of EVLP for lung transplant programs around the world. Steen has a buffered formulation, and its largest constituent is human serum albumin which maintains a physiologically relevant colloid osmotic pressure, minimizing lung damage.[9] Dextran 40 in the solution reduces endothelial-leukocyte interactions.[10] Steen solution has been shown to maintain stable lung function during EVLP for up to 12 hours.[11,12]

Despite this, some degree of ischemia reperfusion injury (IRI) is inevitable during reperfusion with EVLP. In fact, it is well established that proinflammatory cytokines measured in the perfusate and tissue increase significantly over time even in successful perfusions[13,14] and prolonged EVLP eventually causes progressive lung injury.[15,16] IRI is characterized by acute inflammation and increased oxidative stress and contributes to the development of PGD after lung transplantation.[17]

As a better understanding of the pathophysiological events that occur during EVLP continues to evolve, it is critical that the scientific advancement of perfusion technology accompanies it. In light of this, a modified organ evaluation solution has been developed. Unlike original Steen, the modified Steen (mSteen) contains new components, such as acetyl salicylic acid (SA), retinoic acid (RA), and methylprednisolone, which possess antioxidative and anti-inflammatory properties. Indeed, research has already shown the potential benefits of perfusing with solutions with antioxidative properties, such as modified Custodiol-N, which contains iron chelators and deferoxamine.[18]

This study sought to evaluate the effectiveness of a mSteen solution in comparison to the original Steen solution, which is in widespread use clinically. By using a porcine EVLP and transplantation model, functional parameters and markers of inflammation and organ injury were compared following perfusion with either Steen or mSteen.

Methods

Further methods are provided in the Supplement.

Ethical considerations

The study complies with the European Directive 2010/63/EU as amended by Regulation (EU) 2019/1010 and the Swedish Board of Agriculture's regulations and general guidelines for experimental animals SJVFS2019:9 L150. Animal care complies with the European Convention for the production of vertebrate animals used for Experimental and other Scientific purposes. Ethical approval was granted (M174-15 and 15906/2020) and the Food and Drug Administration guidance Utilizing Animal Studies to Evaluate Organ Preservation Devices (May 8, 2019) was considered throughout the study.

Porcine lung procurement

Swedish domestic pigs of both sexes (30-70 kg) were randomized among 3 study groups; unperfused Control (N = 4), Steen (N = 6-8), or mSteen (N = 6-8). Donor pigs from all groups were anesthetized via intramuscular injection of ketamine 20 mg/kg body weight, 100 mg xylazine, and 0.5 mg atropine. Before thoracotomy, pigs were also injected with 4 μg/kg fentanyl or 0.4 mg/kg midazolam. Euthanasia was performed via ventricular fibrillation following sternotomy, before immediate retrieval. Donor lungs were retrieved following standard procurement guidelines and as previously described for porcine models.[19] Lungs were flushed with 3 liter cold Perfadex Plus, after which the Control lung group underwent no intervention. This was to model the baseline state of the lung before ischemic injury. The Steen and mSteen group underwent 24 hours cold static storage in Perfadex Plus in a refrigerator maintained at 4°C to 6°C before undergoing EVLP. This was to ensure that lungs from healthy young pigs were representative of the extended criteria human lungs which would undergo EVLP in a clinical setting.

Porcine ex vivo lung perfusion

EVLP was performed using the XVIVO Perfusion System (XPS) for 4 hours using a minimal intervention strategy. The XPS was set up and primed according to the manufacturer's instructions for use with 2 liter of Steen or mSteen. The first hour of EVLP consisted of a warm-up phase according to the Toronto protocol as detailed in the XPS instructions for use. Then each perfusate was exchanged with the corresponding fresh perfusate solution. Thereafter, a pressure-controlled system was adopted, which maintained a physiologically relevant pulmonary artery pressure (PAP) of 13 to 15 mm Hg and left atrial (LA) pressure of 3 to 5 mm Hg by adjustment of flow. Pulmonary vascular resistance (PVR), flow as a percentage of estimated cardiac output (CO), peak airway pressure, and dynamic compliance were measured for functional assessment. PAP, left atrial pressure, LA temperature, and tidal volume were also recorded during EVLP. For the last hour, the heater cooler was set to 15°C to cool the system as per clinical practice for transplantation.

Novel perfusate formulation

Acetyl SA was added as an anti-inflammatory agent to stabilize the proinflammatory environment generated during perfusion, while antioxidative RA was added to target the oxidative stress associated with reperfusion during EVLP. Corticosteroids are immunomodulatory and anti-inflammatory and are often added to the circuit during perfusion as part of the standard protocol at various centers. Methylprednisolone was added to mSteen due to its improved potency compared to hydrocortisone[20] and simplification and standardization for the user by having it pre-existing in formulation.

The composition of the 2 different Steen solutions is listed in Table 1.

Table 1 Ingredients in Steen and mSteen

Component (g/liter)	mSteen	Steen
NaCl	4.44	5.03
D-glucose monohydrate	1.19	1.98
KCl	0.34	0.34
NaH_2PO_4	0.17	0.19
$CaCl_2$	0.22	0.22
$MgCl_2$	0.24	0.24
Tris	0.24	-
$NaHCO_3$	1.26	1.26
Arginine	0.26	-
HSA (25%)	70	70
Dextran 40	5	5
Dextran 1	0.5	-
$C_9H_8O_4$ (acetylsalicylic acid)	0.1	-
$C_{20}H_{28}O_2$ (retinoic acid)	0.0005	-
$C_{22}H_{30}O_5$ (methylprednisolone)	0.0035	-

Abbreviations: mSteen, modified Steen; HSA, human serum albumin.

For EVLP, perfusate was supplemented with 5 ml (25 mg/ml) Imipenem/Cilastatin and 1 ml (5000 IU) heparin. Steen also received 2 ml (50 mg/ml) Solu-Cortef (hydrocortisone) as per standard protocol at Igelösa; however, as mSteen already contains low amounts of methylprednisolone within its formulation, the mSteen group received no hydrocortisone.

Porcine lung transplantation

The left lung was transplanted into a blood type compatible recipient pig. Following reperfusion, the native right lung was removed by sequential clamping of each lobe until removal of the entire lung by approximately 1.5 hours postreperfusion. Ventilator and physiological parameters (pressure and heart rate) were recorded hourly during the 6 hours of recipient survival following reperfusion, before euthanasia. The study workflow and sampling are presented diagrammatically in Figure 1.

Sample collection

Lower lobe tissue samples were collected from the same area for every lung and stored in RNA later for qPCR and RNA-sequencing. Ten milliliter perfusate was also collected hourly from the venous line during EVLP and used for blood gas analysis or centrifuged at 2,000g for 10 minutes and stored for biomarker analysis. Arterial blood samples were collected hourly following left lung transplantation and used for blood gas analysis.

RNA-sequencing

3′ RNA-sequencing was performed using a smaller cohort of lung biopsies (Steen/mSteen; N = 5, Control; N = 4). Library preparation was done using the QIAseq UPX 3′ Transcriptome kit and sequenced using the NextSeq500. DESeq2 was used to calculate differential expression. Before gene set enrichment analysis (GSEA) and Ingenuity Pathway Analysis (IPA), porcine Ensembl gene IDs were converted to human orthologs using bioDBnet:dbOrtho.

Real-time quantitative PCR

Tissue was homogenized using QIAZOL (Qiagen) and the Tissue Lyser II (Qiagen). RNA was isolated using the RNeasy Mini kit and RNase-free DNase kit (Qiagen). Complimentary deoxyribonucleic acid synthesis was performed using the Tetro complimentary deoxyribonucleic acid synthesis kit (Bioline). Gene expression was quantified using real-time quantitative polymerase chain reaction (RT-qPCR) with Taqman gene expression assays (*ACTB*, Ss03376563_uH; *IL6*, Ss03384604_u1; *TNFAIP3*, Ss04954198_m1; Applied Biosystems) and Taqman Gene Expression Master Mix 2X (Applied Biosystems). qPCR was carried out using the Step One Plus Real-time PCR system (Applied Biosystems) using the comparative CT (ΔΔCT) method and normalizing to β-actin.

Enzyme-linked immunosorbent assay

The Porcine IL6 DuoSet enzyme-linked immunosorbent assay (ELISA) (DY686, R&D) and Porcine tumor necrosis

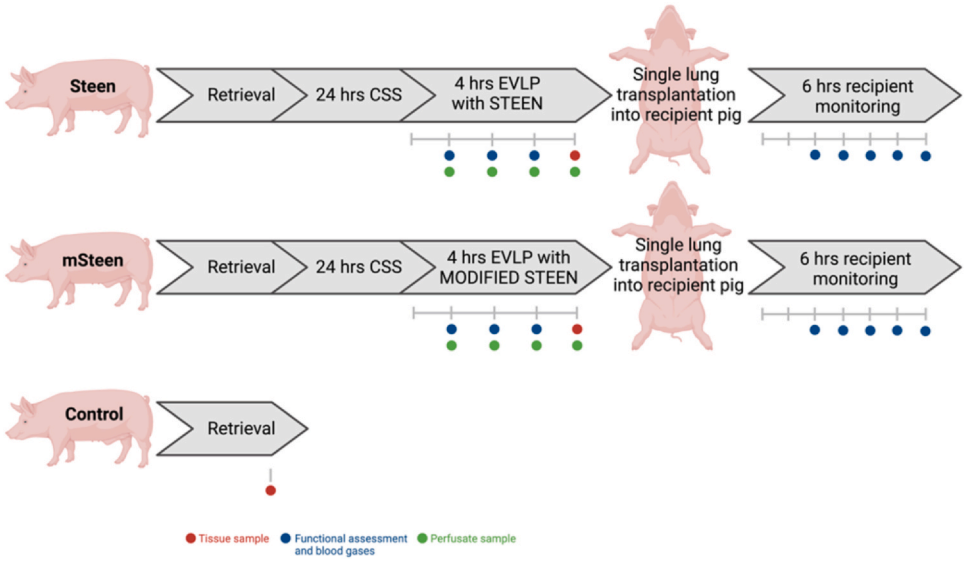

Figure 1 Diagram of workflow and sampling. Porcine lungs were retrieved as described and randomly allocated to groups (Steen, N = 8; modified Steen, N = 8; Control, N = 4). For the unperfused Control group, left lower lobe biopsies were taken immediately following retrieval and used for RNA-sequencing and validation. Lungs allocated to the Steen and modified Steen groups underwent 24 hours CSS in Perfadex plus before 4 hours EVLP with either Steen or modified Steen, respectively. Following cooling, the left lung was then transplanted into a recipient pig, which was monitored for 6 hours. Functional parameters and blood gases were recorded during EVLP and following transplantation. Right lower lobe biopsies were taken post-EVLP and used for RNA-sequencing and validation in a smaller cohort (first N = 5 per group). Sequential perfusate samples were also collected during EVLP and used for validation. CSS, cold static storage; EVLP, ex vivo lung perfusion.

factor-α (TNF-α) DuoSet ELISA (DY690B, R&D) were used and plates were read at an absorbance of 450 nm.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 10.0.3). Normality was determined using a Shapiro Wilk test. For parametric data, the mean and standard deviation (SD) are presented, while nonparametric data are presented as the median with interquartile range. Statistical tests are described for each experiment individually.

Results

Lungs perfused with mSteen had improved physiological function compared with lungs perfused with Steen

Physiological parameters were recorded throughout EVLP, excluding the 1-hour warm-up and 1-hour cooling period, to assess lung function in response to Steen or mSteen. PVR was significantly reduced in lungs perfused with mSteen compared to Steen (Figure 2A; $p = 0.0391$). mSteen perfused lungs also allowed a significantly higher flow rate ($p = 0.0001$),

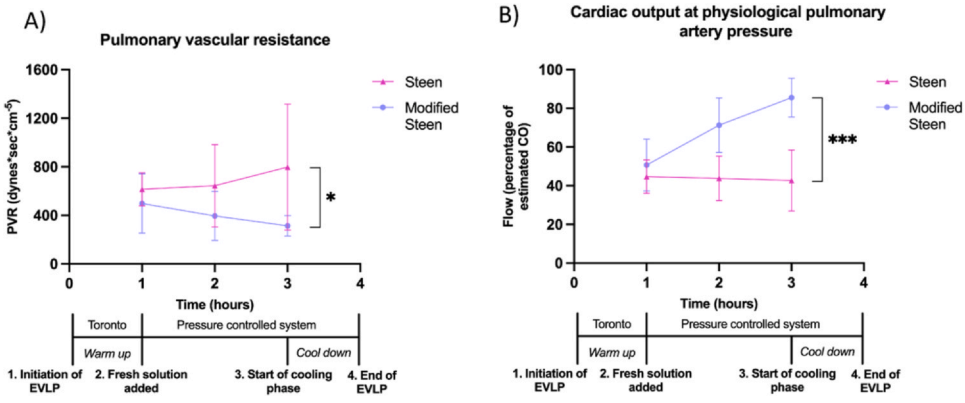

Figure 2 Physiological parameters during EVLP. **(A)** PVR and **(B)** flow as a percentage of estimated CO at PAP of 13 to 15 mm Hg for lungs perfused with Steen or modified Steen was measured throughout EVLP, excluding the 1-hour static warm-up and 1-hour cooling period. N = 8 per group. Mean is presented with SD. A 2-way repeated measures ANOVA was used to calculate statistical significance between modified Steen and Steen overall. Depicted is the ANOVA *p*-value for the column factor which describes whether there is a significant difference between the treatment groups (Steen vs mSteen). *$p < 0.05$, ***$p < 0.001$. ANOVA, analysis of variance; CO, cardiac output; EVLP, ex vivo lung perfusion; mSteen, modified Steen; PAP, pulmonary artery pressure; PVR, pulmonary vascular resistance; SD, standard deviation.

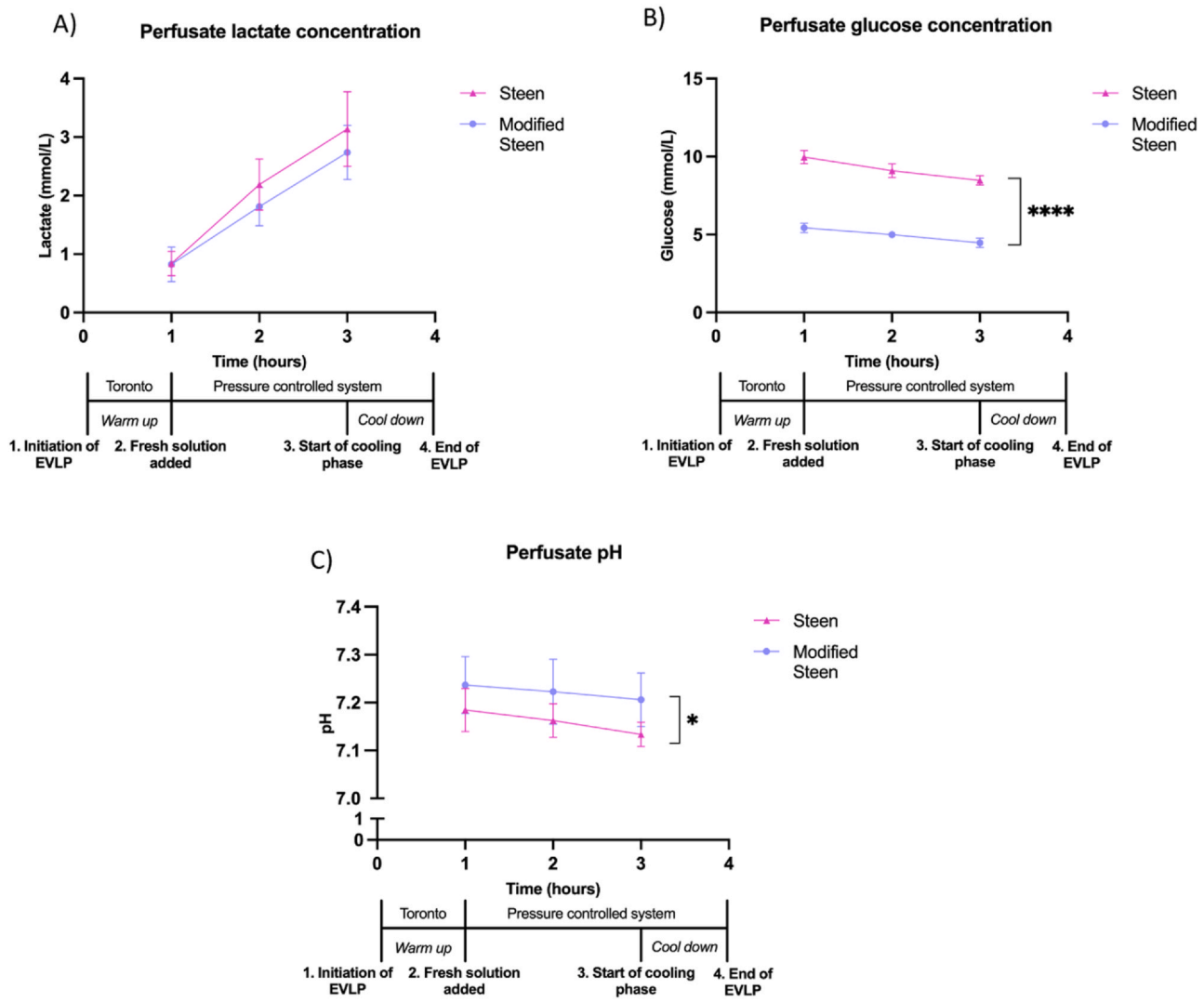

Figure 3 Perfusate blood gas measurements during EVLP. **(A)** Lactate concentration, **(B)** glucose concentration, and **(C)** pH for lungs perfused with Steen or modified Steen were measured throughout EVLP, excluding the 1-hour static warm-up and 1-hour cooling period. N = 8 per group. Mean is presented with SD. A 2-way repeated measures ANOVA was used to calculate statistical significance between modified Steen and Steen overall. Depicted is the ANOVA *p*-value for the column factor which describes whether there is a significant difference between the treatment groups (Steen vs mSteen). *$p < 0.05$, ****$p < 0.0001$. ANOVA, analysis of variance; EVLP, ex vivo lung perfusion; mSteen, modified Steen; SD, standard deviation.

while maintaining a physiological PAP of 13 to 15 mm Hg (Figure 2B). There was no significant difference in dynamic compliance, peak airway pressure, or the arterial partial oxygen pressure/the inspired oxygen fraction (P/F) ratio (Figure S1A-C, respectively). There was a trend toward reduced perfusate lactate concentration (Figure 3A) and a significantly higher pH in the mSteen group (Figure 3C, $p = 0.0230$). Perfusate glucose was lower in the mSteen group due to a reduced concentration in its formulation (Figure 3B, $p = 0.0001$).

RNA-sequencing revealed upregulation of certain inflammatory genes post-EVLP compared with unperfused Control lungs

RNA-sequencing was performed on post-EVLP and Control lung biopsies. Hierarchal clustering and principle component analysis (PCA) did not reveal distinct clustering of Steen vs mSteen, but showed separation between Control and post-EVLP (Figure 4A and B).

Compared with Control, Steen post-EVLP had 436 differentially expressed genes (DEGs), with several immune genes such as *BIRC3, TNFAIP3, SERPINE1, GADD45A, DDX21*, and *ICAM1* being the most significant (Figure S2Ai). mSteen post-EVLP also showed upregulation of some immune-related genes among the most significant, such as *GADD45A, TNFRSF12A, STX11*, and *DEPP1*, but showed a fewer amount of DEGs compared to Control than Steen (391 DEGs, Figure S2Aii). When comparing mSteen post-EVLP with Steen post-EVLP, only 1 gene was significantly differentially expressed. *CYP26B1*, involved in regulation of RA levels in the body,[21] was significantly upregulated in mSteen samples (Figure S2B, $p = 0.0099$).

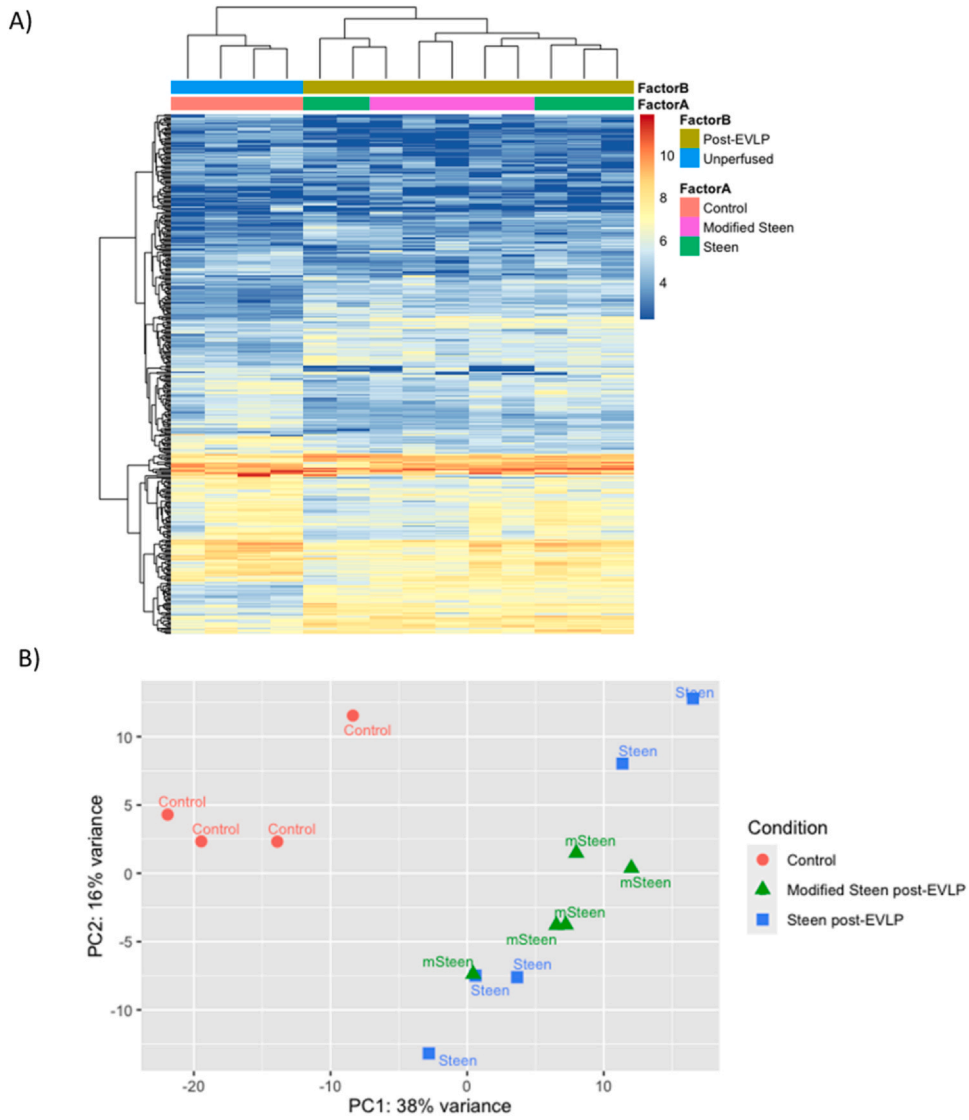

Figure 4 Gene expression data from RNA-sequencing. **(A)** Hierarchal clustering of samples in a heatmap using the top 400 genes with the highest variance across samples and **(B)** PCA plot of samples using all genes. A variance-stabilized transformation was applied to raw counts before hierarchal clustering and PCA. Control, N = 4; Steen, N = 5; mSteen, N = 5. EVLP, ex vivo lung perfusion; mSteen, modified Steen; PCA, principle component analysis.

Cold storage and EVLP were associated with positive enrichment of inflammatory gene sets

GSEA was carried out to identify differential enrichment of gene sets between groups. When comparing Steen post-EVLP with Control, 27 gene sets were significantly positively enriched, while only 21 gene sets were significantly positively enriched in mSteen post-EVLP vs Control. One pathway was significantly negatively enriched in Steen post-EVLP vs Control, while mSteen post-EVLP showed a total of 3 significantly negatively enriched pathways (Figure 5Ai and Aii; FDR < 0.05). Both Steen and mSteen post-EVLP showed enrichment of inflammatory pathways, such as TNF-α signaling via nuclear factor (NF)-κB, inflammatory response, apoptosis, and interleukin-6 (IL-6) Janus kinase/signal transducer and activator of transcription 3 signaling compared with Control. However, these were enriched to a lesser extent in mSteen (Figure 5B).

Perfusion with mSteen significantly reduced enrichment of various inflammatory gene sets compared to Steen

When directly comparing post-EVLP samples, 2 pathways were significantly positively enriched and 12 pathways were significantly negatively enriched in mSteen compared to Steen (FDR < 0.05, Figure 5C). The top 2 most negatively enriched pathways were inflammatory pathways; Epithelial Mesenchymal Transition (FDR = 0.0030) and the TNF-α signaling via NF-κB (FDR = 0.0040) pathways. Figure 5Di and Dii shows the top 50 leading edge genes for the top 2 significantly negatively enriched pathways. In this case, z score describes how much the count for that sample varies from the mean of all samples for a given gene. Positive z scores indicate an increased expression compared to the mean and negative z scores indicate reduced expression. Generally, z scores for mSteen samples

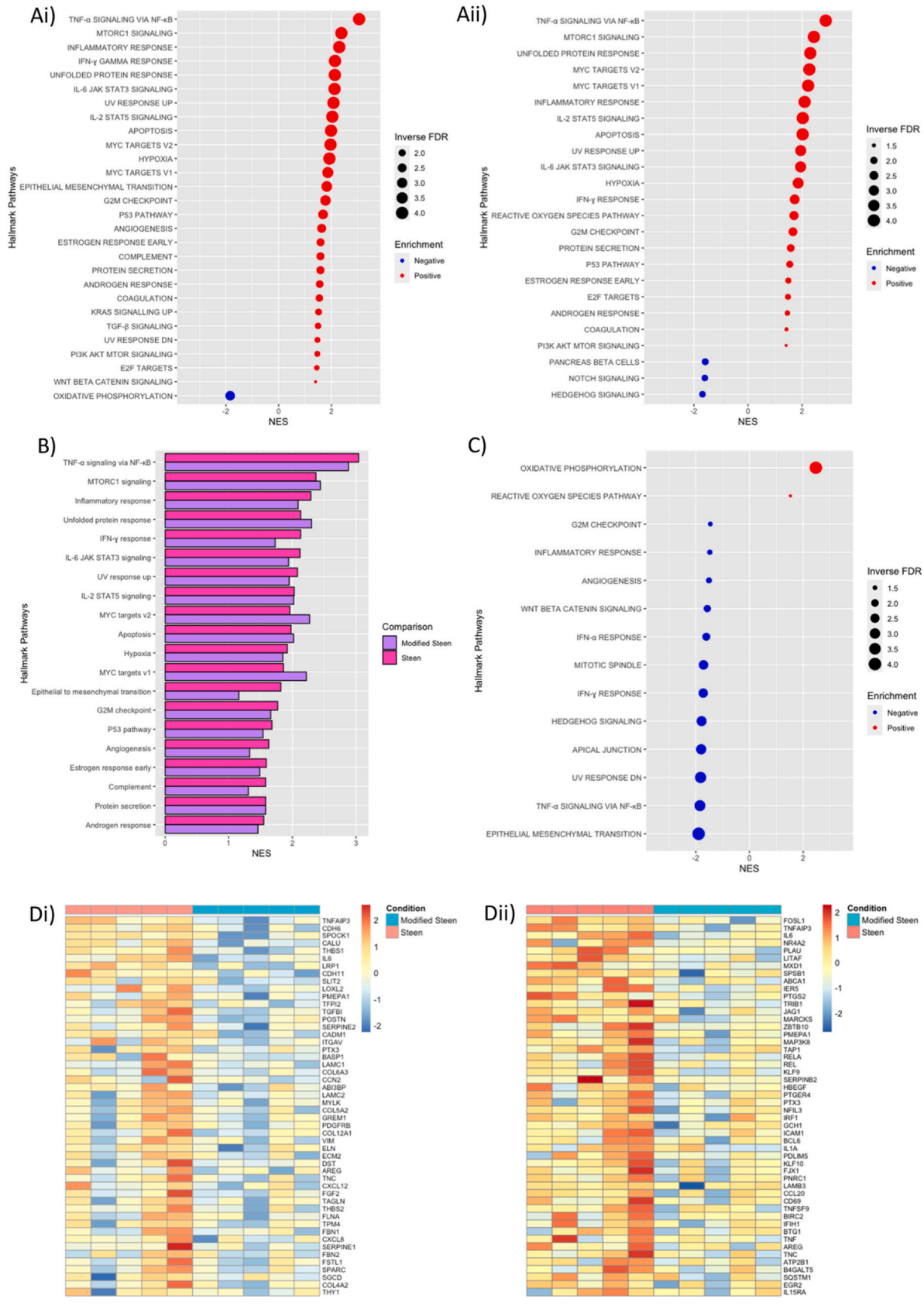

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Figure 5 Gene set enrichment analysis. **(Ai)** Graph depicting pathways that were significantly positively (red) or negatively (blue) enriched in lungs perfused with Steen compared to Control. **(Aii)** Graph depicting pathways that were significantly positively (red) or negatively (blue) enriched in lungs perfused with modified Steen compared to Control. Pathways shown had an FDR *p*-value of < 0.05. The graph shows NES along the x axis and hallmark pathways along the y axis and the size of each point corresponds to the inverse of the FDR *p*-value. **(B)** Comparison of normalized enrichment scores between Steen post-EVLP compared with Control (pink) and modified Steen post-EVLP compared with Control (purple). Depicted are the top 20 pathways for Steen vs Control. The graph shows NES along the x axis and hallmark pathways along the y axis. Depicted are pathways which had an FDR *p*-value of < 0.05 between at least 1 of the comparisons. **(C)** Graph depicting pathways that were significantly positively (red) or negatively (blue) enriched in lungs perfused with modified Steen when compared to lungs perfused with Steen. Pathways shown had an FDR *p*-value of < 0.05. The graph shows NES along the x axis and hallmark pathways along the y axis and the size of each point corresponds to the inverse of the FDR *p*-value. **(D)** Heatmaps of the top 50 leading edge genes involved in the core enrichment of the hallmark. **(Di)** Epithelial to mesenchymal transition and **(Dii)** TNF-α signaling via NF-κB pathways. These pathways were the most significantly negatively enriched in modified Steen vs Steen post-EVLP. Depicted are z scores of each sample for each gene. For heatmaps, z scores were calculated using normalized TPM counts to give an indication of how much the count for that sample varies from the mean for a given gene. Red = positive z score. Blue = negative z score. N = 4 (Control), N = 5 (Steen), N = 5 (modified Steen). AKT, protein kinase B; EVLP, ex vivo lung perfusion; FDR, false discovery rate; IFN-α, interferon-α; IFN-γ, interferon-γ; IL-2, interleukin-2; IL-6, interleukin-6; JAK, janus kinase; KRAS, kirsten rat sarcoma viral oncogene homolog; MTOR, mammalian target of rapamycin; MTORC1, mammalian target of rapamycin complex 1; NES, normalized enrichment score; NF-κB, nuclear factor-κB; PI3K, phosphoinositide 3 kinase; STAT, signal transducer and activator of transcription; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; TPM, transcript per million; UV, ultraviolet; WNT, wingless-related integration site.

appeared lower than z scores for Steen samples for genes involved in the core enrichment of these inflammatory pathways.

Cold storage and EVLP were associated with activation of inflammatory pathways, although to a lesser extent following perfusion with mSteen

IPA was carried out to predict the activation of enriched pathways. Due to the stringency of the gene thresholds set for IPA, a direct comparison between mSteen and Steen post-EVLP was not made as there was only 1 gene that met cut offs. Instead, separate core analyses were performed, comparing each post-EVLP group with unperfused Control. These were then indirectly compared using a comparative analysis. IPA generates a z-score which refers to the level of activation (+) or inhibition (-) of a pathway.

Figure 6Ai and Aii shows the top 20 most significant pathways ($p < 0.05$) ordered by z-score for Steen post-EVLP vs Control and mSteen post-EVLP vs Control, respectively. When comparing Steen with Control, among the most activated pathways were inflammatory pathways, such as the tumor microenvironment pathway ($p < 0.0001$) and the IL-6 signaling pathway ($p < 0.0001$), and cellular stress pathways, such as the unfolded protein response pathway ($p < 0.0001$). In contrast, the most activated pathways for mSteen vs Control were dendritic cell maturation ($p = 0.0017$), type I diabetes mellitus signaling ($p < 0.0001$), and high motility group box 1 signaling ($p < 0.0001$), while endothelial nitric oxide synthase signaling ($p = 0.0028$) and peroxisome proliferator-activated receptor signaling ($p < 0.0001$) were inhibited.

A comparative analysis was then carried out to compare the core analysis of Steen post-EVLP vs Control with the core analysis of mSteen post-EVLP vs Control. Figure 6B depicts a comparison of the z-scores for significant pathways identified. The pathways with the most difference between Steen vs Control and mSteen vs Control were the tumor microenvironment pathway, IL-6 signaling, tumor necrosis factor receptor 1 (TNFR1) signaling, and acute phase response signaling, all of which were activated to a lesser extent in mSteen vs Control than Steen. Figure 6Ci-iv shows a breakdown of the genes involved in each pathway and the log2 fold change (FC) when comparing each post-EVLP group to Control. Various inflammatory genes, such as *IL6*, *TNFAIP3*, NF-κB transcription factor complex genes, and NF-κB inhibitor genes in these top pathways showed higher FC compared to Control in Steen samples than mSteen.

IL6 and *TNFAIP3* gene expression was lower in lungs perfused with mSteen than Steen and corresponded with reduced IL-6 and TNF-α accumulation in perfusate over time

IL6 is a well-known proinflammatory cytokine gene and *TNFAIP3* is a primary response gene induced following TNF-α exposure of endothelial cells.[22] As genes that played a significant role in pathways identified in both GSEA and IPA, both were validated using qPCR.

RNA-sequencing showed that *TNFAIP3* gene expression was significantly increased compared to Control for Steen post-EVLP (Figure S2Ai; log2FC = 2.62, $p < 0.0001$) and mSteen post-EVLP (Figure S2Aii; log2FC = 1.80, $p < 0.0001$), albeit to a greater extent for Steen. Interestingly, for *IL6*, while Steen showed a significant increase compared to Control (Figure S2Ai; log2FC = 3.98, $p < 0.0001$), mSteen was not significantly different (Figure S2Aii).

Accordingly, visualization of the counts per million (CPM) values generated using RNA-sequencing for both *IL6* (Figure 7Ai) and *TNFAIP3* (Figure 7Bi) showed that Steen post-EVLP samples had increased expression, while Control and mSteen post-EVLP samples were comparably much lower. qPCR data confirmed these findings; Steen post-EVLP samples had significantly higher expression of both *IL6* ($p = 0.0117$, Figure 7Aii) and *TNFAIP3* ($p = 0.0146$, Figure 7Bii) compared to Control, while mSteen post-EVLP samples were not significantly different to Control.

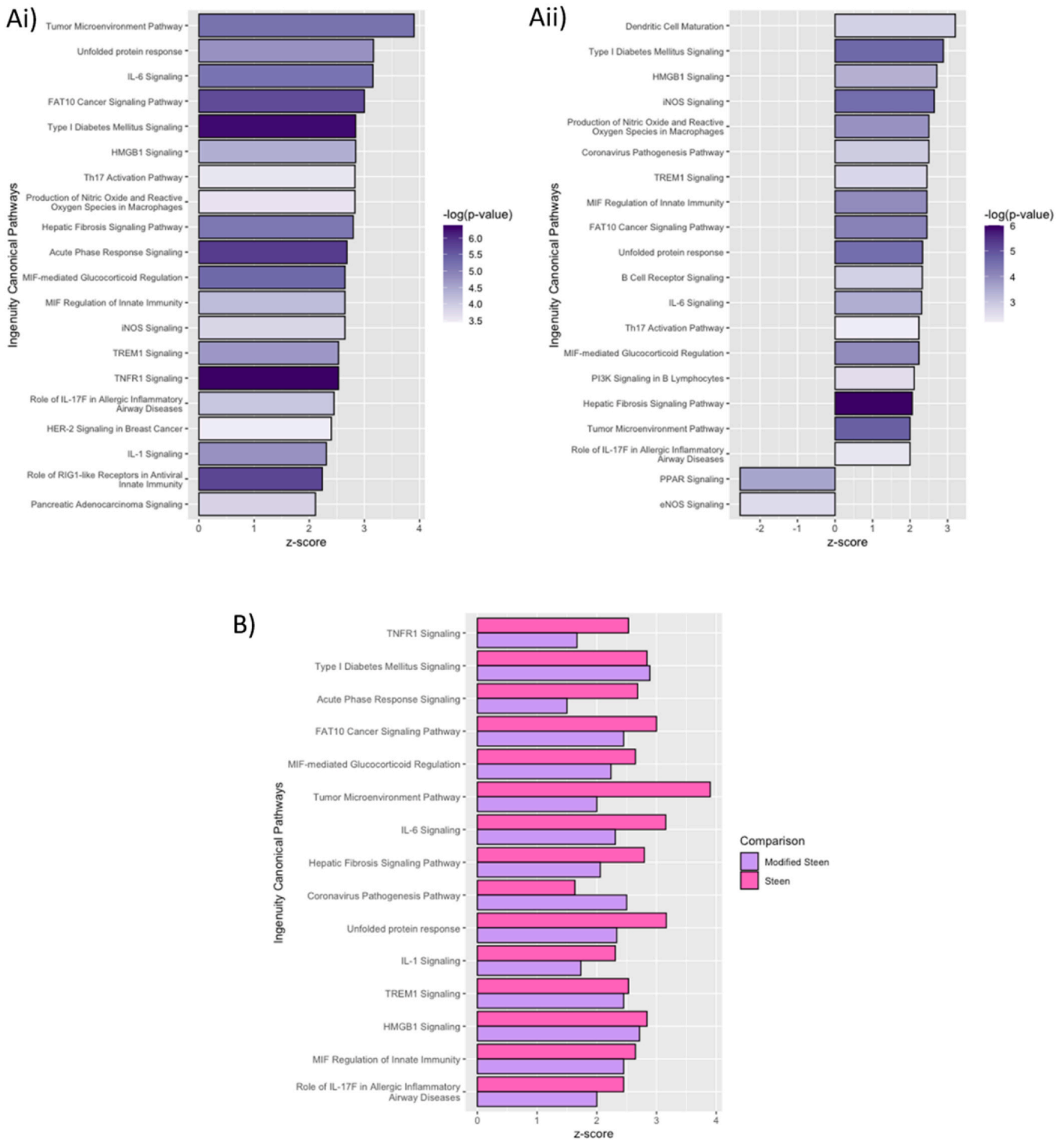


Figure 6 Ingenuity Pathway Analysis. For IPA, gene thresholds of a fold change ratio of ≥1 or ≤−1 and an FDR q-value < 0.05 were set. Shown are the top 20 most significant pathways with z-scores which were ≥2 or ≤−2 for **(Ai)** Steen post-EVLP samples compared with Control samples and **(Aii)** modified Steen post-EVLP samples compared with Control samples. The graphs show the z-score along the x axis and ingenuity canonical pathways along the y axis. Bars are colored according to inverse of the log *p*-value. **(B)** Comparison of z-scores between Steen post-EVLP compared with Control (pink) and modified Steen post-EVLP compared with Control (purple). Shown are the 15 most significant pathways for Steen versus Control which had a z-score of ≥2 or ≤−2 for at least 1 of the comparisons to Control. The graph shows z-score along the x axis and ingenuity canonical pathways along the y axis. Comparison of fold change of genes in **(Ci)** the Tumor Microenvironment Pathway, **(Cii)** IL-6 signaling pathway, **(Ciii)** TNFR1 Signaling Pathway, and **(Civ)** the Acute Phase response pathway between Steen post-EVLP compared with Control (pink) and modified Steen post-EVLP compared with Control (purple). Pathways selected were 4 of the most significant with the greatest difference between comparisons. The graphs show log2 fold change along the x axis and genes along the y axis. N = 4 (Control), N = 5 (Steen), N = 5 (modified Steen). *p*-values were calculated as part of the IPA core analysis using Fischer's exact test. eNOS, endothelial nitric oxide synthase; EVLP, ex vivo lung perfusion; FAT-10, human leukocyte atigen-F adjacent transcript 10; FDR, false discovery rate; HER-2, human epidermal growth factor receptor 2; HMGB1, high mobility group box 1; IL-1, interleukin-1; IL-6, interleukin-6; IL-17F, interleukin-17F; iNOS, inducible nitric oxide synthase; IPA, Ingenuity Pathway Analysis;

MIF, macrophage migration inhibitory factor; PI3K, phosphoinositide 3 kinase; PPAR, peroxisome proliferator-activated receptor; RIG1, retinoic acid-inducible gene 1; Th17, T helper 17; TNFR1, tumor necrosis factor receptor 1; TREM1, triggering receptor expressed on myeloid cells 1.

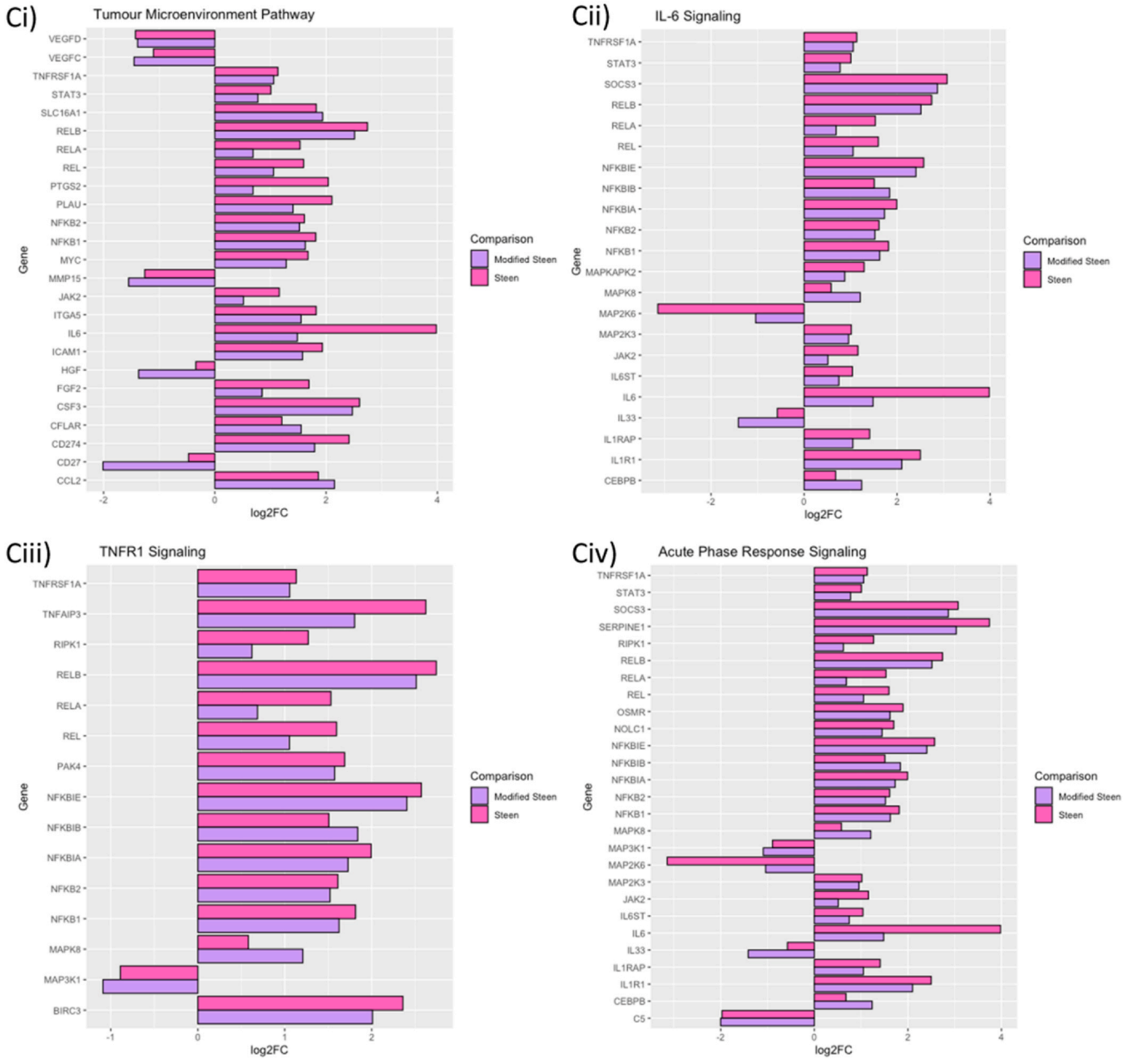
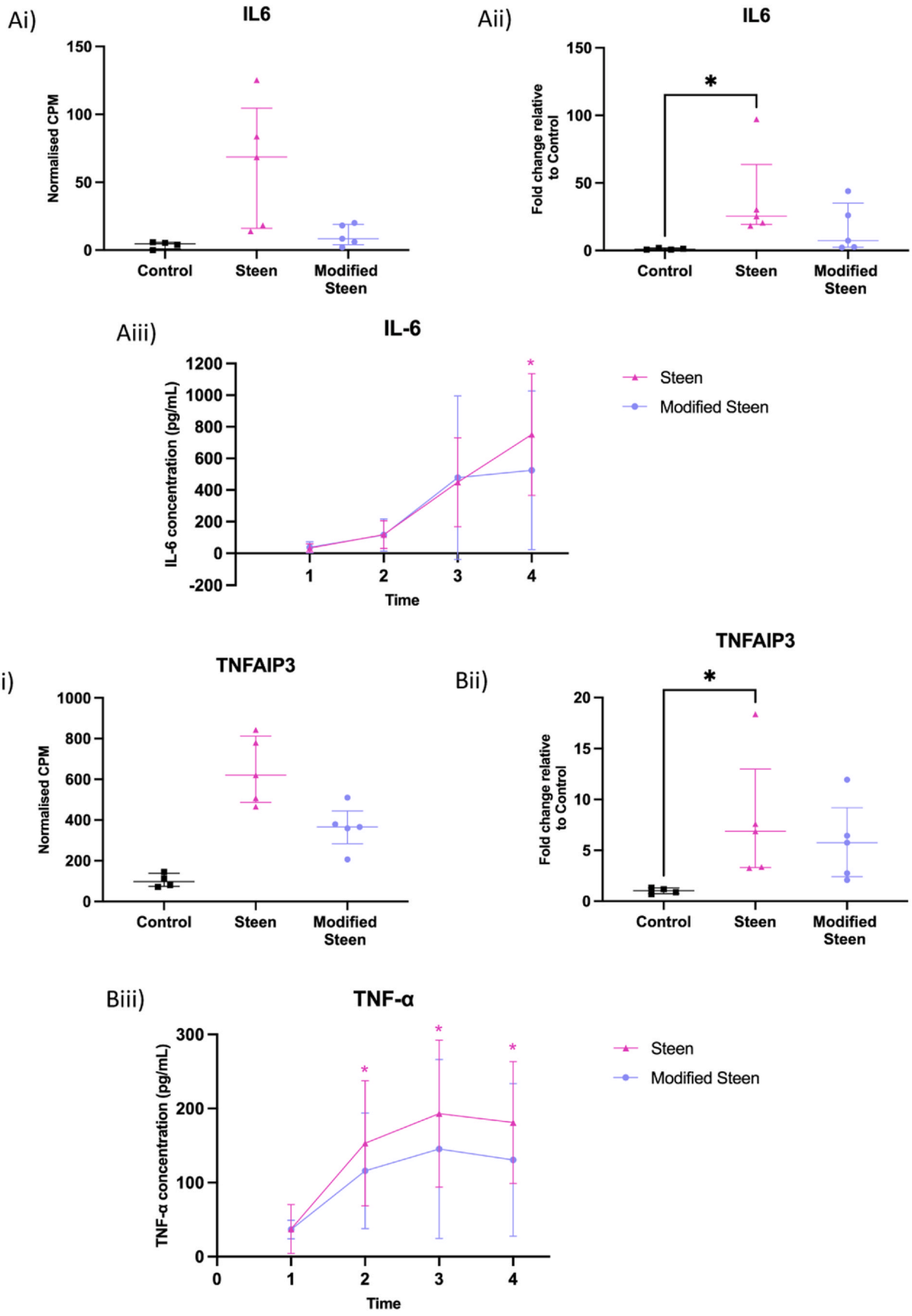


Figure 6 (*continued*)

Protein expression of IL-6 and TNF-α was examined in perfusates from lungs perfused with Steen or mSteen. Both cytokines started at similar levels immediately following solution exchange. Multiple comparisons revealed a significant increase in IL-6 at 4 hours ($p = 0.0272$) and TNF-α at 2 ($p = 0.0324$), 3 ($p = 0.0305$), and 4 ($p = 0.0102$) hours compared with 1 hour in Steen samples, while there were no significant differences between time-points in the mSteen group (Figure 7Aiii and Biii, respectively).

Perfusion with mSteen improved lung function following single lung transplantation

To assess the function solely of the transplanted lung following reperfusion in the recipient, P/F ratio, peak airway pressure, and mean PAP were monitored after the complete removal of the native right lung. While both peak airway pressure and mean PAP showed no significant difference (Figure 8B and C, respectively), lungs perfused with mSteen



(caption on next page)

Figure 7 Validation of RNA-sequencing using qPCR and ELISA. As some of the top leading edge genes in the epithelial to mesenchymal transition and TNF-α signaling via NF-κB pathways, *IL6* and *TNFAIP3* genes were validated using qPCR, before measuring protein levels of IL-6 and TNF-α in perfusate using ELISA. Depicted is normalized CPM from RNA-sequencing for **(Ai)** *IL6* and **(Bi)** *TNFAIP3* and gene expression using qPCR for **(Aii)** *IL6* and **(Bii)** *TNFAIP3* for Control, lungs perfused with Steen and lungs perfused with modified Steen. For qPCR data, gene expression is depicted as relative to Control. N = 4 (Control), N = 5 (Steen), N = 5 (modified Steen). Median is presented with interquartile range. To calculate statistical significance for qPCR, a nonparametric 1-way ANOVA with Dunn's multiple comparisons test was used. *$p < 0.05$. **(Aiii)** IL-6 and **(Biii)** TNF-α in perfusate measured in pg/ml over time during perfusion with Steen or modified Steen. Perfusate samples were taken hourly immediately following the solution exchange after the first hour of heating. N = 5 per group (except missing value due to lost sample). Mean is presented with SD. To determine statistical significance over time, a 2-way mixed effects analysis with Dunnett's multiple comparisons test was used, which compared each time point against 1 hour for each group. This was used instead of repeated measures to accommodate for missing values due to a missing perfusate sample (first repeat in modified Steen group, timepoint 3 hours). *(pink) $p < 0.05$ each timepoint vs 1 hour for Steen. ANOVA, analysis of variance; CPM, counts per million; ELISA, enzyme-linked immunosorbent assay; IL-6, interleukin-6; NF-κB, nuclear factor-κB; qPCR, quantitative polymerase chain reaction; SD, standard deviation; TNF-α, tumor necrosis factor-α.

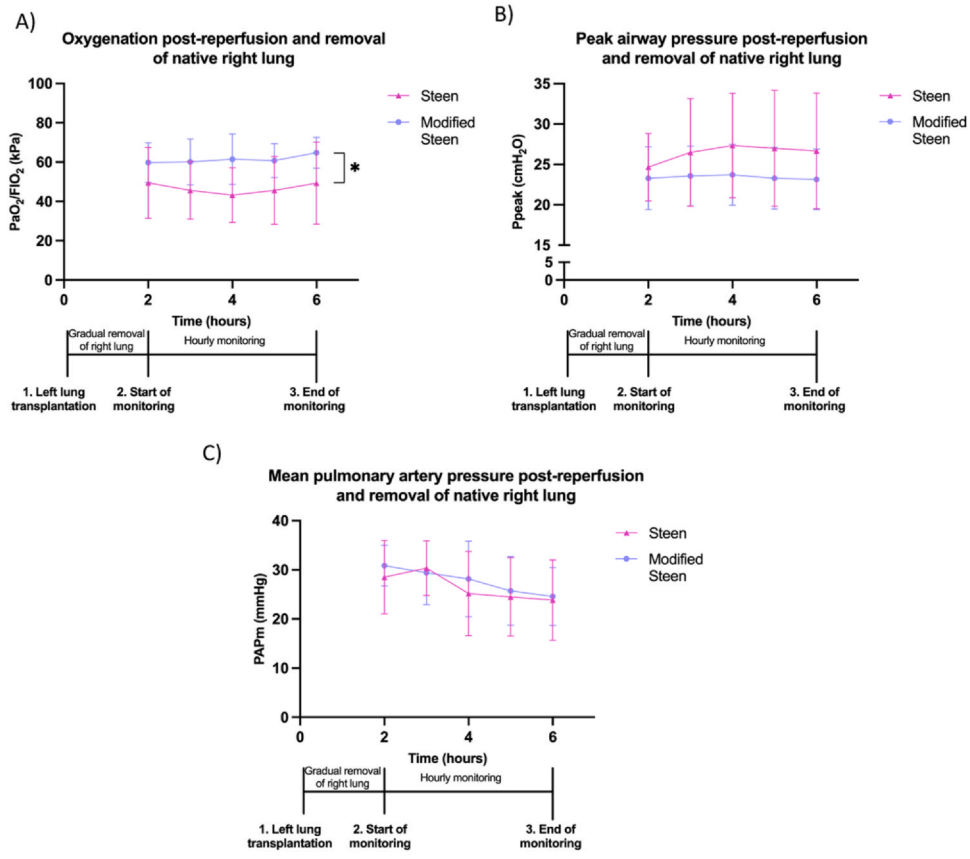


Figure 8 Lung function post-transplantation. The native right lung was removed gradually lobe by lobe starting immediately after transplantation of the donor left lung, achieving complete removal at around 1.5 hours post-transplant. Since the time of removal slightly varied between cases, blood gases and ventilator parameters were recorded hourly starting from 2 hours post-transplant. **(A)** The measured P/F ratio, **(B)** peak airway pressure, and **(C)** PAPm was monitored until 6 hours post-transplant, when the recipient pig was euthanized. N = 6 (Steen), N = 7 (modified Steen). From the N = 8 EVLPs in each group, the surgeon was unavailable to carry out the transplantation of 1 lung per group and 1 pig died shortly after reperfusion in the Steen group. Mean is presented with SD. A 2-way mixed effects analysis was used to calculate statistical significance between modified Steen and Steen overall. This was used instead of repeated measures to accommodate for missing values. Depicted is the ANOVA *p*-value for the column factor which describes whether there is a significant difference between the treatment groups (Steen vs mSteen). *$p < 0.05$. ANOVA, analysis of variance; EVLP, ex vivo lung perfusion; mSteen. modified Steen; PAPm, mean pulmonary artery pressure; P/F, arterial partial oxygen pressure/the inspired oxygen fraction; SD, standard deviation.

showed significantly higher P/F ratios in the recipient pig compared to lungs perfused with Steen ($p = 0.0462$, Figure 8A). There were no significant differences in arterial pH or lactate and glucose concentration (Figure S3).

Discussion

Discussion

In this study, a mSteen solution was evaluated using a porcine EVLP and transplantation model with an extended cold ischemic time to model extended criteria donation. First, we demonstrate that perfusion of lung grafts with mSteen in our model is both feasible and superior to the current clinically used original Steen solution. Second, we define the inflammatory processes that occur following cold storage and EVLP using transcriptomic analysis. In this study, mSteen reduced inflammatory gene and protein changes in lung grafts, which contributed to superior lung physiological function both during EVLP and following transplantation.

During EVLP, a higher perfusate pH and a trend toward reduced lactate suggested improved control of tissue acidosis with mSteen. Perfusion with mSteen also resulted in improved lung function during EVLP compared to Steen, with mSteen lungs exhibiting a significantly lower PVR. Research has suggested a negative correlation of PVR during EVLP with post-transplant outcome.[23,24] Indeed, this current study showed higher P/F ratios in recipient pigs transplanted with lungs perfused with mSteen than Steen, indicating that these lungs had an improved oxygenation capacity.[25] Perhaps due to their improved function, lungs perfused with mSteen also showed a better capacity to withstand higher flow rates during EVLP, while maintaining a PAP of 13 to 15 mm Hg at a mean CO of 85.57% by the end of perfusion. It was therefore unsurprising that mSteen-perfused lungs responded better to 100% CO following transplantation into the recipient pig through improved oxygenation. An important consideration of this is that the use of mSteen may offer protective benefits for protocols using higher flow rates, such as the Lund protocol which uses 100% CO.[26]

Unsupervised analysis revealed distinct clustering of post-EVLP samples and unperfused Control samples. Four hundred and thirty-six and 391 genes were differentially expressed in Steen and mSteen post-EVLP vs Control, respectively, and various DEGs were involved in acute inflammation. Accordingly, it has been shown that cytokine gene expression in lung tissue is increased following cold ischemia[27] and EVLP[28-30] and that local inflammatory responses increase with cold ischemic time duration and significantly after reperfusion.[31] GSEA revealed that the top significantly positively enriched pathway for both Steen and mSteen post-EVLP vs Control was TNF-α signaling via NF-κB. TNF-α is key in initiating the inflammatory cascade during IRI and has been suggested to be one of the most important mediators for complete lung IRI development.[32,33] Our GSEA data corroborate these studies. In addition, IPA identified activation of inflammatory and oxidative pathways, such as the tumor microenvironment pathway, unfolded protein response, IL-6 signaling, and production of nitric oxide and reactive oxygen species in macrophages following cold storage and EVLP in line with the characteristic pathology of IRI.

mSteen is formulated with the addition of anti-inflammatory and antioxidative RA, SA, and methylprednisolone. When directly comparing mSteen and Steen post-EVLP samples, only 1 gene was found to be significantly differentially expressed. *CYP26B1* was upregulated in mSteen perfused lungs and is involved in the metabolism of RA.[21] RA has been shown to modulate toll-like receptor 4/NF-κB signaling pathway, causing downregulation of nitric oxide synthase 2 and TNF-α.[34] Similarly, both SA and methylprednisolone have been shown to exert anti-inflammatory properties primarily through inhibition of NF-κB.[35-38]

In line with this, TNF-α signaling via NF-κB was one of the most significantly negatively enriched pathways in mSteen post-EVLP compared with Steen post-EVLP following GSEA. This could indicate that the limitation of lung injury achieved via perfusion with mSteen is primarily exerted through inhibition of transcription factor NF-κB, which is usually initiated early on in IRI to activate proinflammatory cytokine genes. Indeed, alongside TNF-α, NF-κB is one of the most important mediators of IRI[32,33] and its blockade during EVLP has been shown to be protective.[39,40] Furthermore, the comparative analysis performed using IPA identified reduced activation of inflammatory pathways in mSteen post-EVLP vs Control compared to Steen post-EVLP versus Control. While the genes involved in these pathways were primarily inflammatory genes, such as proinflammatory cytokine genes *IL6* and *TNFAIP3*, what was most notable was the involvement of NF-κB transcription factor complex genes and NF-κB inhibitor genes in these pathways. These genes generally had higher FCs from Control in Steen compared to mSteen post-EVLP, again suggesting a role for NF-κB in the protection mediated by mSteen.

Furthermore, in support of IPA and GSEA findings, qPCR analysis identified that gene expression of *IL6* and *TNFAIP3* was comparable between Control and mSteen post-EVLP, while Steen post-EVLP samples showed significantly increased expression compared with Control. Correspondingly, we also found that protein levels of IL-6 and TNF-α in perfusate significantly increased over time in Steen, while mSteen did not. Typically, EVLP is associated with the accumulation of inflammatory cytokines, with several papers showing increases in IL-6[29,41] and TNF-α[13,14] release over time. However, by inhibiting the crucial transcription factor NF-κB, mSteen seemed to ameliorate this inflammatory expression, release, and accumulation.

In this current study, we identify possible targets for therapeutic intervention by identifying inflammatory gene expression changes during cold storage and EVLP. We also demonstrate the superiority of novel perfusate solution mSteen compared to the current clinically used perfusate solution, Steen. Overall our data suggest that mSteen has the potential to improve post-transplant outcomes after EVLP of marginal

organs by limiting inflammatory injury during perfusion. The clinical implications of this are meaningful, especially given that ECD organs which might be assessed using EVLP are already associated with increased incidences of PGD and poorer recipient outcomes.[3,4] mSteen could also facilitate longer and more stable perfusion for advanced assessment and therapeutic strategies and have benefits for pre-existing protocols, such as the Lund technique. Extending EVLP duration with a reduced risk of iatrogenic damage could also offer longer preservation times, providing logistical benefits. Our gene and protein expression data suggest that organ protection by mSteen seems to be primarily mediated through NF-κB inhibition during EVLP.

Limitations

This study not only assesses the effects of mSteen during EVLP but progresses to assess impacts on graft function following transplantation in a large animal model. Although promising, this preliminary data would require larger sample sizes to draw firm conclusions and testing using human organs would be required before clinical adoption. One of the main limitations perhaps lies in the design of the unperfused Control group. While we wanted a control group to represent the most ideal donor situation, that is, lungs with no ischemic injury, we recognize that this is not feasible in clinical practice. However, our control group serves as a good point of comparison to understand the transcriptomic changes that occur during both cold storage and EVLP. Furthermore, as part of the standard center protocol, hydrocortisone was added into the perfusate of the Steen group at the start of EVLP, which could potentially have reduced the magnitude of difference between the Steen and mSteen groups.

CRediT authorship contribution statement

J.G., A.S., E.H., A.F., and S.A. were involved in research design. J.G., A.S., and E.H. conducted the experiments. J.G. analyzed the data and interpreted the results. J.G. drafted the manuscript. A.F. and S.A. reviewed and edited the manuscript.

Disclosure statement

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Andrew J. Fisher reports a relationship with XVIVO Perfusion AB that includes funding grants. Andrew J. Fisher reports a relationship with Therakos that includes consulting or advisory, funding grants, speaking and lecture fees, and travel reimbursement. Andrew J. Fisher reports a relationship with Pfizer Inc. that includes funding grants. Andrew J. Fisher reports a relationship with Chiesi Pharmaceuticals Inc. that includes funding grants. Andrew J. Fisher reports a relationship with Sanofi SA that includes consulting or advisory. Andrew J. Fisher reports a relationship with Altavant Sciences, Inc. that includes consulting or advisory. Andrew J. Fisher reports a relationship with International Society for Heart and Lung Transplantation that includes board membership. Jenny Gilmour reports a relationship with XVIVO Perfusion AB that includes funding grants. Simi Ali reports a relationship with XVIVO Perfusion AB that includes funding grants. Simi Ali reports a relationship with EPSRC Centre for Doctoral training in Molecular Sciences for Medicine that includes board membership. Simi Ali reports a relationship with Northern Counties Kidney Research Fund that includes board membership. Emilia Henriksson reports a relationship with XVIVO Perfusion AB that includes employment, equity or stocks, and travel reimbursement. Anne-Li Sigvardsson reports a relationship with XVIVO Perfusion AB that includes employment, equity or stocks, and travel reimbursement. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhlto.2024.100091.

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