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engineered ex-situ perfused organs



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Current developments in artificial organs and engineered ex-situ perfused organs

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Cristiano Amarelli — Department of Cardiac Surgery and Transplants - Monaldi, Azienda dei Colli, Italy

Cecile Legallais — Centre National de la Recherche Scientifique (CNRS), France



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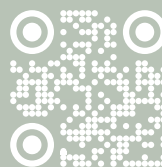
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*CORRESPONDENCE

Cristiano Amarelli,
✉ cristiano.amarelli@ospedaleiccoli.it

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Editorial: Current developments in artificial organs and engineered *ex-situ* perfused organs

Cristiano Amarelli^{1*}, Jutta Arens², Cécile Legallais³ and Thierry Berney^{4,5}

¹Department of Cardiac Surgery and Transplants, Monaldi, Azienda Ospedaliera dei Colli, Naples, Italy,

²Department of Biomechanical Engineering, University of Twente, Enschede, Netherlands, ³Université de technologie de Compiègne, CNRS, BMBI (Biomechanics and Bioengineering), Compiègne, France,

⁴Department of Nephrology, Transplantation and Clinical Immunology, Hospices Civils de Lyon, Groupement Hospitalier Centre, Lyon, France, ⁵Faculty of Natural Sciences and Medicine, Ilia State University, Tbilisi, Georgia

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artificial organs, machine perfusion, hypothermic, normothermic, reconditioning

Editorial on the Special Issue

Current developments in artificial organs and engineered *ex-situ* perfused organs

Introduction

Despite the steady increase in solid organ transplant activity worldwide, a substantial and persistent mismatch remains between the growing prevalence of end-stage organ failure and the limited availability of donated organs. The unmet needs extend well beyond the population of wait-listed and transplanted patients [1], considering that less than 10% of the global needs for transplantation are currently met. Reconditioning of discarded organs, artificial and bioartificial solutions, and xenotransplantation have been proposed for replacing almost every failed organ.

The recent rise of *ex vivo* machine perfusion (EVMP) has provided an opportunity to recondition marginal-quality organs and prevent their discard. This technology also opens a window of opportunity for intervention to assess or repair the organ's function during the perfusion period (for example, using gene therapy strategies) and even to reprogram organs failing due to inherited diseases [2].

This special issue was designed to collect review and original articles addressing current developments in artificial organ development and *ex situ* organ perfusion (heart, lung, kidney, or liver) for various purposes: transportation, assessment, reconditioning, repair, and research model development.

As machine perfusion is already established as a method for reconditioning kidneys and livers [3–5], it is no surprise that a vast majority of submitted and published articles came from the field of thoracic organ transplantation [6, 7], where the unmet needs are greater.

The value of machine perfusion

A review from Lund University covers the efforts made to expand the donor pool including bioengineering techniques and the use of *ex vivo* graft perfusion (Niroomand et al.). It also

covers modifications and treatments that have been tested in clinical trials, in addition to research efforts in both abdominal and thoracic organs. Overall, this article discusses recent innovations in machine perfusion, but organ bioengineering advances are discussed extensively, reviewing how these strategies can improve and increase the quality of donor organs. The main focus is on the lung and the heart, but abdominal organs (liver, kidney, pancreas) are also covered to provide an updated and broad review.

EVMP directly addresses ischemia–reperfusion injury (IRI) and primary graft dysfunction (PGD), which represent the principal barriers to the wider adoption of DCD hearts. The extensive review by Tessari et al. from the University of Padova describes a large number of animal models of EVMP in the context of DCD heart transplantation (Tessari et al.), highlighting the crucial role of preclinical research in the development of the field.

As for the need for pre-clinical models, system miniaturization and single-organ study design can simultaneously enhance experimental rigor, ethical compliance, and translational relevance in *ex situ* lung perfusion research. The group from the University of Hannover developed a low-volume EVMP model in the rat for double- and single-lung application, enabling cost-effective optimal compliance “reduction” of the 3R principles of animal research (Replacement, Reduction, and Refinement) (Katsirntaki et al.). Most relevant perfusion parameters confirmed the model’s validity, with homogeneous lung perfusion evidenced by uniform lung surface temperature, and histological examination confirmed intact lung architecture without infarcts or hemorrhages.

The group at the University of Bari is one of the frontline centers striving to expand the heart donor pool using reconditioning strategies. They have reviewed use of veno-arterial extracorporeal membrane oxygenation (VA ECMO) to ensure organ perfusion and provide the transplanted heart with adequate rest while recovering from IR (Giovannico et al.). The review describes how mechanical circulatory support may turn severe PGD into a reversible condition. They state that, in the new era of heart transplantation, early use of VA-ECMO increases patient survival.

Assessment of the graft during EVMP

One of the added values of EVMP has been the possibility to assess graft function, thereby predicting graft outcome and protecting the recipient from the risk of unpredictable events after transplantation [8]. (Van der Hoek et al.) propose that integrating medical imaging into *ex vivo* human-sized organ perfusion fundamentally enhances organ condition assessment by providing both spatially resolved and functional insights that complement traditional viability markers. The systematic review from the University of Twente shows that medical imaging is a powerful tool to assess organ condition in perfused hearts, kidneys, and livers. Laser speckle contrast imaging,

ultrasound, computed tomography, and magnetic resonance imaging have been convincingly used to identify local ischemic regions and quantify intra-organ perfusion. New imaging techniques, such as ³¹P magnetic resonance spectroscopy and near-infrared spectroscopy, may enable detailed investigation of the graft’s metabolic activity. Scalability and imaging costs for organ assessment remain major concerns for clinical adoption.

An original study from Erasmus University, in a porcine model of normothermic heart EVMP, proposes a novel approach using electrophysiological (EP) parameters derived from electrical mapping as an alternative to biomarkers for assessing post-ischemic cardiac performance (Amesz et al.). This technique offers an objective adjunct (and potential improvement) over the criticized lactate-based DCD heart assessment. Potential voltages, slopes, and conduction velocities measured on the left and right ventricles were computed from unipolar electrograms and compared between groups. Potential voltages and slopes were decreased in hearts with severe warm ischemia. The study concludes that EP parameters could aid in decision-making about the transplantability of DCD hearts.

The University of Groningen team demonstrated in an ovine proof-of-concept that pressure–volume (PV) loop analysis can be used during normothermic ESHP to functionally assess left-ventricular (LV) performance (Ertugrul et al.). This technique is a further attempt to move beyond the reliance on biochemical markers to assess perfusion. This method uses a physiological “working-mode” test to quantify LV function through end-systolic elastance (ESE) measured at 60 and 120 min. The system allows adjustment of preload and afterload to the left ventricle. By increasing preload and measuring ESE, left ventricular function can be reliably assessed, supporting better graft selection for both DBD and DCD donor hearts.

A systematic review of non-randomized controlled trials on patients undergoing transplantation with reconditioned lungs via EVMP was published by the University of Torino, aiming to assess the association between levels of proinflammatory biomarkers (adhesion molecules, chemokines, cytokines, damage-associated-molecular-patterns, growth-factors, metabolites) during EVMP and development of grade 3 PGD within the first 72 h post-transplant (Costamagna et al.). Meta-analysis revealed that the chemokine panel has the strongest association rather than relying solely on traditional physiologic/biochemical monitoring. Pooled chemokine panels measured at the beginning or end of the perfusion period are associated with the development of grade 3 PGD within the first 72 h after lung transplantation, supporting biomarker-guided risk stratification during EVMP.

An interesting protocol for a paired-lung pilot study is presented by two organ donor centers from Spain (Grando et al.). It will include seven organ donors after brain death or after controlled cardiac death. The left lung will be preserved in static cold storage (SCS) and the right lung will be perfused with normothermic EVMP. Samples from bronchoalveolar lavage, perfusion and preservation solutions, and lung biopsies will be collected from both lungs. The study is designed to test whether EVMP reshapes the lung microbiome, and whether microbiome shifts align with changes in local inflammatory activation.

The University of Innsbruck reports on a study evaluating mitochondrial function by high-resolution respirometry as a viability assessment of liver grafts (Hofmann et al.). They observed that in livers taken off normothermic EVMP for diagnostic biopsy and preserved in SCS conditions thereafter, mitochondrial respiration was stable up to

Abbreviations: AAV, adeno-associated virus; DBD, Donation after brain death; DCD, Donation after circulatory death; ESE, End-systolic elastance; EP, Electrophysiological; ESHP, *Ex situ* heart perfusion; EVMP, *Ex vivo* machine perfusion; IRI, Ischemia-reperfusion injury; NRP, Normothermic regional perfusion; PGD, Primary graft dysfunction; SCS, Static cold storage; VA-ECMO, Venoarterial extracorporeal membrane oxygenation; VCA, Vascularized composite allograft.

4 h but decreased afterwards. They conclude that SCS can be safely applied to extend the biopsy measurement window for up to 4 h to determine organ quality, and that human liver respiration degrades beyond 4 h of SCS following normothermic machine perfusion.

Intervention to improve graft quality during EVMP

EVMP not only allows the reconditioning of marginal grafts but also provides a window of opportunity for various types of interventions to further improve graft quality. The combination of intervention and assessment methods during EVMP is a key toward maximizing marginal donor organ utilization.

Dawn Bowles' lab at Duke University delivered a muscle-tropic recombinant adeno-associated virus (AAV), AAV-SLB101, to porcine hearts during a brief normothermic EVMP period (Dewan et al.). *Ex situ* normothermic heart perfusion can serve as a scalable "gene-delivery window" to transduce a donor heart with a muscle-tropic AAV vector, achieving early-onset, durable myocardial transgene expression after transplant that lasts up to 120 days, with minimal off-target transgene expression. The authors propose that this exploratory study will serve as a critical foundation for translational studies using therapeutic transgenes to improve outcomes in heart transplantation.

Boffini et al. propose that EVMP outcomes may be limited by gravity-dependent fluid accumulation and ventilation-perfusion (V/Q) mismatch in the supine position—particularly in the dorsal lung regions (Boffini et al.). They designed a tilting dome that allows the lung block to be progressively moved from 0° to 90° during perfusion, aiming to maintain physiologic function while potentially reducing dorsal congestion. This feasibility study, conducted in a porcine model without a control group, showed that the lungs maintained satisfactory gas-exchange parameters throughout the 4-h experimental period. Histology showed neither edema nor congestion; granulocytic infiltration was sporadic in the lung interstitium and absent from the alveoli; no hemorrhage or microthrombosis was detected.

A group from Astana has studied the impact of VA ECMO, including bronchodilator administration, hemofiltration, and cytokine removal using a Cytosorb membrane, on lung preservation in a porcine model (Faizov et al.). In their study, ECMO and hemofiltration maintained lung function for at least 24 h, as supported by a study from the Bartlett group a few years ago [9]. Additional cytokine removal significantly improved organ quality over 24 h of EVMP.

A look at vascular composite allografts (VCA)

One of the frontiers of EVMP is the potential to expand its use to VCA transplantation [10], including for the preservation of uterine grafts [11]. The scarcity of literature reflects the relatively low level of clinical activity in this field and the need for clear targets to define its effectiveness.

A study from Harvard Medical School reported the impact of subnormothermic EVMP on prolonged preservation of VCA tissues in a porcine hind limb allotransplantation model. (Goutard et al.) demonstrate that oxygenated, acellular subnormothermic

machine perfusion can preserve VCAs for 24 h and still support successful allotransplantation, whereas time-matched SCS leads to severe post-transplant muscle degeneration and early failure. This preclinical evidence provides a strong translational proof of principle that EVMP can meaningfully extend the VCA preservation window.

Finally, a group from Aachen has developed an artificial womb (Heyer et al.). This artificial organ is not designed as an implantable device, but as an *ex vivo* "bridge-to-life" for severely prematurely born infants. This extremely interesting paper lacks *in vivo* data but describes the performance achieved in adapting to the physiologic growth of an extremely preterm neonate over a 4-week period, envisioning its future possible adoption. Results of the *in vitro* tests showed a temperature constancy of 36.8 °C without pressure loss. A filtration and disinfection system was designed and has proven strong disinfection capabilities, effective filtering of metabolic waste, and the ability to avoid phospholipid washout.

Conclusion

This collection of articles demonstrates the vitality and ongoing improvements in the field of organ reconditioning, repair, and replacement using advanced technologies. Research on EVMP is progressing rapidly, especially for thoracic organs, and is entering the field of VCA, but it also offers an impressive source of pathophysiological insights. So far, technical challenges have prevented the clinical application of EVMP to the pancreas on a convincing basis, but research is ongoing [12], as graft preservation remains a main focus for reshaping the field. These strategies are only one of the avenues studied, developed, and implemented to narrow the ever-increasing gap between the number of quality donor organs and the number of patients living with end-stage organ failure and on the waiting list for a kidney, liver, heart, or lung.

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Enhanced Donor Lung Viability During Prolonged *Ex Vivo* Lung Perfusion Using ECMO Technology

Linar Faizov¹, Yerik Zuparov¹, Aiyim Turarova¹, Zhuldyz Nurmykhametova^{1*}, Aidyn Kuanyshbek¹, Rymbay Kaliyev¹, Anatoly Sergienko², Timur Lesbekov¹ and Yuriy Pya¹

¹Department of OR with Mechanical Circulatory Support, National Research Cardiac Surgery Center, Astana, Kazakhstan,

²Department of Bioengineering, National Research Cardiac Surgery Center, Astana, Kazakhstan

The shortage of donor organs remains a significant obstacle to addressing the growing demand for lung transplantation. We have developed a mobile EVLP circuit utilizing extracorporeal membrane oxygenation (ECMO) technology. The goals of our study was to evaluate the safety of a cellular-based EVLP system and to assess the efficacy of continuous cytokine removal and the application of bronchodilators during prolonged EVLP. A total of 29 pigs, aged 6–18 months and weighing 93 ± 13.1 kg (mean $\pm$ SD), were used in the study. The lungs were randomly allocated into the following groups: Cytosorb group ($n = 12$), were perfused with an EVLP circuit containing a Cytosorb cartridge, and Control group ($n = 12$), where lungs were perfused with an EVLP circuit without a Cytosorb cartridge, Bronchodilator group ($n = 5$) consisted of lungs that were nebulized with a combined bronchodilator every 4 h after the initiation of ventilation and Control group ($n = 5$) inhaled the control solvent under the same settings. Our study demonstrates that whole blood based perfusate with continuous ultrafiltration for functioning lung in machine perfusion can be straightforward and efficient for at least 24 h. Additional cytokine removal had led to significant improvement of organ quality over 24 h of EVLP.

Keywords: normothermic machine perfusion, Cytosorb, *ex vivo* lung perfusion, ECMO technology, bronchodilation

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*Correspondence

Zhuldyz Nurmykhametova,
✉ zhzyknuir@icloud.com

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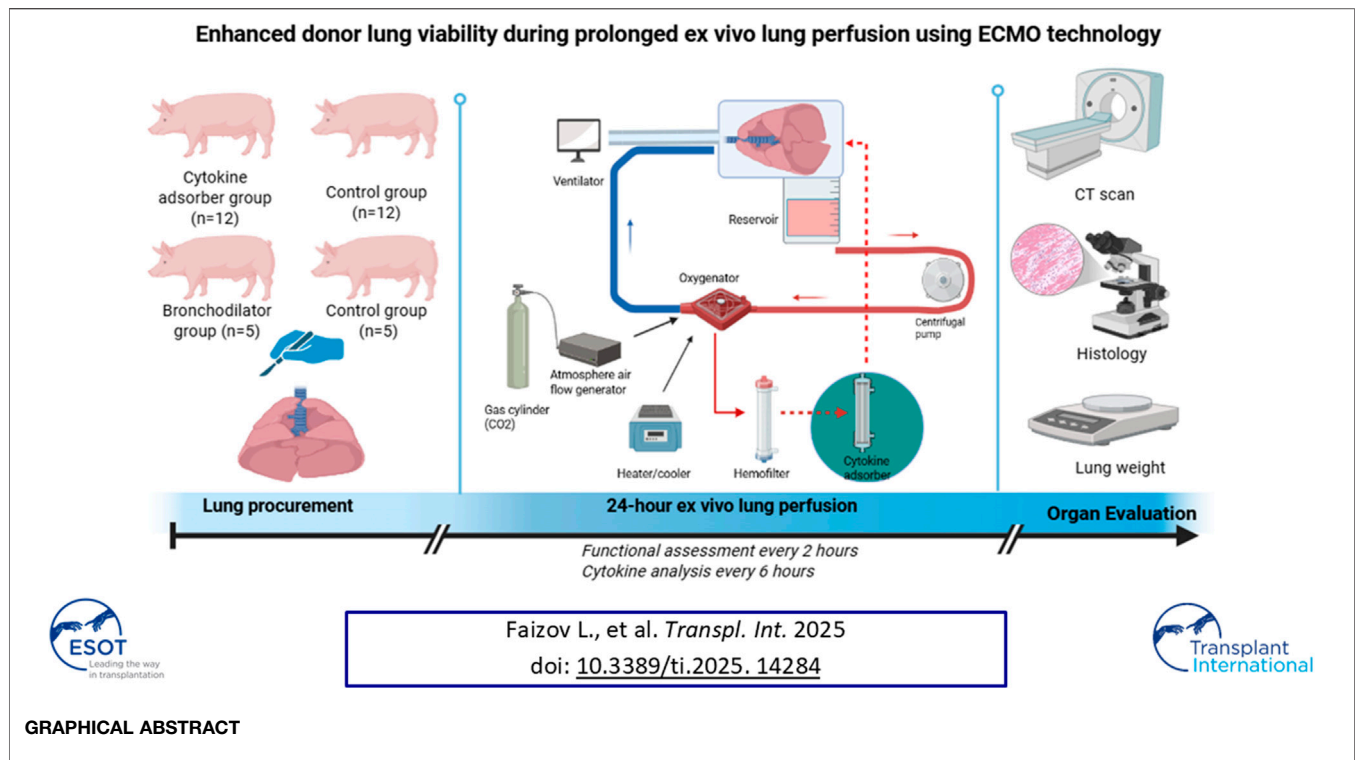
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INTRODUCTION

The shortage of donor organs remains a significant obstacle to addressing the growing demand for lung transplantation (LTx). Despite an annual increase in the number of lung transplants performed, approximately 18.8% of transplant candidates are removed from the waiting list each year due to the lack of suitable donor lungs [1]. *Ex vivo* lung perfusion (EVLP) technology, introduced globally by Steen et al. in the mid-2000s, has revolutionized the field by expanding the donor pool without compromising recipient outcomes [2, 3]. This innovative technique preserves lungs in a physiological state before transplantation, allowing for their evaluation, preservation, and reconditioning. Unlike cold static preservation, normothermic perfusion sustains the metabolic activity and viability of lung cells for extended periods. Various EVLP protocols are now in clinical use, employing different types of perfusates. Research suggests that cellular perfusates offer advantages over acellular perfusates, including reduced edema formation [4–6], lower levels of inflammatory cytokines [7], and decreased expression of genes associated with acute lung injury (ALI) [8, 9]. The TransMedics Organ Care System (OCS) has demonstrated effective use of donor blood for *ex vivo* lung preservation, reducing ischemic time, minimizing blood wastage, and enhancing the efficiency of organ retrieval and transplantation processes [10]. However, despite



its significant benefits, the high costs associated with this technology and its consumables may limit its broader adoption [11, 12]. Primary graft dysfunction (PGD) is a major cause of transplant-related morbidity and mortality, primarily driven by ischemia-reperfusion injury (IRI), which triggers oxidative stress, inflammation, and alveolar damage [13–15].

To address these challenges, we developed a mobile EVLP circuit based on extracorporeal membrane oxygenation (ECMO) technology. The primary goal of this study was to evaluate the safety of a novel, easily transportable, cellular-based EVLP system. A secondary goal was to assess the efficacy of continuous cytokine removal and the application of bronchodilators during prolonged EVLP.

MATERIALS AND METHODS

Animal Model

Experiments were conducted in accordance with the guidelines of the National Research Cardiac Surgery Center's Animal Care and Use Committee (Protocol No 2022/01-121). A total of 29 pigs (*Sus scrofa* ssp. domesticus), aged 6–18 months and weighing 93 ± 13.1 kg (mean $\pm$ SD), were used in the study. Prior to surgery, food was withheld for each pig for 24 h and water for 12 h. Thiopental sodium (10–15 mg/kg), administered through an angiocatheter to induce an anesthesia. Animals were intubated and mechanical ventilation was initiated in volume control mode with a tidal volume of 8 mL/kg, peak inspiratory pressure (PIP) between 18–20 cmH₂O, and positive end-expiratory pressure (PEEP) of 5 mmHg. Ventilation settings were adjusted to

maintain normocapnia, ensuring that the partial pressure of carbon dioxide (PaCO₂) remained within the target range of 35 mmHg–45 mmHg. Anesthesia was maintained using isoflurane, titrated to achieve a minimum alveolar concentration (MAC) of 0.5%–1.5%. Vital parameters, including heart rate, invasive blood pressure, central venous pressure, oxygen saturation, and end-tidal CO₂, are monitored continuously.

Lung Procurement

A median sternotomy was performed, followed by exsanguination via right atrial cannulation. Systemic anticoagulation was achieved with an intravenous bolus of 50,000 IU heparin sodium (Chempharm, Kazakhstan). Prior to lung procurement, a recruitment maneuver was applied. The porcine lungs were harvested with the trachea clamped at a peak inspiratory pressure (PIP) of 25 cmH₂O. The pulmonary arteries were flushed in both antegrade and retrograde directions with 1,000 mL of Plasmalyte solution (Baxter Polska, Warszawa) to remove residual blood. After harvesting, a collapse test was performed to evaluate the biomechanical properties of the lungs. To ensure safe cannulation of the pulmonary artery to the EVLP circuit, a 3–4 cm graft was attached to elongate the vessel. Following this, the lungs were attached to the EVLP system and perfused for 24 h. Reperfusion of the *ex vivo* lungs began approximately ± 50 min after aortic cross-clamping.

Ex Vivo Lung Perfusion

The EVLP system was primed with blood from the donor pig, human Albumin 20% - 500 mL (Biopharma, Ukraine) and

diluted to achieve a mean hematocrit of 10%–15% (2% standard error, hereafter SE), making a total volume of approximately 1L. The heat exchanger integrated in the oxygenator was used to keep the perfusate at normothermia and facilitated gas exchange using 0.1–0.2 L per minute (LPM) of atmosphere air flow with mixed CO₂ 30–50 mL/min. The system was then de-aired and circulation started at a low flow 0.5 L/min, than perfusion flow during 24 h was 0.8–1.0 L/min. Pharmacological support provided via controlled on demand infusion of glucose, insulin, sodium nitroprussid and sodium bicarbonate, methylprednisolone 250 mg (Pfizer, USA). Hemoglobin and glucose were checked every 30 min during EVLP. Similarly, the perfusate was maintained at a normal range of pH = 7.4 (SD 0.1).

Active zero balance ultrafiltration with Plasmalyte solution (Baxter Polska, Warszawa) as a plasma substitution solution was used to maintain the hematocrit and electrolytes in the target normal range.

The EVLP circuit utilized extracorporeal membrane oxygenation (ECMO) technology. The system included a chamber designed for the harvested lung, equipped with an integrated reservoir to facilitate passive drainage of pulmonary venous blood. Continuous perfusion was maintained using a centrifugal pump, with the perfusate flow rate set at 30% of the estimated cardiac output. To deoxygenate the perfusate, carbon dioxide was intermittently (30–50 mL/min) introduced into the oxygenator. The perfusate temperature was regulated at 37 °C using a heat exchanger (made in our institution) to optimize cellular metabolism during perfusion. A hemofilter (B.Braun A vitrum AG, Germany) was integrated into the circuit with a flow rate of 30 mL/min to allow continuous hemodialysis of the perfusate across all experimental groups. After mounting the lung, the filtration flow was maintained at 0.03 L/min for the duration of the experiment. Fluid loss due to ultrafiltration was compensated by adding Plasmalyte solution. The ultrafiltration process helps remove potentially deleterious substances, including activated complement factors, which may contribute to inflammation or lung injury. Additionally, a cytokine adsorber (CytoSorb 300, CytoSorbents Inc. New Jersey, USA) was included in the Cytosorb group. The main differences between our ECMO-based EVLP system and established protocols are summarized in **Table 1**.

The trachea was connected to a mechanical ventilator (Zisline MV300, ZetMedical Ltd, USA) via a silicone tube. Pressure-controlled ventilation was applied with a positive end-expiratory pressure (PEEP) of 5 cmH₂O, an inspiratory pressure of 6 cmH₂O, a respiratory rate of 10 breaths/min, and an FiO₂ of 21%. Peak inspiratory pressure (PIP) was limited to 25 cmH₂O to minimize the risk of barotrauma. A humidification system (VADI VH-1500, VADI Medical Technology Co., Ltd, Taiwan) was employed during mechanical ventilation to maintain adequate airway moisture.

Randomization

The lungs were randomly allocated into the following groups:

1. Cytosorb group (n = 12), where lungs were perfused with an EVLP circuit containing a Cytosorb cartridge, and Control

group (n = 12), where lungs were perfused with an EVLP circuit without a Cytosorb cartridge.

2. The Bronchodilator group (n = 5) consisted of lungs that were nebulized with a combined bronchodilator (Berodual® N, Boehringer Ingelheim, Kiev, Ukraine) every 4 h after the initiation of ventilation. The active components of the bronchodilator included an anticholinergic agent, Ipratropium bromide (20 µg), and a β₂-adrenoceptor agonist, Fenoterol hydrobromide (50 µg). The corresponding Control group (n = 5) inhaled the control solvent under the same settings.

Organ Evaluation

Functional assessments were conducted every 2 h, measuring pulmonary artery pressure (PAP), peak airway pressure, dynamic compliance (C_{dyn}), the ratio of partial pressure of oxygen to the fraction of inspired oxygen (PaO₂/FiO₂), and blood gas levels. The lungs were weighed before the initiation of EVLP to establish a baseline and reweighed after the perfusion process to evaluate weight gain.

Radiologic Assessment

Following 24 h of perfusion, computed tomography (CT) scans were performed to evaluate lung structure. CT scans were performed using a multi-detector computed tomography (MDCT) system (Siemens Healthineers AG, Germany). Images were independently reviewed by two radiologists blinded to group assignments to minimize bias. Lung injury was assessed using a standardized scoring system [16] defined as follows: 1—absence of consolidation; 2—Minimal consolidation, involving 1%–25% of the lung; 3—Moderate consolidation, affecting 26%–50% of the lung; 4—Extensive consolidation, covering 51%–75% of the lung; and 5—Near-total consolidation, with 76%–100% of the lung affected. The total score was then calculated and presented.

Microscopic Assessment

Lung tissues were collected from the same lower lobe at the end of the EVLP procedure for histological analysis. Tissue samples were fixed in formalin, embedded in paraffin, and sectioned into thick slices. The sections were stained with Hematoxylin and Eosin to assess cellular architecture and lung injury. Microscopic examination was conducted using light microscopy. Histological lung injury was scored based on a standardized injury scale [17]. Lung injury scores were quantified by a blinded investigator. The lung injury score was obtained by summing the scores of five independent variables: intra-alveolar edema, interstitial edema, neutrophil infiltration in the alveolar or interstitial spaces, and the presence of hyaline membranes. Scores for each sample were assigned based on the extent of damage observed: 0 – none, 1 -mild, 2 – moderate, 3 – severe, 4- diffuse.

Cytokine Level Monitoring

Cytokine levels, including interleukin-6 (IL-6) and interleukin-8 (IL-8), were analyzed using blood samples drawn from the EVLP circuit at 6-h intervals over the 24-h perfusion period.

TABLE 1 | Comparative overview of the ECMO-based EVLP system and conventional EVLP platforms. Adapted from Menander [11].

Parameter	Our protocol (ECMO-based EVLP)	OCS lung system	Toronto Protocol
Perfusion Circuit	Modified ECMO circuit (centrifugal pump, oxygenator, heat exchanger)	Portable OCS circuit (pulsatile flow)	XVIVO circuit (centrifugal pump, oxygenator, heat exchanger)
Priming Solution	Plasmalyte + Albumin + donor blood	Proprietary OCS solution + packed red blood cells (PRBC)	Steen solution
Flow Initiation	Low flow 0.5 L/min → target 0.8–1.0 L/min	Automated gradual ramp-up	10%–40% of predicted cardiac output, adjusted stepwise
Gas mixture	Controlled air flow gas generator (21% O ₂ , CO ₂)	Automated oxygenation and ventilation	8% CO ₂ , 6% O ₂ , 86% N ₂
Ventilation Mode	Conventional ventilator, lung-protective settings	Integrated ventilator, lung-protective settings	Integrated ventilator, lung-protective settings
Perfusion Pressure Target	Pulmonary artery pressure <20 mmHg	Pulmonary artery pressure ~10–20 mmHg	Pulmonary artery pressure <20 mmHg
Anticoagulation	Heparin 50,000 IU (initial)	Heparinized priming solution	Heparin bolus and maintenance doses
Preservation Solution	Plasmalyte	OCS solution	Perfadex or Steen Solution
Pre-flush			
Assessment Duration	Up to 24 h	Up to 6–8 h	Up to 12 h
Unique Feature	Adaptation of clinical ECMO circuit for EVLP	Portable, fully integrated, transportable	Widely established research and clinical protocol

Statistics

All results are expressed as mean ± standard deviation (SD). The Shapiro-Wilk test was used to assess the normality of data distribution. Longitudinal data trends between groups were analyzed using a two-way analysis of variance (ANOVA). Comparisons of continuous outcomes between groups at specific time points were conducted using Mann-Whitney U tests. A p-value of <0.05 was considered statistically significant. All analyses were performed using GraphPad Prism version 10.4 software.

RESULTS

The primary aim of this study was to establish and evaluate an innovative, alternative EVLP system based on an ECMO circuit. Our results demonstrate that this ECMO-based EVLP setup enabled stable perfusion and ventilation of donor lungs over 24 h, maintaining physiological parameters and functional stability comparable to established EVLP protocols.

Functional Assessment

Pulmonary artery pressure (PAP), peak airway pressure, dynamic compliance (C_{dyn}), and the ratio of partial pressure of oxygen to the fraction of inspired oxygen (PaO₂/FiO₂) were recorded at 2-h intervals throughout the EVLP period. Gas exchange efficiency, as measured by the PaO₂/FiO₂ ratio, remained consistently above 300 in both groups over the 24-h perfusion period, indicating lung viability. However, PaO₂/FiO₂ ratio was significantly higher in the Cytosorb group than control (p < 0.05; **Figure 1A**). Moreover, lung mechanics, as determined by dynamic compliance, was significantly better in the Cytosorb group (p < 0.05; **Figure 1B**). Pulmonary artery pressure demonstrated similar trends over time and no difference was observed between groups (P = 0.9; **Figure 1C**).

Peak airway pressure (**Figure 2A**) was significantly lower in the Bronchodilator group compared to the Control group (p < 0.05). Dynamic compliance (**Figure 2B**) significantly improved in the Bronchodilator group (94.8 ± 0.8 mL/cmH₂O) compared to

the Control group (86.6 ± 3.2 mL/cmH₂O, p < 0.05) by the end of the 24-h perfusion period. Notably, dynamic compliance in the Bronchodilator group showed a steady upward trend, suggesting progressive improvement in lung mechanics.

Radiological Assessment

The CT images in the Cytosorb group showed better-preserved lung tissue and less edema, while the control group exhibited more areas of consolidation (**Figure 3**). The differences in lung injury between the two groups were statistically significant (Cytosorb: 3.5 ± 1.1 vs. Control: 4.6 ± 1.0; p < 0.05).

Histological Analysis

Histological lung injury scores were significantly lower in the Cytosorb group compared to the control group (Cytosorb: 2.1 ± 0.9 vs. Control: 3.2 ± 1.3; p < 0.05). Microscopic examination of the Cytosorb group revealed well-preserved alveolar architecture with minimal disruption to the alveolar-capillary barrier. In contrast, lung injury in the control group was characterized by more extensive interstitial edema at the end of EVLP. Notably, no evidence of alveolar or interstitial hemorrhage was observed in any of the samples (**Figure 4**).

Weight Gain

Lung weight was measured before and after the 24-h EVLP period (**Figure 5**). Baseline lung weight did not differ significantly between the 2 groups (Cytosorb 559 ± 45g, Control 556 ± 69g; p = 0.89). By the end of the run, the Cytosorb group exhibited the least weight gain than the Control group (11.5% and 42.2 respectively, p < 0.01).

Cytokine Analysis

Cytokine analysis revealed that levels of IL-6 and IL-8 were significantly lower in the Cytosorb group compared to the control group during EVLP, indicating a potential reduction in inflammatory response (**Figure 6**). These findings provide insight into the modulation of inflammation and immune response during prolonged perfusion.

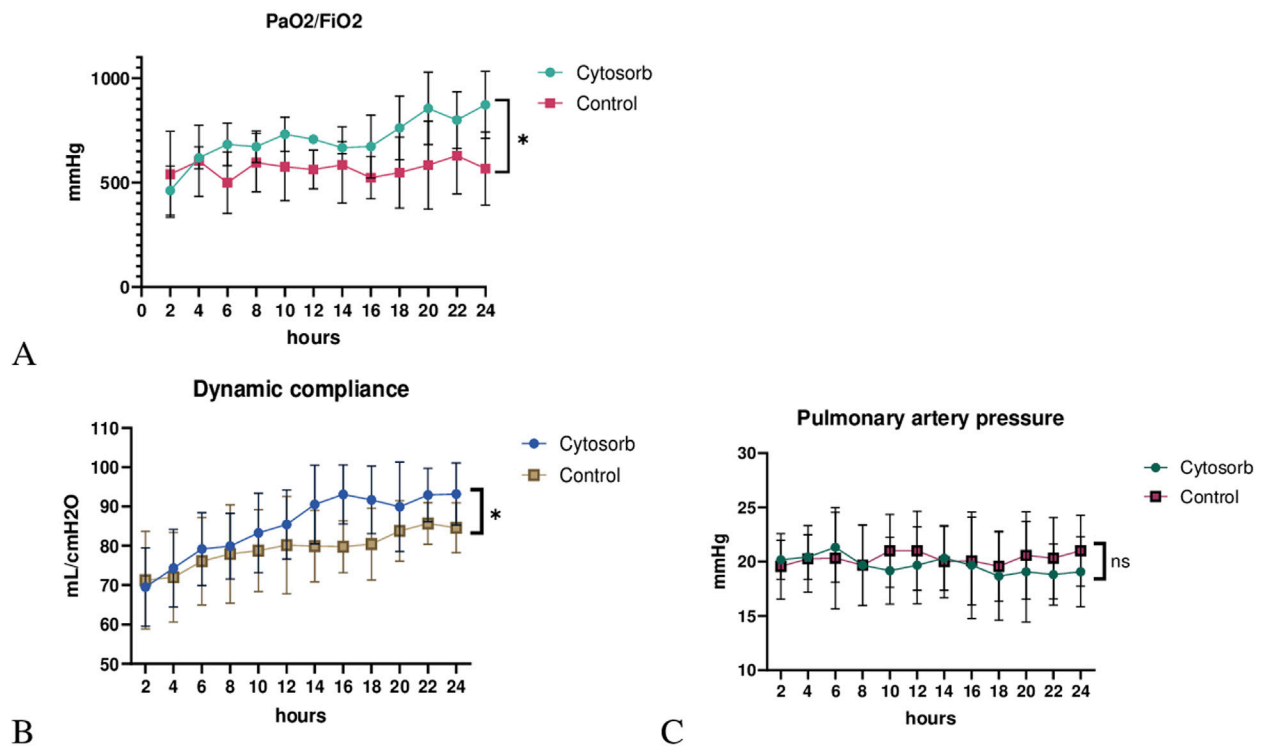


FIGURE 1 | Lung physiology during *ex vivo* lung perfusion. **(A)** The PaO₂/FiO₂ ratio was significantly higher, and **(B)** dynamic compliance was markedly improved in the Cytosorb group compared to the Control group. **(C)** Pulmonary artery pressure (PAP) showed no significant difference between groups. Results are shown as mean ± standard deviation, analyzed using 2-way ANOVA (**p* < 0.05).

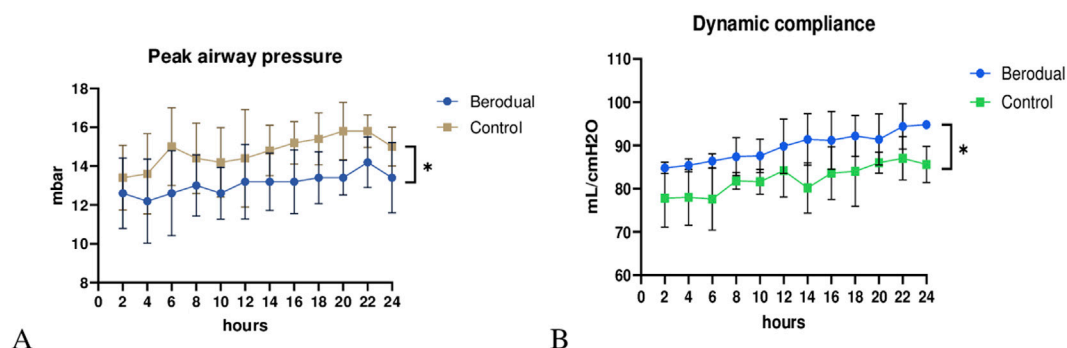


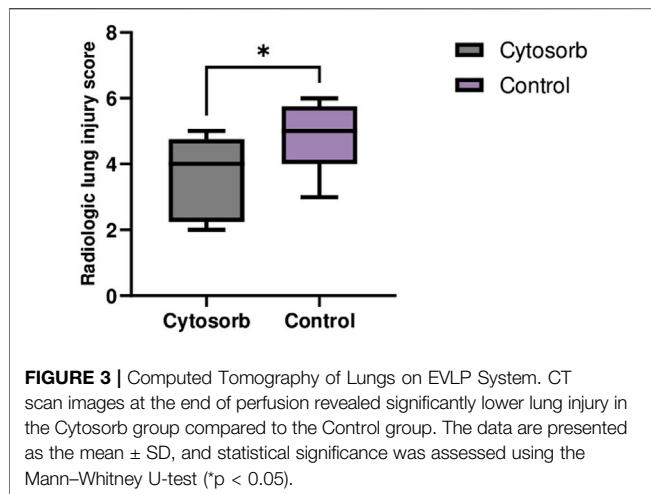
FIGURE 2 | Functional assesment during *ex vivo* lung perfusion. Berodual group had significantly lower peak airway pressure **(A)** and higher dynamic compliance **(B)** than the Control group (**p* < 0.05).

DISCUSSION

The use of EVLP has significantly advanced the field of lung transplantation by providing a platform to assess and optimize donor lungs before transplantation. Functional parameters such as oxygenation capacity, pulmonary vascular resistance, and lung compliance during EVLP have been shown to correlate with post-transplant graft function. Studies have demonstrated

that lungs meeting EVLP criteria for transplantation exhibit favorable early post-transplant outcomes, including lower rates of primary graft dysfunction and improved short-term survival [18].

In this study, we explored the use of an ECMO-based system as an alternative platform for EVLP. Unlike conventional EVLP circuits, which rely on custom-built perfusion setups and specialized equipment, the use of an ECMO system offers



several potential advantages for donor lung preservation, including simplified circuit assembly, clinical familiarity, widespread availability, and integration into transplant logistics.

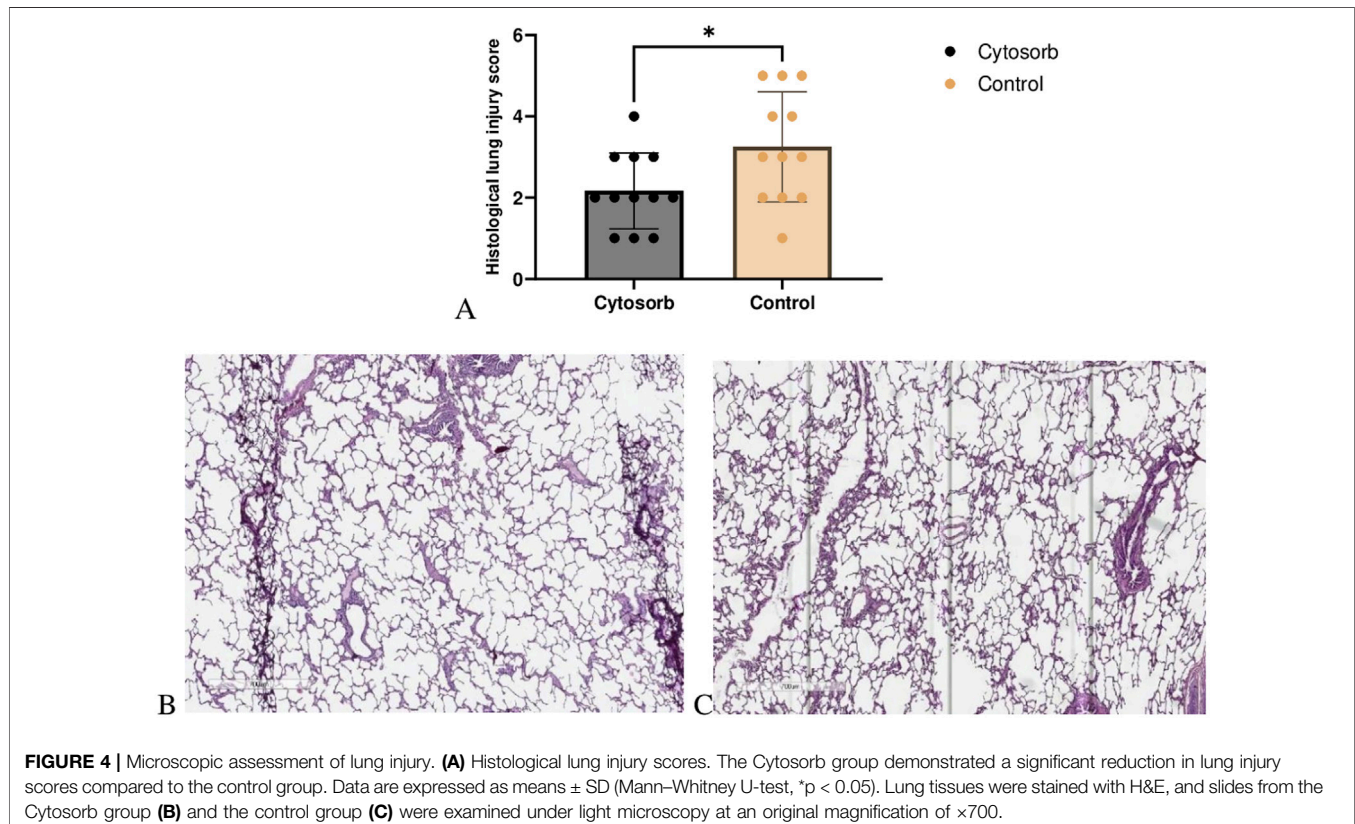
While the effects of adjunctive therapies such as Cytosorb adsorption and bronchodilator administration have been reported in the literature, the application of these interventions in an ECMO-based *ex vivo* perfusion model represents a novel approach. Our findings suggest that this system could serve as a reliable alternative to traditional EVLP

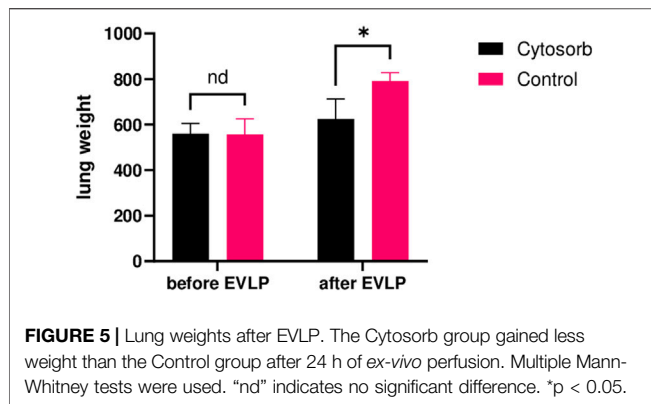
machines and may contribute to expanding the donor lung pool by improving preservation strategies.

Cytokines, signaling proteins released during IRI, intensify inflammation by attracting leukocytes to the injury site [19]. Elevated levels of cytokines, particularly interleukin-6 (IL-6) and interleukin-8 (IL-8), in bronchoalveolar lavage fluid immediately after lung transplantation, are associated with poor short-term and long-term graft outcomes [20]. During *ex vivo* lung perfusion (EVLP), increased IL-8 levels have been correlated with the development of PGD [21]. EVLP offers a valuable platform for reducing ischemia-reperfusion injury by allowing therapeutic interventions during lung evaluation and treatment [22].

The findings from this study highlight the potential of combining advanced technologies, such as the Cytosorb cartridge to improve outcomes during EVLP. The Cytosorb group exhibited superior lung mechanics, as evidenced by a significantly higher $\text{PaO}_2/\text{FiO}_2$ ratio and improved dynamic compliance. These outcomes align with previous research with acellular perfusate indicating the efficacy of cytokine filtration in mitigating inflammation and reducing lung injury during EVLP [23]. Clinically, cytokine filtration has demonstrated the potential to improve oxygenation and reduce the incidence and severity of PGD following lung transplantation [24, 25].

Another promising approach to attenuate IRI during EVLP involves the use of β_2 -adrenoreceptor agonist inhalation [26]. β_2 -adrenergic receptors (β_2 -AR) are densely distributed throughout the lungs and play a critical role in regulating various cellular





functions. In airway smooth muscle, β_2 -AR activation induces bronchodilation by increasing cyclic adenosine monophosphate (cAMP) levels. In the alveolar epithelium, β_2 -AR stimulation enhances ion transport, aiding in the clearance of excess alveolar fluid. Furthermore, activation in type II alveolar cells promotes surfactant production, which is essential for maintaining lung compliance [27]. In endothelial cells, β_2 -AR activation regulates nitric oxide-dependent vasodilation and reduces vascular permeability [28]. Additionally, β_2 -AR stimulation in mast cells suppresses airway inflammation by inhibiting histamine release [29].

Anticholinergic agents, which inhibit muscarinic receptors, also contribute to pulmonary protection. In addition to bronchodilation, these agents have demonstrated protective effects in experimental models of lung IRI. They mitigate oxidative stress [17], reduce neutrophil migration to sites of inflammation [16, 30], and downregulate matrix metalloproteinase-9 (MMP-9) activity [16], suggesting potential benefits beyond their primary role in bronchodilation.

Reconditioning lung grafts with nebulized β_2 -adrenomimetics during EVLP has shown promising results. Studies in canine models indicate that inhalation of β_2 -AR agonists during EVLP significantly reduces lung injury [26] and improves post-transplant

graft function [31]. However, the combined use of β_2 -adrenomimetics and anticholinergic agents during extended EVLP remains unexplored.

Our study demonstrates that whole blood based perfusate with continuous ultrafiltration for functioning lung in machine perfusion can be straightforward and efficient for at least 24 h. Additional cytokine removal had led to significant improvement of organ quality (morphological and functional) over 24 h of EVLP. By the end of EVLP, perfusate sodium concentrations were in physiological levels in contrast to the study of Iskander et al [23].

During this study we did not observe neither metabolite accumulation, nor electrolyte imbalance in perfusate. There are critical thresholds of these parameters as the primary driver of the deterioration in compliance over EVLP have been established in previous works [9]. All groups demonstrated stable electrolyte, metabolic and immunological homeostasis. The latter effect was more pronounced in immunomodulation (Cyto-Sorb) group in terms of weight gain, lung injury scores by CT and microscopic assessment, lung physiology.

Overall, our results strongly support significant morphological and functional benefit of continuous ultrafiltration in combination with cytokine removal and bronchodilation during 24 h EVLP. These effective tools enable future therapeutic possibilities for extended criteria donation, long term organ transportation, and even organ banking.

Limitations

One of the limitations of our study was the 24 h limited EVLP. Further studies looking at metabolism, immunomodulation are needed to extend perfusion significantly beyond 24 h. Our current study did not test combining bronchodilators with Cytosorb, but we recognize its potential clinical significance. Future research should investigate the synergistic effects of this combination during EVLP to determine whether it enhances lung compliance, reduces inflammation, and ultimately improves post-transplant graft function and success rates. Another limitation was absence of LTx after EVLP. Although our study does not include post-transplant follow-up, the functional

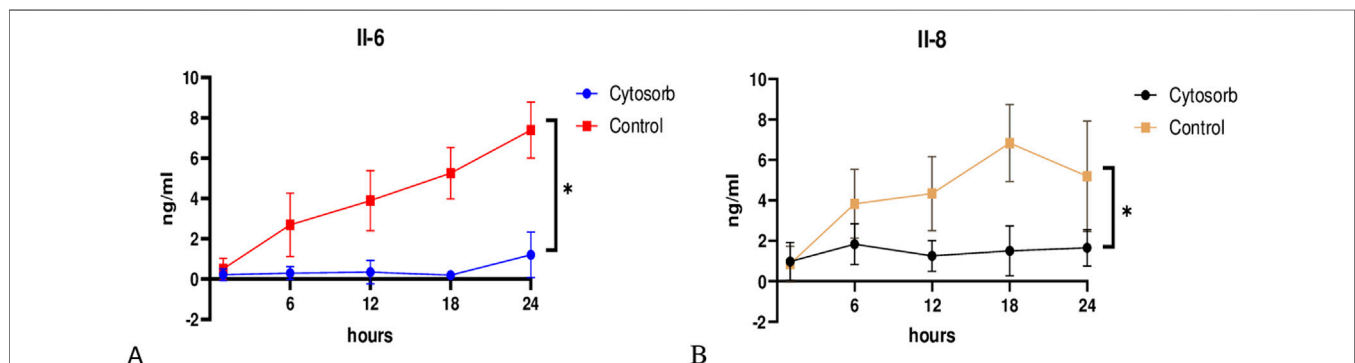


FIGURE 6 | Cytokine profile during EVLP. Cytosorb group significantly reduced interleukin IL-6 (A) and IL-8 (B) levels compared to the control group throughout the perfusion. Data are presented as mean $\pm$ standard deviation. Two-way analysis of variance (ANOVA) with * $p < 0.05$ was used for comparisons.

viability observed during EVLP suggests a strong potential for successful graft performance *in vivo*. Future research incorporating LTx and post-transplant follow-up is needed to validate these findings further and enhance the clinical relevance of EVLP as a reliable predictor of post-transplant outcomes. Our group is constantly progressing on this issue.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was approved by National Research Cardiac Surgery Center's Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

LF - concept/design, YZ - data collection, AT - approval of the article and to data analysis/interpretation, AK - data collection, ZN - data analysis/interpretation, critical revision of article, data collection, RK - data collection, AS - data collection, data analysis/

interpretation, TL - data analysis/interpretation, critical revision of article, and YP - critical revision of article, approval of article.

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CONFLICT OF INTEREST

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Ex-Vivo Heart Perfusion Machines in DCD Heart Transplantation Model: The State of Art

Chiara Tessari*, Giovanni Lucertini, Mariangela Addonizio, Veronica Geatti, Daniela Bacich, Nicola Pradegan, Assunta Fabozzo, Roberto Bianco, Giuseppe Toscano, Vincenzo Tarzia and Gino Gerosa

Cardiac Surgery Unit, Cardio-Thoraco-Vascular and Public Health Department, Padova University Hospital, University of Padua, Padova, Italy

The Donation-after-Circulatory-Death (DCD) heart transplantation program increases donor pool but resulting in more serious ischemic-related myocardial injury (IRI), leading to higher incidence of primary graft dysfunction (PGD). *Ex-vivo* machine perfusion (EVMP) for DCD heart is being considered a useful aid in improving grafts number and quality assessment, aiming to better outcomes. In this review we will analyze the role of EVMP techniques in the context of DCD with special attention to their clinical aims and results and future perspectives. A review of available clinical and pre-clinical studies involving EVMP with DCD donation model was performed. Thirty-four original articles about preclinical studies were found. First studies were designed to evaluate graft function in DCD hearts after EVMP, while recent research focus on possible therapies that could be associated with EVMP. Twenty-one original articles about clinical studies were found with the Organ-Care-System (TransMedics) as MP used. Outcomes, such as survival rates or rejection episodes, are comparable to outcomes from donation-after-brain-death. EVMP in the setting of DCD heart transplantation can be a valid tool for organ preservation and transport. The role of pre-clinical research will be crucial to reduce IRI, achieve organ reconditioning and reduce incidence of PGD.

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*Correspondence

Chiara Tessari,
✉ chiara.tessari@unipd.it

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Keywords: heart transplantation, donation after cardiac death, ischemia and reperfusion injury, *ex vivo* heart perfusion, *ex vivo* heart preservation

BACKGROUND

According to most recent worldwide guidelines, heart transplantation (HT) remains the gold standard for treatment of patients with end-stage heart failure (ESHF) [1, 2]. Nevertheless, the discrepancy between organ availability and request is responsible for an unacceptable high awaiting risk mortality [3]. According to the OPTN/SRTR 2021 Annual Data Report, pretransplant mortality rate in US is still 8.6 deaths per 100 patient-years [4]. HT candidates are becoming significantly older, with high levels of morbidity and thus being often bridged to transplantation with mechanical circulatory support [5, 6]. In addition, donor characteristics are changing towards older donors with a worse risk factors profile,

Abbreviations: CSS, Cold Static Storage; DBD, Donation after brain death; DCD, Donation after circulatory death; ESHF, end-stage heart failure; EVHP, *ex-vivo* heart preservation; HMP, hypothermic MP; IRI, ischemia-reperfusion injury; MP, Machine Perfusion; NMP, normothermic MP; NRP, normothermic regional perfusion; PGD, primary graft dysfunction; VA-ECMO, veno-arterial extracorporeal membrane oxygenation.

resulting in a higher incidence of poorer organ quality, especially in European countries [7]. This increases the need of accepting marginal organs for marginal candidates [8].

Multiple strategies aimed to increase donor pool and improve procured graft quality are being explored, as shown in **Table 1**. In addition to increasing the absolute number of viable organs, the main etiopathological target of these techniques and technologies is to reduce total ischemia time, the burden of ischemia-reperfusion injury (IRI) and primary graft dysfunction (PGD) [9, 10]. In particular, technologies called *ex-situ* or *ex-vivo* heart preservation (EVHP) technologies, allow the preservation of donor hearts outside the body, and can be used to assess the donor organ as well as to reduce total ischemia time.

On the other hand, it has been estimated that the use of Donation after Circulatory Death (DCD) hearts could increase the donor pool by approximately 30%–48% [10–13]. The DCD heart transplantation program is already a reality: it consists in using grafts from patients who do not fulfill brain death criteria but have no hope of recovery. In these situations, organ procurement occurs after circulatory arrest. This creates an inevitable phase of warm ischemia [absent in donation after brain death (DBD)] resulting in more serious myocardial IRI, thereby leading to higher incidence of PGD [14–17]. The lack of evidence pertaining best practices in different steps of DCD heart procurement makes it an evolving field. As a matter of fact, in 2020 DBD still represented 97% of donor organs used in the US [4]. *Ex-situ* machine perfusion (MP) for DCD heart is being considered a useful aid in improving grafts number and quality assessment, aiming to better patients’ outcomes. Optimal strategies for cardiac graft assessment are still under evaluation [18]. In this review we will analyze the role of EVHP techniques in the context of DCD with special attention to their clinical aims and results and future perspectives.

METHODS

A review of available studies involving EVHP in human and animal procured with DCD donation model was performed. PubMed was searched to select studies related to this topic. The search terms were: (Transpl*[TIAB] AND (*Ex-vivo* [TIAB] OR *Ex-situ* [TIAB] OR Xvivo [TIAB] OR OCS [TIAB] OR Organ Care System [TIAB] OR perfus* [TIAB] OR preserv*[TIAB]) AND (myocard*[TIAB] OR cardia*[TIAB] OR Heart [TIAB]) AND (DCD [TIAB] OR circula* [TIAB])). The last search was performed up to January 2024. Two authors independently assessed the qualification of the references. A third review author resolved any disagreements

between the 2 review authors when necessary. The full text of the retrieved articles and their references were assessed to identify if the studies were adequate to the objective of this review. Statement of Institutional Review Board and Ethics board approval, and Statement of Human and Animal Rights were also checked before inclusion of the study.

A relevant study in this review met the following inclusion criteria: an original study on any kind of DCD with unique data, conducted with EVHP of the grafts in both clinical or preclinical settings, full text written in English only and reported details on the system used and on outcomes. If overlapping data were presented in different publications from the same data source, only the most detailed and relevant articles were included. Abstracts from meetings and other forms of publication were excluded.

Pre-Clinical Studies Results

Supplementary Table reports the results of the review on original pre-clinical studies involving EVHP in the context of DCD. A total of 34 original articles were identified from 2013 to 2023, involving 774 DCD hearts perfused with MP after procurement. The overall study objectives can be classified into three categories.

- Primarily graft evaluation
- Primarily protocol optimization
- Primarily therapeutic approaches for reconditioning.

Historically, early studies aimed to evaluate graft function in DCD pre-transplant settings. The main objectives of these studies were to assess cardiac viability and performance following a period of warm ischemia, as well as to identify valid indicators of graft injury/quality. Through organ reperfusion, the goal was to identify criteria that could distinguish grafts amenable to heart transplantation from those that were not. The first reported study, dating back to 1997, utilized a custom-made MP device with an erythrocyte-enriched perfusate solution to investigate the effects of normothermic ischemic insults of varying durations [19]. In this study, coronary flow response during reperfusion was considered a key parameter for assessing recovery of power output. Subsequent studies identified additional parameters reflecting organ damage and predictive of myocardial function quality, such as direct hemodynamic or echocardiographic parameters, in particular systolic function, diastolic function, left-ventricular end systolic pressure-volume relationship, left ventricular maximal developed pressure, preload recruitable stroke volume, isovolumic relaxation constant [20–45], histological markers [23, 24, 26, 27, 29, 31, 32, 38–41, 45–47], vascular and microvascular analysis [25–27, 32, 48, 49], laboratory markers indicating metabolism, oxidative stress, etc. of whom the most and easiest used was lactate level [21–24, 30–33, 38–43, 45–48, 50–55] and gene and protein expression [26, 43, 44, 55]. These early studies laid the groundwork for the second category of studies, which focused on the optimization of the primary graft preservation protocol. These studies aimed to identify the most effective organ preservation protocol, including optimal preservation temperature, cardioplegic solution type, and enrichment of the solution with erythrocytes, albumin, etc.

TABLE 1 | Strategies for increasing donor pool and improving procured graft quality.

Donor selection	Graft management
Marginal donors	Graft preservation
Marginal hearts	Pre-transplant graft evaluation
Optimization of allocation policies	Pre-transplant graft “therapy”
Long distance procurement	
DCD (Donation after circulatory death)	

TABLE 2 | Comparison of perfusion strategies and clinical outcomes in human studies using EVMP in DCD.

Study Design	Study Groups	Perfusion Time (min)	Total Preservation Time (min) total time out of body	Perfusate used Cardioplegia used	Myocardial functional assessment	Outcomes	Article highlights	Reference
Case series	3 DCD	254 min	352 min	Standard OCS perfusate	Lactates trend	30 days survival: 100% (n = 3) moderate cellular rejection: 66.6% (n = 2) Post-operative ECMO: 66.6% (n = 2)	<i>First successful clinical DCD heart transplantations with donor organs procured at a distance necessitating reanimation, resuscitation, and transportation with use of an ex-vivo cardiac perfusion device</i>	[56]
Retrospective single-center	13 DCD, 9 transplanted (all donor hearts were transported on the OCS, except for a single heart, where, uniquely, the donor and the recipient were within the same hospital) 20 DBD	205 min	functional WIT of 17.3 min (range, 11–21 min) before cardioplegic infusion. After cardioplegia, an additional 23–28 min of ischemia was encountered while explanting and instrumenting the heart on the OCS before blood reperfusion	Standard OCS perfusate St Thomas' cardioplegic solution 4 C	Lactates trend	100% survival rate 2/9 requiring MCS (22.2%) No episodes of rejection		[57]
Case series	2 DCD OCS with long-term LVAD support	patient 1 360 min patient 2,307	cold ischemic time 13 min + WIT 13 min cold ischemic time 12 min + WIT 21 min	Standard OCS perfusate 1L Custodial cardioplegic solution (4C) + 10,000 UI heparin +2500 UI of erythropoietin +50 mg glyceryl trinitrate	Lactates trend	30-day survival: 100% (n = 2) PGD incidence 0% Post-operative ECMO: 0%	<i>Successful DCD short-term outcomes, despite adverse donor and recipient risk factors, including bridge to transplantation with an implantable LVAD.</i>	[58]
Retrospective single-center	DCD OCS (n = 33/45 DCD heart retrievals), 12 hearts did not progress to circulatory arrest within 30 min from WLS >33 Hearts Retrieved >23 Hearts transplanted +10 Hearts not transplanted (n = 2 OCS failure, n = 8 inadequate recovery)	276 ± 67 min	81 ± 33 min (cross clamp time)	Standard OCS perfusate Cardioplegia St. Thomas' solution + glyceryl trinitrate (100 mg/L) + erythropoietin (5,000 U/l)	Lactates trend, subjective evaluation of ventricular contractility	Overall survival was 95% at 1 month (n = 22) MCS 39,1% (n = 9) PrimPGD 4.3% (n = 1) Antibody-mediated rejection within the first 12 months of transplant was DCD 0.2 ± 0.7 vs. DBD 0.1 ± 0.4 episodes (p > 0.1); acute cellular rejection (ISHLT 2R), DCD 0.8 ± 1.6 vs. DBD 1.0 ± 1.6 episodes (p > 0.1) A total of 7 patients had early acute kidney injury (30%), 4 of whom required short-term hemodialysis (17%)	<i>Despite a higher requirement for MCS for delayed graft function, primarily in recipients with ventricular assist device support, overall survival and rejection episodes are comparable to outcomes from contemporary brain-dead donors</i>	[59]
Retrospective single-center	50 DCD heart offers >39 were declined, 3 donors did not proceed to cardiac arrest within the protocol specified time period 8 DCD hearts were retrieved with the OCS, 7 successfully transplanted	median 263 min IQR (242–296)	34 min (IQR 31–39 min). WIT	Standard OCS perfusate Leucocyte depleting filter Cardioplegia: 500 mL St Thomas crystalloid cardioplegia +50 mg glyceryl trinitrate +2,500 units of erythropoietin	Lactates trend	30-day survival 100% 90-day survival rate was 86% Postoperative MCS with an intra-aortic balloon pump was required in 2/7 (29%) cases and ECMO was required in 3/7 (43%)		[60]
Retrospectively matched, observational cohort study single-center	A. 128 potential DCD donors 78% (100) suffered from cardiac arrest within 4 h –75/100 underwent OCS (18/75 declined)	NRP median 181 IQR (153–200)	warm ischemic time NRP 23 min (21–28)	Standard OCS perfusate Cardioplegia: 500 mL of St. Thomas's solution +2,500 IU of	Lactates trend	DCD vs. DBD no difference in 30 days survival (97% DCD vs. 99% DBD, p = 1.00) or 1 year (91% DCD vs. 89% for, p = 0.72) no difference in post-operative	<i>During OCS Langendorff coronary perfusion system allows the heart to beat but not eject and is therefore incapable of a functional assessment</i>	[12]

(Continued on following page)

TABLE 2 | (Continued) Comparison of perfusion strategies and clinical outcomes in human studies using EVMP in DCD.

Study Design	Study Groups	Perfusion Time (min)	Total Preservation Time (min) total time out of body	Perfusate used Cardioplegia used	Myocardial functional assessment	Outcomes	Article highlights	Reference
	–25/100 underwent TA-NRP (3/25 declined; 19/25 OCS; 3/25 CSS) –79/100 DCD heart transplantation B. 164 DBD heart transplantations were undertaken, with 50% of heart transplantations performed; CSS preservation	DPP median 251 IQR (221–294)	DPP 36 min (32–43)	erythropoietin +50 mg of glyceryl trinitrate cold crystalloid cardioplegia		ECMO, (15% DCD and 6% DBD for PGD ($p = 0.12$)) no difference in number of treated rejection episodes within the first year after transplantation DPP vs. TA-NRP All recipients in the TA-NRP group remained alive for over the 5 years of the program (survival 100%). For recipients in the DPP group, 30-day survival was 96% ($p = 1.00$), and 1-year survival was 86% ($p = 0.15$). Survival was comparable between the 2 retrieval techniques Similar rates of any postoperative MCS (37% vs. 26%, $p = 0.58$). 18% of DPP and only 5% TA-NRP heart recipients required ECMO support for PGD after HTx ($p = 0.27$)		
Retrospective single-center	DCD OCS ($n = 49/69$ DCD heart retrievals); 17 (35%) were declined >32 of the retrieved hearts (62%) were finally accepted for transplantation	281 ± 68 (no ECMO) 306 ± 60 (ECMO)	cold ischaemic time (29 ± 5) + (281 ± 68) + warm ischaemic time (24 ± 6) Cold ischaemic time (27 ± 6) + (306 ± 60) + warm ischaemic time (23 ± 3)	Standard OCS perfusate cold crystalloid St Thomas' cardioplegic solution containing 100 mg/L of glyceryl trinitrate and 5,000 Units/L of erythropoietin	Lactates trend	At 5 years of follow-up, the 1-, 3-, and 5-year survival was 96%, 94%, and 94% for DCD hearts compared with 89%, 83%, and 82% respectively for DBD hearts Immediate post-implant requirement for ECMO support for delayed graft function 31% ($n = 10$); no difference in rejection rates when compared with standard criteria DBD hearts	Higher lactate values (>5 mM) were acceptable as long as there was a down-trending lactate level with an arteriovenous differential in favor of lactate metabolism as an energy source rather than its accumulation indicating signs of myocardial injury	[61]
Case series	Patient 1 DCD Patient 2 DCD	276 241	46 (total cross clamp time) 69 (total cross clamp time)	Not specified	Ex-vivo coronary angiography	Not specified	First reports of successful transplantation of DCD allografts following ex vivo coronary angiography for the exclusion of significant CAD. External assessment of the coronary arteries alone may have resulted in the exclusion of otherwise viable organs for transplantation. This presents itself as a potentially organ-sparing procedure	[62]
Retrospective single-center	6 DCD + OCS 9 DBD + CSS	280	warm ischemic time was 28.5 min	1,500 mL of donor blood + heparin 500 mL of cold crystalloid cardioplegia	Lactates trend, subjective evaluation of ventricular contractility	1-year survival 100% Significant rejection during in-hospital-stay: DCD 2 (33.3%), DBD, 0 (0.0%) ($p = 0.143$) Need for renal replacement therapy: DCD 1 (16.7%), DBD 2 (22.2%) ($p = 1.00$)	DCD heart transplant can be performed safely with excellent short-term survival in children; no significant difference in major outcomes compared to a DBD cohort	[63]

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TABLE 2 | (Continued) Comparison of perfusion strategies and clinical outcomes in human studies using EVMP in DCD.

Study Design	Study Groups	Perfusion Time (min)	Total Preservation Time (min) total time out of body	Perfusate used Cardioplegia used	Myocardial functional assessment	Outcomes	Article highlights	Reference
Retrospective single-center	1237 DCD procurements met inclusion criteria (normothermic MP 109, CSS 1128)	Not specified	Not specified	standard OCS perfusate	Not specified	Discharge LV systolic dysfunction grade (mild): DCD 1 (16.7%), DBD 3 (33.3%) (p = 0.6) Discharge RV systolic function grade worse than mild: DCD 1 (16.7%), DBD 1 (11.1%) (p = 1) no difference between groups in median total WIT (24.0 min vs. 24.0 min, p = 0.89), but the MPH group demonstrated shorter median operative WIT (circulatory arrest to cross-clamp; 8.7 min vs. 10.9 min, p = 0.003) Total actual hearts yield 79; total expected mean yield 3.86; ratio (95%CI): 20.45 (20.45-20.45); p = 0.000 Overall organ yield of DCD heart donors was observed to be 33% higher than expected (O:E 1.33; 95% CI: 1.22-1.45)	<i>MPH use in DCD procurements does not lead to delays in WIT and does not negatively affect organ yield of other concurrently procured organs</i>	[64]
Retrospective single-center	31 DCD OCS 16 DBD OCS 38 DBD cold storage		Cold ischemic time DCD-OCS 94.5 ± 11.0 vs. DBD-OCS 89.2 ± 13.6 vs. DBD-CSS 127.3 ± 63.6; p < 0.050	Standard OCS perfusate	Not specified	6-month survival DCD-OCS 96.8% vs. DBD-OCS 100% vs. DBD-CSS 94.7%; p = 0.637 Post-op MCS: 51.6% DCD vs. 31.3% DBD OCS vs. 15.8% DBD CSS; p < 0.050 PGD: 19.5% DCD-OCS vs. 0% DBD-OCS vs. 5.3% DBD-CSS; p < 0.050 6-month Significant rejection >2R: DCD-OCS 25.8% vs. DBD-OCS 18.8% vs. DBD-CSS 34.2%; p = 0.489 Dialysis at time of discharge: DCD-OCS 6.5% vs. DBD-OCS 0% vs. DBD-CSS 2.6%; p = 0.493	<i>There were no differences in cardiac MRI findings between the three transplant types, including presence of gadolinium hyperenhancement after transplant (all p > 0.050)</i>	[65]
Case report	1 DCD	423	WIT 24 min	1.2 L of donor blood been passed through a leucocyte filter with 500 mL of TransMedics Priming Solution containing buffered electrolytes + mannitol, multi-vitamins, antibiotics, albumin, steroids amino acids, dextrose-insulin, and low-dose adenosine 1L of cold del Nido cardioplegia	Lactates trend	Not specified	<i>Expanding the donor acceptance criteria to include more distant donor locations and enrolling recipients with extended criteria (e.g., heart retransplantation) is feasible</i>	[66]

(Continued on following page)

TABLE 2 | (Continued) Comparison of perfusion strategies and clinical outcomes in human studies using EVMP in DCD.

Study Design	Study Groups	Perfusion Time (min)	Total Preservation Time (min) total time out of body	Perfusate used Cardioplegia used	Myocardial functional assessment	Outcomes	Article highlights	Reference
Retrospective multicenter	157 DCD wit TA-NRP 673 DBD	Not specified	14.9 ± 7.6 (SD)	Not specified	Lactates trend	Survival similar in the taNRP group when compared to DBD. 30-day survival 96.8% ([92.5%–98.6%] 95% CI, n = 156), 1-year survival 93.2% ([87.7%–96.3%] 95% CI, n = 72) and 5-year survival 84.3% ([69.6%–92.2%] 95% CI, n = 13) 12.8% DCD patients required postoperative MCS after transplantation 12.7% for DBD group (p = 1) 7% experienced acute rejection warranting treatment The association between using either CSS or MP in patients receiving taNRP and survival did not differ significantly between groups (HR = 0.33 [0.06; 1.76], p = 0.196) The use of MCS early after surgery differed significantly between the two groups (p = 0.0311). 10.3% CSS required MCS (n = 14.0), vs. 28.6% in MP (n = 6)	<i>taNRP offers an effective method of organ preservation and procurement</i>	[67]
Retrospective multicenter	128 DCD 103 retrieved 74 transplanted (23 declined) 297 DBD	ECMO for PGD 309 ± 56 no ECMO 276 ± 56	ECMO warm + cold ischemic time 48 ± 11 no ECMO warm + cold ischemic time 58 ± 13	standard OCS perfusate approximately 1.2–1.5L of donor blood before the administration of cardioplegia. tirofiban in addition to heparin to prevent leucocyte filter clotting 1L of cold crystalloid cardioplegia	Lactates trend and subjective evaluation of ventricular contractility	1- and 5-year survival of DCD HTx recipients was 94% and 88%, comparable to that of a contemporary cohort of DBD: 87% and 81% (p-value non significant) The requirement for ECMO for severe primary graft dysfunction (sPGD) occurred in 12 of 74 (16%) of cases overall. ECMO requirement was significantly lower in the contemporary cohort (4/51, 8%) compared to the initial cohort (8/23, 35%) (P = 0.0064) Patients that required ECMO for sPGD also had a significantly higher cardiopulmonary bypass time compared to those who did not; 254 (228–301) versus 158 (132–183) min, respectively (P < 0.0001) incidence of PGD: 8% (4/51) global dysfunction of the heart was observed during early postoperative period, probably resulting from long perfusion of the donor heart, and the fact that the organ was harvested from a marginal donor. Therefore, a decision was made to initiate central ECMO. The recipient had two revisions due to bleeding, and was successfully weaned from ECMO on the third day	<i>Survival is comparable to that of traditional BD donors; asystolic warm ischemic time has an important role to play in initial organ dysfunction; tirofiban is a safe addition to the blood collection protocol and helps reduce filter clotting, and, poor lactate profiles on NMP may indicate an underlying pathology</i>	[13]
Case report	1 DCD, patient with a left ventricular assist device	Not specified	17h 03 min (total cross clamp)	Not specified	Lactates trend			[68]

(Continued on following page)

TABLE 2 | (Continued) Comparison of perfusion strategies and clinical outcomes in human studies using EVMP in DCD.

Study Design	Study Groups	Perfusion Time (min)	Total Preservation Time (min) total time out of body	Perfusate used Cardioplegia used	Myocardial functional assessment	Outcomes	Article highlights	Reference
Retrospective multicenter	A. 68 OCS B. 51 Conventional	381 min	115 min pre e post OCS ischemia 228 min ischemic non OCS	standard OCS perfusate	Lactates trend	Grade 1R rejection was observed according to endomyocardial biopsy, but the patient was hemodynamically stable, so he did not require additional treatment Similar 30 days survival (A: 92.4% vs. B: 90.2%; $p = 0.745$) postoperative MCS (%) (A: 25.0 vs. B: 39.2; $p = 0.112$) and postoperative dialysis (chronic) (%) (A: 4.4 vs. B: 27.5; $p < 0.001$) were numerically better in OCS, without any difference in the occurrence of early rejection (<1R A: 23.5; B: 25.5, $p = 0.83$)	OCS heart allowed safe transplantation of surgically complex recipients with excellent 1-year outcomes, despite long preservation times and unfavourable donor characteristics	[69]
Unblinded, randomized, controlled trial, multicenter	90 DCD 80 transplanted 90 DBD 86 transplanted	Not specified	Not specified	standard OCS perfusate cold crystalloid del Nido cardioplegia solution (containing Plasma-Lyte A, mannitol, magnesium sulfate, sodium bicarbonate, potassium chloride, and lidocaine)	Lactates trend	Risk-adjusted 6-month survival in the as-treated population was 94% (95% confidence interval [CI], 88–99) among DCD, as compared with 90% (95% CI, 84–97) among DBD (least-squares mean difference, –3 percentage points; 90% CI, –10 to 3; $P < 0.001$ for noninferiority [margin, 20 percentage points]) No substantial between-group differences in the mean per-patient number of serious adverse events associated with the heart graft at 30 days after transplantation PGD moderate 6% DCD vs. 5% DBD; severe 15% DCD vs. 5% DBD.	Risk-adjusted survival at 6 months after transplantation with a donor heart that had been reanimated and assessed with the use of extracorporeal nonischemic perfusion after circulatory death was not inferior to that after standard-care transplantation with a donor heart that had been preserved with the use of cold storage after brain death	[70]
Retrospective, single center	122 DCD 21 OCS 101 TA-NRP followed by CSS 263 DBD 10 OCS 253 CSS	Not specified	Not specified	Not specified	Not specified	No significant differences between groups in 1-year survival (94% DCD vs. 92% DBD, $p = 0.50$), incidence of severe PGD (6% DCD vs. 6% DBD, $p = 0.99$), treated rejection during the first year (13% DCD vs. 18% DBD, $p = 0.21$), or likelihood of cardiac allograft vasculopathy at 1 year after transplantation (15% DCD vs. 14% DBD, $p = 0.96$)	In the largest single-center comparison of DCD and DBD HTx to date, outcomes among DCD recipients are noninferior to those of DBD recipients	[71]
Retrospectivemulticenter	215 potential DCD hearts were offered of which 98 (46%) accepted 57 (27%) donor hearts retrieved and 50 (23%) DCD transplanted	258 (216–306)	warm ischemic time 28 (24–34) + cold ischemic time 13 (9–19)	standard OCS perfusate St Thomas' cold crystalloid + erythropoietin + glyceryl trinitrate	Lactates trend	No difference in the 30-day survival rate between DCD and DBD (94% vs. 93%) or 90 days survival (90% vs. 90%) respectively 1 year survival 84% DCD vs. 84% DBD, $p = 0.91$		[72]

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TABLE 2 | (Continued) Comparison of perfusion strategies and clinical outcomes in human studies using EVMP in DCD.

Study Design	Study Groups	Perfusion Time (min)	Total Preservation Time (min) total time out of body	Perfusate used Cardioplegia used	Myocardial functional assessment	Outcomes	Article highlights	Reference
	179 DBD					Higher rate of ECMO use post-DCD heart transplants compared to DBD (40% vs. 16%, $p = 0.0006$) Hemofiltration 60% DCD vs. 45% DBD, $p = 0.08$ Treated rejection episode in 30 days 8% DCD vs. 14% DBD, $p = 0.41$; in 90 days 9% DCD vs. 24% DBD, $p = 0.06$ Among 558 DCD procurements, recovery occurred in 89.6%, and 92.5% of recovered hearts were utilized for transplant NRP was also associated with higher odds for heart utilization after recovery compared with DPP (OR, 3.79; 95% CI, 1.40-10.24; $P = 0.009$)	NRP procurements have a higher yield for DCD heart transplantation compared with direct procurement and perfusion	[73]
Retrospective UNOS registry	558 procurements 65% DPP (363) 25% NRP (195)	Not specified	Not specified	Not specified	Lactates trend			

BVAD, Biventricular Assist Device; CAD, coronary artery disease; CPB, Cardio-Pulmonary Bypass; CSS, cold static storage; CRRT, continuous renal replacement therapy; DBD, donor after brain death; DCD, donor after circulatory death; ECMO, ExtraCorporeal Membrane Oxygenation; HTx, Heart transplant; IABP, Intra Aortic Balloon Pump; LVAD, Left Ventricular Assist Device; LV, left ventricle; MCS, Mechanical Circulatory Support; NA, not applicable; NRP, Normothermic Thoracoabdominal Regional Perfusion; OCS, Organ Care System; PGD, Primary graft dysfunction; RV, right ventricle; WIT, warm ischemic time. At the moment the only clinical approved MP, is OCS (Organ Care System).

Additionally, these studies sought to identify organ evaluation metrics that could predict organ quality for transplant use. Various parameters were identified, including laboratory indices of myocardial damage, oxidative stress, histological markers such as tissue edema and electron microscopy alterations, and the maintenance of adequate echocardiographic or hemodynamic parameters.

Progressive advances in this field shifted research focus from graft evaluation to potential therapies that could be associated with MP to preserve myocardial function and oxygen consumption, reduce ischemia-reperfusion injury (IRI) mechanisms, and facilitate organ reconditioning. Among the therapeutic approaches explored were the use of CytoSorb filters [43], IL-11 [45], methylprednisolone [40], Intralipid [50], autologous mitochondrial transplantation [41], melatonin [42], the NLRP3 inflammasome inhibitor Mcc950 [55], and HSP90 inhibitors [44]. In the early studies, the rat model was predominantly used, whereas later, more advanced translational studies employed porcine models.

Human Studies Results

Table 2 presents the results of the review on original human studies involving EVHP in the context of DCD. We identified 21 original articles from 2013 to 2023, involving 866 DCD hearts perfused with MP after procurement and subsequently transplanted. A total of 268 DCD hearts were declined for transplantation. All studies used the OCS Heart as the sole MP device in controlled DCD heart transplantation settings. Initially, the purpose of using MP was to reduce ischemia time in distantly-procured transplantations or in recipients with adverse risk factors, such as those on ventricular assist device support [13, 59, 61, 65].

Parallel to these studies, parameters were examined that characterize DCD grafts in comparison to DBD settings [57, 62, 64, 66, 67, 69–71] or between DCD grafts preserved via cold storage versus those preserved using MP [12, 60, 69, 71, 72]. Among the most relevant parameters, Dhital et al. demonstrated a differential role of lactates during OCS in DCD hearts, showing that higher lactate values (>5 mM) in DCD grafts were still acceptable as long as there was a downward trend in lactate levels and an arteriovenous differential favoring lactate metabolism as an energy source, rather than its accumulation, which would indicate myocardial injury [61]. Additionally, studies have been published examining diagnostic insights into DCD organs under MP, such as coronary angiography or cardiac magnetic resonance imaging [62, 65]. Both short- and long-term outcomes, including survival rates and rejection episodes, were found to be comparable to outcomes from DBD donors [13, 56–72].

DISCUSSION

In this review the role of EVHP for heart preservation in DCD clinical settings resulted in evaluation of grafts before HT, extended *ex-situ* preservation of the graft before implantation, and resuscitation of the graft. However, in DCD pre-clinical settings EVHP role becomes deeply investigate biological

mechanisms causing IRI in order to test potential treatment to reduce or prevent it, improving cardiac recovery, and decreasing the risk of PGD or delayed graft failure.

Ex-Vivo Heart Perfusion Technologies

In DCD the inevitable warm ischemia time (time between withdrawal of life support and circulatory arrest) makes the already energy-depleted heart poorly tolerant of additional cold ischemia caused by transport with well-established cold static storage (CSS) [10]. Alternative ways of organ preservation can be preferred in this context to improve patients' outcomes. *Ex-situ* MP can be divided into normothermic MP (NMP; 34°C–37.5°C), hypothermic MP (HMP; 1–8°C) and subnormothermic MP (sNMP; 20°C–33.5°C) [74]. NMP is defined as heart preservation in a beating, unloaded, metabolically active state on a portable device. Currently, the only commercially available option for clinical practice is the Organ Care System (OCS; TransMedics, Andover, MA) [8, 70, 75]. The OCS device has recently been awarded approval by the Food and Drug Administration for use in DCD transplantation. HMP is defined as heart preservation in a non-beating state with low myocardial metabolism and perfused with oxygenated nutrient-rich perfusate. Current available devices are.

- The XVIVO NIHP system (XVIVO Perfusion AB, Göteborg, Sweden), which is still under clinical investigation (ongoing clinical trial),
- The Paragonix SherpaPerfusion Cardiac Transport System (Paragonix Technologies, Cambridge, MA), still not used in clinical practice with experience limited to swine models,
- The LifeCradle system (Organ Transport Systems, Inc, Frisco, TX), which has been studied in human hearts only *in vitro* settings [10].

Subnormothermic MP at the moment is limited to other organs (liver, kidney).

Pre-Transplant Heart Evaluation

One of the main issues in DCD is graft quality evaluation before implantation. During DCD, organs can either be directly procured (Direct procurement and perfusion, DPP) and Thoracoabdominal normothermic regional perfusion (TA-NRP) can be used. The latter consists in using veno-arterial extracorporeal membrane oxygenation (VA-ECMO) to re-perfuse the allograft in the donor immediately after pronouncement of circulatory death, with the goal of allowing the heart to recover from the warm ischemic injury, allowing for a detailed evaluation of cardiac function and pump function *in situ*. Then, the graft is procured if deemed suitable for use [76] and either maintained with *ex situ* MP or with CSS during transport.

If DPP is chosen, the pre-retrieval function is incompletely assessed. In this setting, NMP enables a quantitative heart assessment via measurement of heart rate and rhythm, pump flow, coronary flow rate, aortic pressure, hematocrit, temperature oxygen saturation and arteriovenous lactate levels trend. Nevertheless, ventricular contractility is evaluated only via subjective visual examination – epicardial echocardiography is technically demanding and not standardized – in the setting of an

unloaded heart (as the left ventricle is actively vented during perfusion in resting mode) [10, 17, 75]. A recent systematic review highlighted the role of coronary angiography performed while the graft was already connected to NMP [77]. So far, nine total cases of ex-situ coronary angiography of donor human hearts plus one experimental porcine model have been reported. This technique is particularly useful in DCD because coronary angiography is not always available due to ethical issues and availability in procurement centers [15, 57]. In some institutions even echocardiogram is not routinely available because it is considered an unacceptable antemortem intervention [59].

Pre-Clinical Studies

Although CSS is universally accepted as a safe and effective method of heart preservation, PGD continues to affect approximately 3% of clinical heart transplants performed worldwide, and accounts for 26% of deaths in the first 30 days after transplantation [78]. It has been demonstrated that a continuous perfusion with oxygen and metabolites is a physiological support of aerobic metabolism needed for the maintenance of cell integrity and vital cell functions during the transport period [79–83]. Other potential advantages are myocardial cooling through the native coronary circulation and the ongoing washout of metabolic byproducts resulting in improvement of myocardial preservation and microvascular protection. In CSS settings there is a change in myocardial metabolism and viability: ATP levels are depleted and fail to recover despite reperfusion with all necessary substrates for energy repletion, markers of oxidative injury (MDA), apoptosis (caspase-3), and endothelial dysfunction (ET-1) are induced and are further exacerbated by reperfusion [23, 51, 84]. In contrast, EVHP preserves ATP and maintains baseline tissue pH. As a result, these hearts show a rapid restoration of ATP and shorter period of acidosis during reperfusion, suggesting decreased oxygen debt and improved microvascular recovery with a strong correlation with systolic function [85]. Although EVHP is demonstrated to preserve systolic function, a consistent concern is represented by edema, which may negatively affect post-transplant diastolic recovery [21, 29, 86]. In addition, MP does not evaluate diastolic function.

Pre-Transplant Heart Therapies

NMP has also been considered in experimental settings to prevent IRI in DCD and subsequently leads to a lower incidence of PGD and delayed graft failure. This has been achieved in pre-clinical scenarios by enriching the MP perfusate with specific molecules interfering with core steps of IRI, as the nucleotide-binding oligomerization domain-like receptor pyrin domain-containing 3 (NLRP3) inflammasome [55]. Oxidative stress can also be targeted by adding melatonin to cardioplegia and perfusate during EVHP [42]. Even cardiac gene therapy is emerging as a promising approach. It can be obtained both with addition of siRNA in cardiac NMP or via adeno-associated virus specific gene transfer to myocytes [87]. Target genes are involved in inflammatory and apoptotic signal proteins (such as C3, nuclear factor κ B-p56, caspase-8, and caspase-3). Additionally, a limited number of studies have reported on the use of mesenchymal stem cells during EVHP due to their anti-inflammatory and immunomodulatory effects both by

cell-to-cell contact and by secreting substances [88]. At the moment the focus of these studies is still on feasibility and safety aspects.

To date, more than 50 DCD heart transplants have been performed in Australia and United Kingdom by means of NMP technology with excellent short-term and long-term (4 years) outcomes, comparable with those of DBD transplantation [12, 13, 59].

However, given the high cost of the MP devices themselves, comparative cost analyses are needed to elucidate their cost effectiveness. The OCS-DCD US trial was the first randomized trial comparing DCD heart transplant to DBD standard criteria heart transplant clinical outcomes [75]: to date, 180 patients (90 DCD vs. 90 DBD) were enrolled and transplanted. One year patient and graft survival were greater than 90%, with a higher rate compared with DBD. Unfortunately, incidence of moderate-to-severe ISHLT PGD was around 20% in DCD versus 9.1% in DBD, raising the idea that warm ischemia (even though <30 min) can negatively affect early graft function. Chew et al. observed that in the recovery of hearts for DCD, WIT was a crucial determinant of outcome [58]. The need for post-transplant mechanical circulatory support rises when the asystole to cardioplegia (AP) time exceeds 15 min. The interval between circulatory arrest and AP time became a determinant of delayed graft function and the need for short-term ECMO. The full recovery of DCD hearts after short-term support using ECMO was suggestive of delayed graft function as opposed to PGD. On the other hand, Coniglio et al. showed that there were no differences in cardiac MRI findings realized <60 days from HT between those who underwent DCD transplantation using OCS device, DBD using OCS device and DBD transported via CSS, including presence of gadolinium hyperenhancement after transplant (all $p > 0.050$) [64].

CONCLUSION

To conclude, EVHP in the setting of DCD heart transplantation model can be a valid tool for organ preservation and transport. The role of pre-clinical research will be crucial to reduce IRI, achieve organ reconditioning and reduce incidence of PGD and delayed graft function.

AUTHOR CONTRIBUTIONS

CT designed and directed the study and wrote the proof; GL, MA, and VG collected the data and contributed in writing the proof; DB contributed in writing the proof and revised correct English; NP, RB, GT, and VT supervised the work and approved the proof; GG conceived and supervised every part of the study and approved the proof. All authors approved the final version and give agreement to be accountable for all aspects of the work.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontierspartnerships.org/articles/10.3389/ti.2025.12987/full#supplementary-material>

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Delivery of a Muscle-Targeted Adeno-Associated Vector Via *Ex Vivo* Normothermic Perfusion Is Efficient, Durable, and Safe in a Preclinical Porcine Heart Transplant Model

Krish C. Dewan^{1†}, Jeng-Wei Chen^{1,2†}, Alejandro A. Lobo¹, Ryan T. Gross¹, Chunbo Wang¹, Karla G. Rivera¹, Keely Dieplin Tran¹, Smith Ngeve¹, Violet G. Johnston¹, David Wendell³, Carolyn K. Glass⁴, Amy Evans⁵, Sam Ho⁶, Paul Lezberg⁷, Widler Casy⁸, Marla Bazile⁸, Kruti Patel⁸, Adam S. Cockrell⁸, Carmelo A. Milano¹ and Dawn Bowles^{1*}

¹Department of Surgery, Duke University Medical Center, Durham, NC, United States, ²Department of Surgery, National Taiwan University Hospital, Taipei, Taiwan, ³Duke Cardiovascular Magnetic Resonance Center, Duke University Medical Center, Durham, NC, United States, ⁴Department of Pathology, Duke University Medical Center, Durham, NC, United States, ⁵Perfusion Services, Duke University Medical Center, Durham, NC, United States, ⁶Gift of Hope Organ and Tissue Donor Network, Itasca, IL, United States, ⁷TransMedics, Inc., Andover, MA, United States, ⁸Solid Biosciences, Charlestown, MA, United States

Normothermic *ex-vivo* organ perfusion (EVP) systems not only provide a physiological environment that preserves donor organ function outside the body but may also serve as platforms for *ex-vivo* organ modification via gene therapy. In this study, we demonstrated that a rationally designed muscle-tropic recombinant AAV, AAV-SLB101, delivered to the donor heart during brief normothermic EVP achieves durable cardiac transgene expression out to 90 and 120 days post-transplant in a porcine preclinical model. Moreover, transgene expression was detectable as early as 48 h post-transplant. Histological and MRI analyses of the donor myocardium showed no functional or structural impact on the allograft and no off-target gene expression in the recipient. This work will serve as a critical foundation to inform translational studies with therapeutic transgenes to improve allo-, xeno-, and auto-heart transplant outcomes.

Keywords: adeno-associated virus vector, gene therapy, heart transplantation, *ex vivo* heart preservation, transgene durability

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*Correspondence

Dawn Bowles,
✉ dawn.bowles@duke.edu

[†]These authors share first authorship

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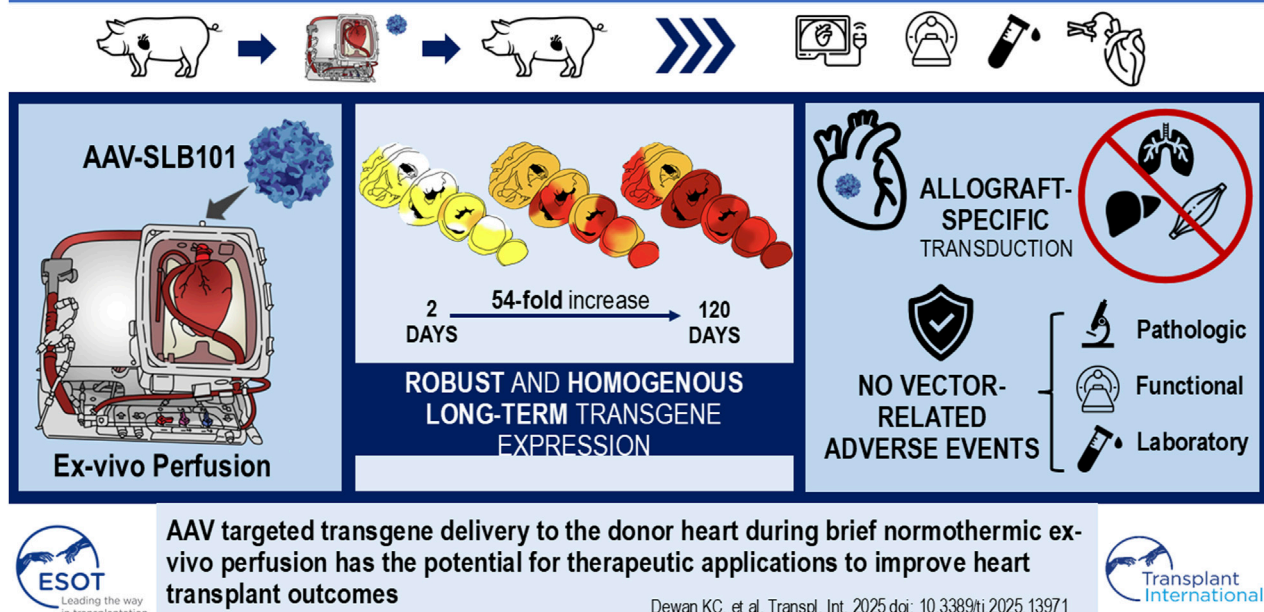
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INTRODUCTION

Heart transplantation (HT) remains the gold standard treatment for patients with advanced heart failure with a median overall survival that exceeds 12 years [1]. Early ischemia-reperfusion injury, primary graft dysfunction, chronic rejection, and debilitating side effects from long-term general immunosuppression remain key limitations. Gene therapy provided to a donor organ

Abbreviations: HT, Heart Transplant; OCS, Organ Care System; EVP, *Ex Vivo* Perfusion; AAV, Adeno-Associated Virus; SLA, Swine Leukocyte Antigen; VGC, Viral genome Copies; CMR, Cardiac Magnetic Resonance Imaging; LA, Left Atrium; SA, Atrial Septum; RA, Right Atrium; LV, Left Ventricle; RV, Right Ventricle; IVS, Interventricular Septum; RLU, Relative Light Units; OCT, Optimal Cutting Temperature compound (OCT); ddPCR, droplet polymerase chain reaction; CBC, complete blood count; CMP, comprehensive metabolic panel; ANOVA, Analysis of Variance.

Delivery of a Muscle-Targeted Adeno-Associated Vector via Ex Vivo Normothermic Perfusion is Efficient, Durable, and Safe in a Preclinical Porcine Heart Transplant Model



GRAPHICAL ABSTRACT

ex-vivo may provide a promising avenue to address these limitations and improve outcomes after HT.

Normothermic *ex-vivo* organ perfusion systems, such as the Organ Care System (OCS™; TransMedics, Inc., Andover, MA), create a physiological environment that perfuses and preserves donor organ function outside the body. While these systems are currently used to decrease ischemic time and facilitate greater organ utilization from longer distances, *ex-vivo* perfusion (EVP) may also serve as a platform for organ modification via advanced therapeutics such as gene therapy [2–8]. The applications of such an approach are numerous, including: i) countering rejection or ischemic-reperfusion injury ii) genetic modification of allogeneic donor hearts to expand the donor pool; iii) genetic modification of pig donor hearts to facilitate xenotransplantation; or iv), autotransplantation after EVP correction of genetic predispositions for advanced heart failure. Importantly, EVP creates an isolated environment for genetic modification of the donor heart, which avoids potential biological complications that could arise in a recipient host from unwanted biodistribution of a heart-specific genetic therapeutic administered systemically.

Adeno-associated viral (AAV) vectors have emerged as the preferred delivery modality for many currently approved gene therapies due to their favorable safety profile, tissue-specific tropism, durable efficacy, and manufacturability [9]. In our previous work using a pig heterotopic HT model, we successfully delivered the firefly luciferase transgene to donor

hearts through EVP with recombinant AAV vectors (SASTG, a myocardial-enhanced AAV3b variant) [8]. At 30-day follow-up, we demonstrated consistent, dose-dependent protein expression and vector DNA in the allograft without off-target effects [8]. It remains unclear how soon after transplantation transgene expression begins and how long transgene expression persists in the allograft.

In this study, we demonstrate robust, early-onset, and durable gene expression from 2 to 120 days after transplantation following delivery of a novel muscle-specific recombinant AAV capsid, AAV-SLB101, in our laboratory's preclinical porcine heterotopic heart transplant model [8, 10–13].

MATERIALS AND METHODS

Animals and Vector

The proprietary AAV-SLB101 capsid, containing a vector expressing firefly luciferase from the CK8 promoter, was obtained from Solid Biosciences Inc., (Charlestown, MA). Vector production is described in the **Supplementary Material**. Female Yucatan pigs (Sinclair Bio Resources, Auxvasse, MO), aged 6–8 months and weighing 20–40 kg, were selected based on matched swine leukocyte antigen (SLA) Class I and Class II genotyping and compatible blood typing. Four pairs of pigs were utilized in this study. This study was approved by the Duke University Institutional Animal Care and Use Committee.

Model of Heterotopic Heart Transplantation and Vector Delivery

The porcine heterotopic HT model has been previously described [8]. The AAV dose used for each donor heart was 1×10^{14} viral genome copies (VGCs). The donor heart was subjected to 2 h of EVP during which perfusate was collected at regular intervals. After EVP, the donor heart was arrested using Del Nido cardioplegia and implanted into the recipient pig's abdomen, with aorto-aortic and pulmonary artery-to-inferior vena cava anastomoses as previously described [8].

Recipient pigs were treated with maintenance immunosuppression, including daily methylprednisolone for 14 days (rapidly tapered from 24 mg/kg twice daily on postoperative day 1 to 1 mg/kg once daily by day 14), daily mycophenolate mofetil 500 mg for 14 days and daily intramuscular injected tacrolimus (0.1 mg/kg to begin titrated from there onward to maintain therapeutic serum trough levels) until termination.

Graft Evaluation

Allograft function was examined twice daily by physical palpation and rated on a four-point subjective scale and point-of-care ultrasound. Both donors and recipients underwent preoperative (3 days prior to surgery) and postoperative (day 12) cardiac MRI (CMR) to assess function, fibrosis, and edema. This was performed via T1 mapping, DE-CMR, and assessment of allograft size and function as described previously [14]. A transcatheter endomyocardial biopsy was performed at 30 and 60 days postoperatively [15]. Blood was drawn at biweekly timepoints for tacrolimus trough levels, laboratory panels (comprehensive metabolic panel, complete blood count with differential, and troponin), and for plasma for future analyses. Plasma was obtained by centrifugation of whole blood at 11,000 rpm and isolation of the supernatant fraction. This was flash frozen in liquid nitrogen and stored at -80°C .

Euthanasia and Terminal Cardiac Tissue Preparation

Under sedation and mechanical ventilation, the allograft and native heart individually received 500 mL of Del Nido cardioplegia and were rapidly explanted into ice cold PBS. Various parts of the allograft and native recipient heart were collected. Each heart was sectioned into five breadloaf-like cross sections labeled 1–5 from base to apex respectively. Section *Introduction* represented both atria. The atria were sampled from three different portions: right atrium (RA), left atrium (LA), and interatrial septum (SA). The ventricular portion was horizontally sliced into four sections from the atrioventricular groove to the heart apex, labeled as ventricular cross sections 2, 3, 4, and 5 (Apex). Ventricular cross sections 2, 3, and 4 were further divided into three portions: anterior wall, middle wall, and posterior wall and were sampled from left ventricle (LV), right ventricle (RV) and interventricular septum (IVS). In total, 31 myocardium

tissue samples from different sections were collected for each heart.

Tissue and Plasma Analysis

Transgene expression and vector transduction of tissue samples from the allograft, native heart, and extracardiac organs were assessed using luciferase activity assays, immunofluorescence staining, and DNA isolation/analysis for vector copy number. Lastly, anti-vector antibody assessment was performed using an ELISA assay. Detailed protocol descriptions of can be found in the **Supplementary Material**.

Statistical Analysis

For descriptive statistics, means and standard deviations were calculated. Comparisons were performed using either Mann–Whitney U, Fisher's exact, and chi-square tests where appropriate. For comparison of continuous variables, Student's t-test or Mann–Whitney U test were used for parametric or non-parametric data respectively. A one-way ANOVA was used for multiple comparisons using Tukey's post-hoc tests. All statistical analyses and graph creation were performed using GraphPad Prism version 10.2.3 (GraphPad Software, San Diego, CA). Statistical significance was defined as $P < 0.05$.

RESULTS

Heterotopic Pig Heart Transplant Perioperative Conditions and Outcomes

The overall study design is summarized in **Figure 1**. Four pigs underwent heterotopic HT. Two pigs encountered perioperative complications (delayed bleeding and primary graft dysfunction) requiring early termination of the experiment at 48 and 24 h respectively. The remaining two pigs survived until the designated study endpoints, with follow-up durations of 90 and 120 days, respectively. The general appearance and activity levels of these two long-term survival pigs were unremarkable throughout the study period. Routine laboratory tests, including complete blood count (CBC) and comprehensive metabolic panel (CMP) remained within normal limits overall (**Supplementary Figures S1, S2**). Troponin was only appropriately elevated in the immediate postoperative period due to surgical manipulation but normalized for the remainder of the study (**Supplementary Figure S7**). Minor elevations in blood urine nitrogen and creatinine in one recipient normalized after reduction of tacrolimus (**Supplementary Figure S2**). There was no evidence of renal impairment or change in urinary output.

Early Peak and Subsequent Decline of Vector DNA in Cardiac Tissue Post-Transplant

The presence of vector DNA in cardiac and extra-cardiac tissues was measured in all pigs using qPCR. To compare the viral genome particles in heart tissues, the atrium, anterior

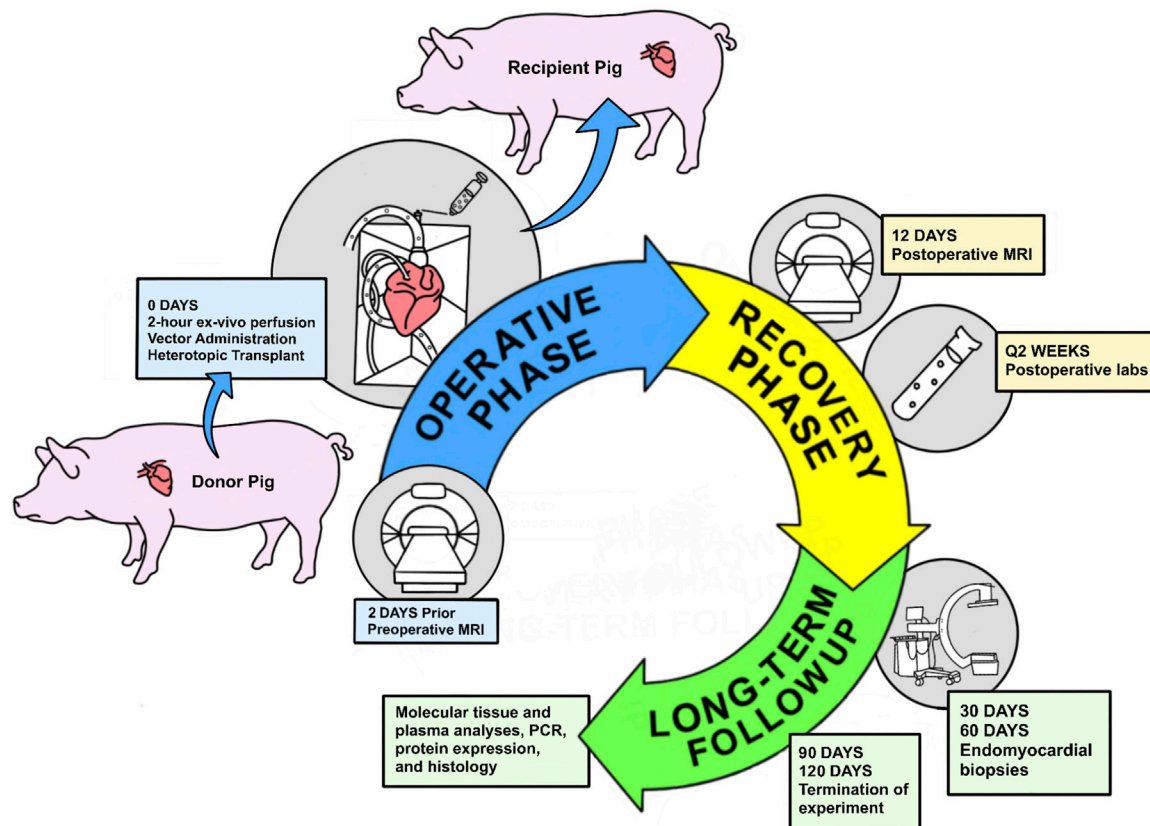


FIGURE 1 | Overview study design of animal experiments and analysis pipeline.

portion of the ventricle, septum, and apex were analyzed (Figures 2A–D). In the allograft, the viral genome DNA copies significantly decreased over time (Figure 2E, $P < 0.001$ by ANOVA, Figure 2G). In the native heart, the average viral genome DNA copies were negligible across different time points (Figure 2F, $P = 0.0382$ by ANOVA). For extra-cardiac organs, most samples had no detectable viral DNA. Only the liver, lung and psoas muscle contained low amounts at the early time points (1512 VGC/ μ g in the lung and 447 VGC/ μ g in the liver at 24 h, 70 VGC/ μ g in liver, and 69 VGC/ μ g in psoas muscle at 48 h). No viral DNA was detected in extra-cardiac organs at 90 and 120 days.

No Change in Vector DNA in the Perfusate Over Time During Ex-Vivo Perfusion

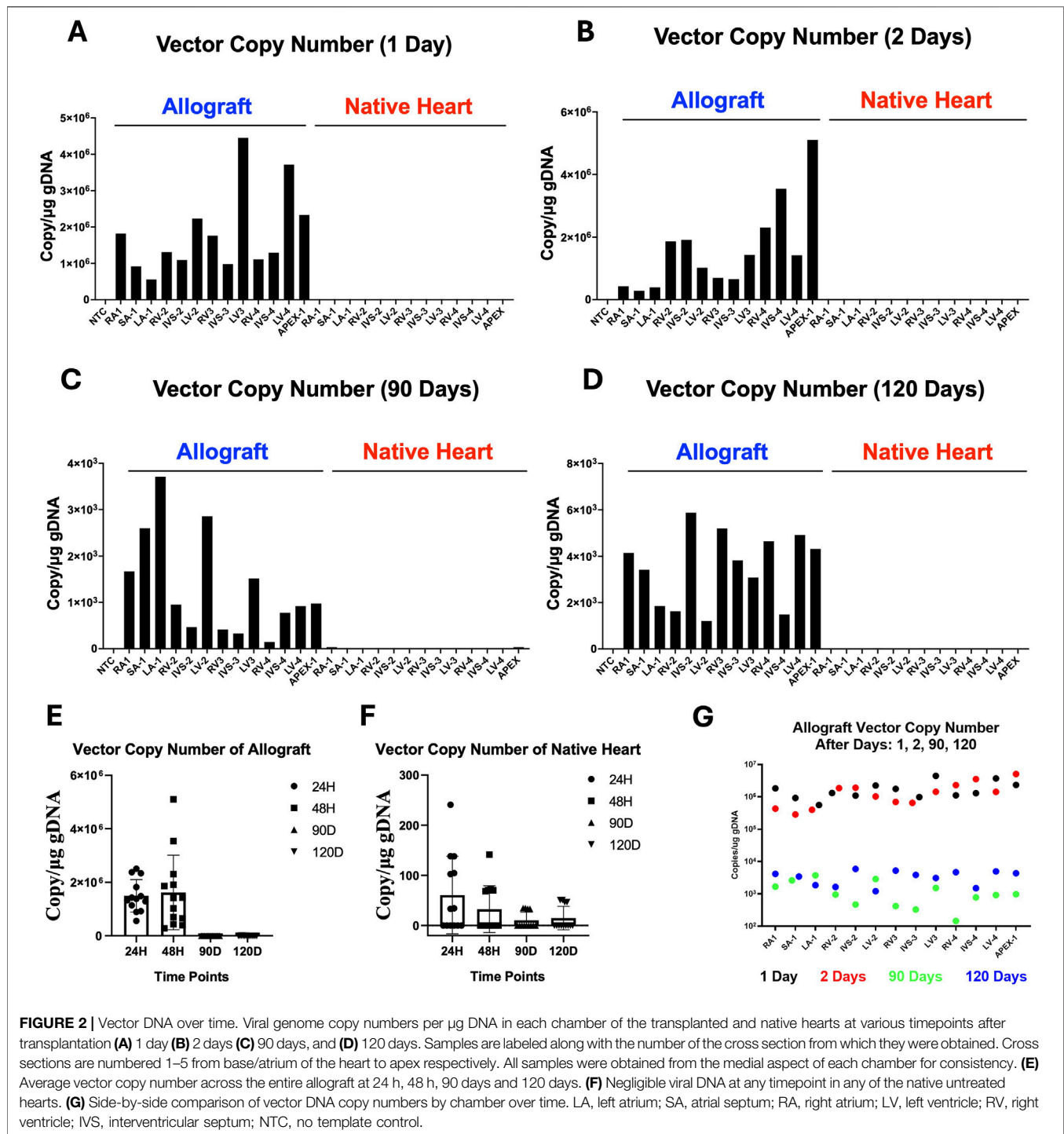
The presence of vector DNA in perfusate from the TransMedics system for all four animals was measured using qPCR. Across all timepoints from 0 to 90 min, there was no significant difference in vector copy numbers ($p = 0.06$; Supplementary Figure S8). A detailed characterization of relevant lab, drug, and timing parameters during the 2 h EVP period are provided in Supplementary Table S1.

Homogenous Durable Transgene Expression and Activity Observed Post-Transplant

Tissue samples from 31 areas of the allograft, including the LA, RA, SA, LV, RV, IVS, and apex, were analyzed for luciferase activity using a luminometer. Complete data sets of individual luciferase activity measurements at different time points and regions of the heart are provided in Supplementary Table S2.

In the allograft of the 24-h pig, no significant luminescence signal was detected in any of the chambers (Figure 3A; Supplementary Table S2). However, in the 48-h pig, enzyme activity was detectable throughout the allograft (Figure 3B; Supplementary Table S2). Immunofluorescence staining of the allograft confirmed the expression of luciferase throughout the allograft, in contrast to the native recipient's heart, which showed no signal on the luciferase assay (Figures 3C–E; Supplementary Figure S4). Allografts examined at 90 days and 120 days post-transplant showed even more robust luciferase activity and immunofluorescence staining (Figure 3E; Supplementary Figures S4, S5).

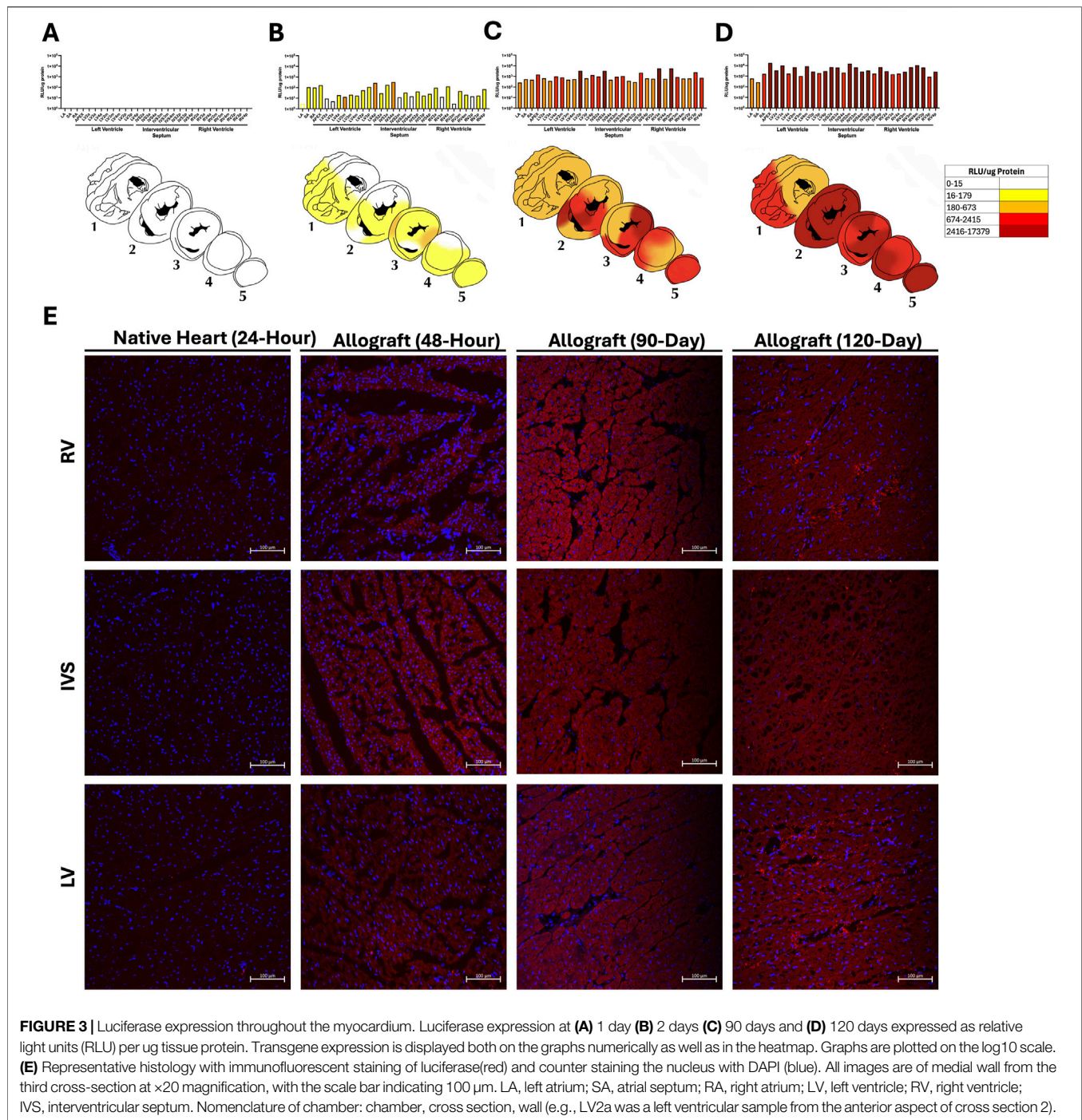
Luciferase activity increased significantly over time, with the luminescence intensity being much higher in the 120-day allograft compared to the 24-h, 48-h, and 90-day allografts



($P < 0.001$ by ANOVA; **Figure 4A**). Luciferase activity increased 54.4-fold in the LV, 334.8-fold in the IVS, 46.5-fold in the RV, and by 11-fold increase in the atria from 2 to 120 days (**Figure 4B**). The same was true of tissues analyzed from the same allograft via endomyocardial biopsies (EMBs) at 30 days and 60 days (septal wall of the RV) along with final collected sections after euthanasia at 90 days (RV wall). The signal intensity at 30 days was 136-fold

stronger than that seen at 48 h ($p < 0.001$; **Figures 4C,D**). Signal intensity was significantly increased at 90 days compared to both 30-day or 60-day timepoints ($P < 0.001$ by ANOVA). There was no statistically significant difference in signal intensity at 60 days compared to 30 days ($P = 0.3124$ by ANOVA) (**Figure 4D**).

The biodistribution of luciferase activity across the allograft was also equal with no significant differences between chambers



of the heart at 48 h ($P = 0.215$, **Figure 4E**). 90 days ($P = 0.73$) or 120 days ($P = 0.10$) (**Figures 4F,G**).

Absence of Off-Target Protein Expression and Demonstrated Safety of Vector Delivery

The recipient's extra-cardiac organs, including the lung, liver, kidney, and psoas muscle, were analyzed for off-target gene expression. The psoas muscle was included due to its proximity to the allograft in this

heterotopic model. Although vector DNA was detected in the liver and psoas muscle of the recipients from the 24-h and 48-h survival studies (**Figure 5B**), both the luciferase enzymatic assay and immunofluorescence staining demonstrated no protein activity or expression in any of the study pigs (**Figures 5A,C**).

To assess the impact of AAV-SLB-101-mediated gene delivery on allograft function, echocardiography, cardiac MRI, and histological analysis of myocardium were performed on the pigs survived to 90 and 120 days respectively.

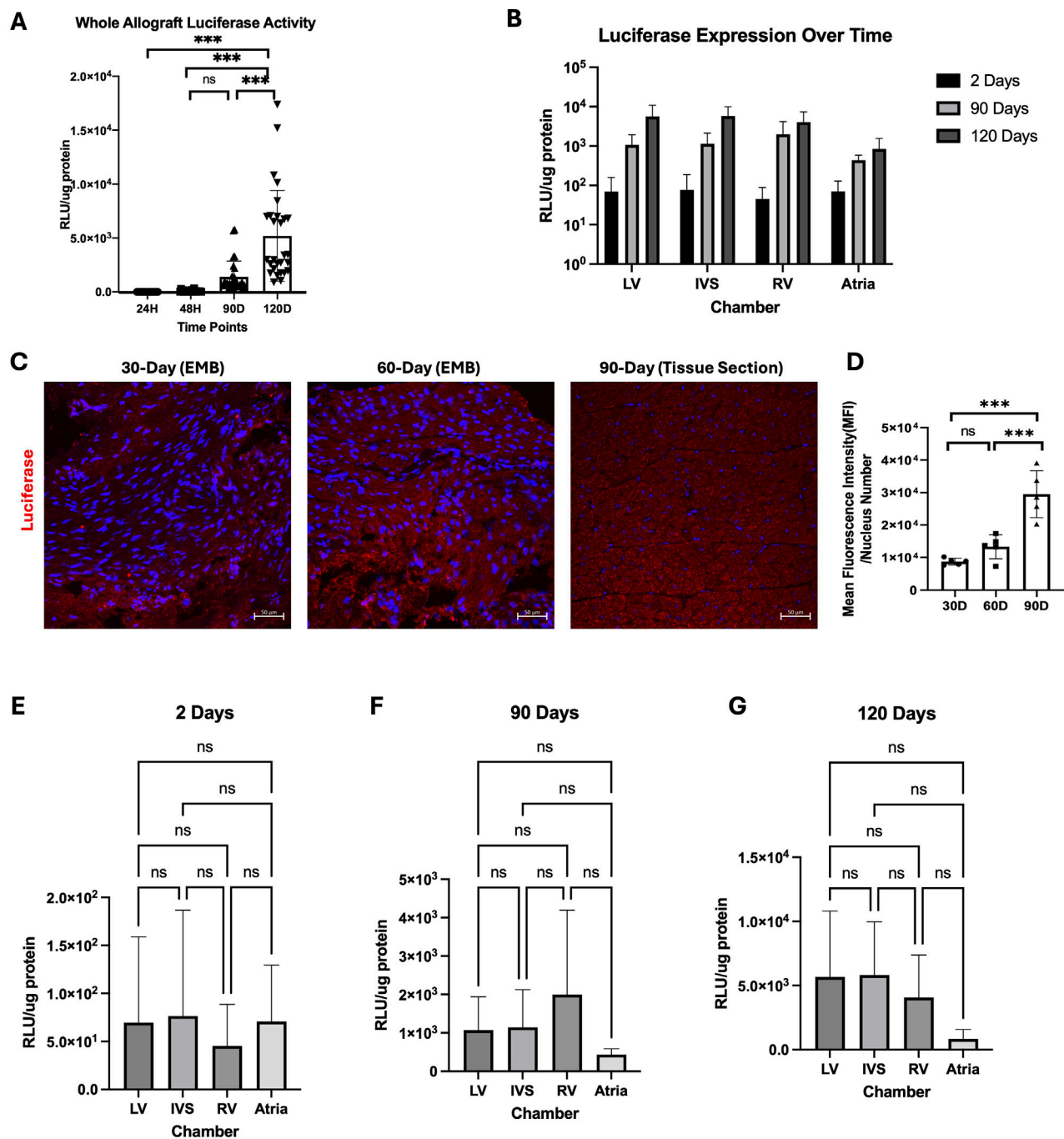
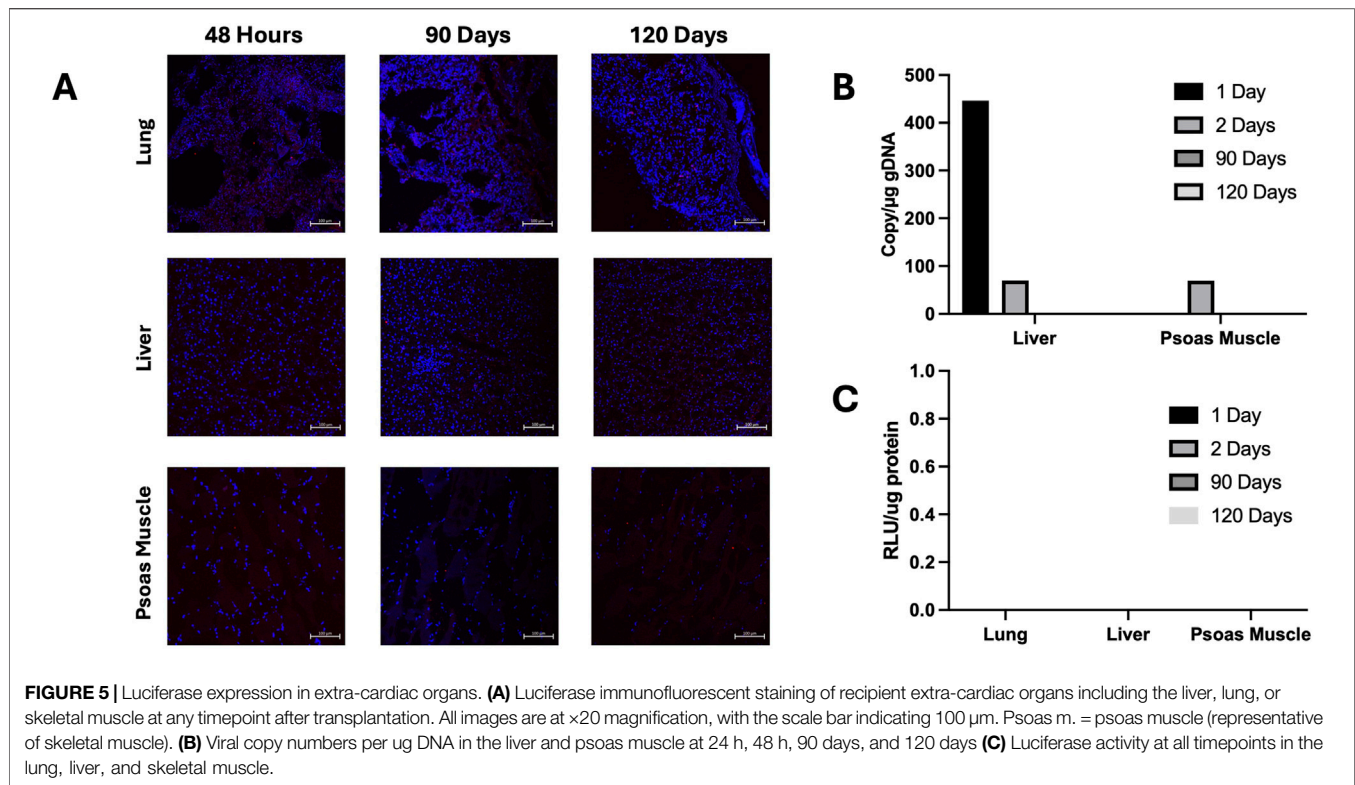


FIGURE 4 | Luciferase biodistribution and progression of expression over time. **(A)** Overall luciferase expression from 1 to 120 days assessed by luciferase activity assay. **(B)** Luciferase expression over time by chamber. Graphs are plotted on the log10 scale. **(C)** Representative luciferase staining of endomyocardial biopsies. **(D)** Mean fluorescent intensity (MFI) per nucleus of luciferase among endomyocardial biopsy samples at 30, 60, and 90 days. Luciferase enzymatic activity across all chambers (atria and ventricles) at all three timepoints from 2 days **(E)** to 90 days **(F)** to 120 days **(G)** post-transplant. RLU values were calculated by pooling values for all samples from each chamber ($n = 3$ atria, $n = 10$ LV, $n = 9$ IVS, $n = 9$, IVS). LV, left ventricle; IVS, interventricular septum; RV, right ventricle.

Echocardiography consistently demonstrated robust allograft contractility throughout the study period. Pre- and post-operative cardiac MRI revealed no signs of fibrosis on delayed-enhancement cardiac magnetic resonance imaging

(DE-CMR) scans and no tissue edema on T1 mapping (**Figures 6A–E**). Additionally, the extracellular volume (ECV) values remained stable before and after the operation (**Figure 6F**). LV mass was calculated by a formula that takes into account



myocardial volume, thickness, and density. Increase in native heart LV mass was commensurate with the growth of the animal itself. Increases in heterotopic allograft mass were more reflective of expected underfilling and resulting “pseudohypertrophy” of the LV rather than a true LV mass increase. Histological examination with H&E staining of the allograft and the recipient organs demonstrated no overt signs of myocarditis, inflammation, rejection, or compromise in structural integrity as reviewed by a board-certified cardiac pathologist (Figures 6G–J; Supplementary Table S4). Additionally, there was neither elevation in cardiac troponin in any recipient, outside of the immediate postoperative period, nor elevation of functional liver laboratory values throughout the study period (Supplementary Figure S7).

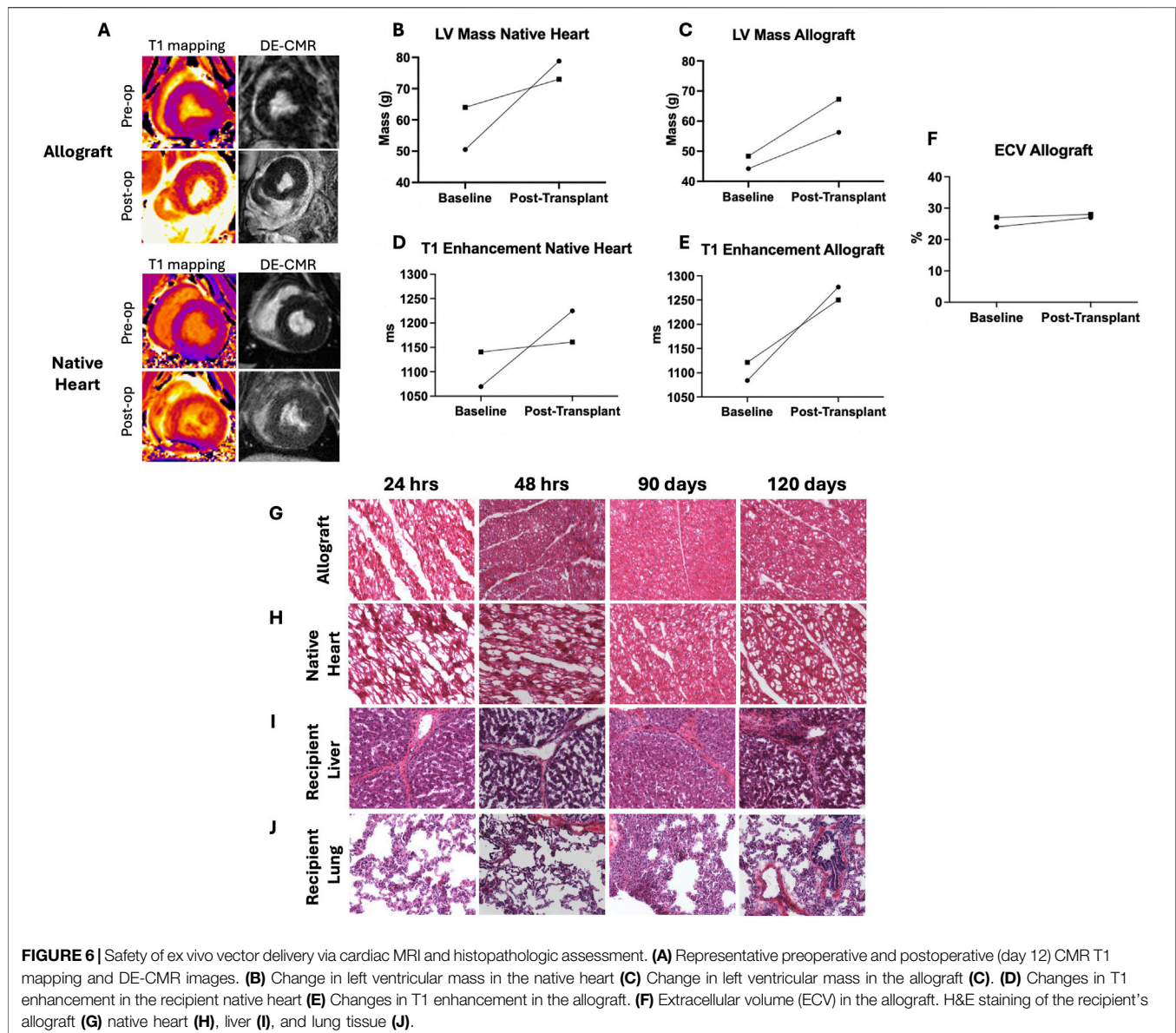
Anti-AAV-SLB101 Capsid Responses Peaked Early and Declined Over Time

The minimally detectable levels of vector DNA in extra-cardiac tissues indicated that minor amounts of vector may leach from the transplanted donor heart into the recipient circulation, which may potentially elicit an antibody response to AAV-SLB101 capsid proteins. The titer of circulating anti-AAV-SLB101 capsid antibodies in plasma of recipient was measured utilizing ELISA (Supplementary Figure S9). There was a 3-fold increase in anti-AAV-SLB101 capsid antibodies in both recipients from baseline to 12 days. For one of the two long-term pigs, the titer declined 46-fold by 120 days to a level comparable to baseline while the other's titer decreased 1.5 fold from peak but remained higher than baseline.

DISCUSSION

This study demonstrated that the muscle-tropic AAV, AAV-SLB101, delivered to the donor heart during a short 2-h normothermic EVP can be effective for achieving early-onset, durable, and targeted cardiac transgene expression without functional or structural impact on the allograft and off-target gene expression. Currently, the Food and Drug Administration (FDA) has approved a total of 14 *ex-vivo* and 29 *in-vivo* gene therapies as safe and effective treatments for genetic disorders [9]. Of these, 89% utilize viral vectors including adenovirus, AAV, herpes simplex virus, γ -retroviral and lentiviral vectors [16]. Among these, AAV vectors have emerged largely as the vector of choice in gene therapies due to their favorable safety profile, durable therapeutic delivery, and precedence for clinical regulatory paths [17]. While a lentiviral vector may allow for longer gene expression to target rapidly-dividing cells (e.g., endothelial cells) or may accommodate larger genetic cargo, cardiomyocytes have very low turnover rates. In addition our proposed therapeutic targets are small enough to be accommodated by an AAV. Therefore, an AAV vector was selected for this study.

There is strong potential for EVP as a platform for gene therapy during organ transplantation [4–11]. *Ex-vivo* administration is attractive in the setting of solid organ transplantation for a number of reasons that we have demonstrated in this preclinical swine model, including: i) quick onset of expression following brief perfusion; ii) homogeneity of transduction throughout the heart; iii)



durability of expression; iv) and mitigation of vector-related adverse events in the recipient due to a near absence of vector load in extra-cardiac tissues. Similar approaches utilizing EVP in preclinical models have been applied to lung, liver, and kidney transplantation [18–26]. Together, these studies support the use of AAV vectors to deliver therapeutics for *ex vivo* donor organ modification before transplant into the recipient with applications including decreasing allograft immunogenicity to mitigate use of general immunosuppressants, prevent ischemia-reperfusion injury, expanding the allogeneic donor pool, and optimizing xenografts for improved transplant outcomes.

AAV-SLB101 was designed with the goal of preferentially targeting and transducing striated muscle cells following systemic administration [12, 13]. In preclinical animal studies, AAV-SLB101 demonstrated effective transduction and biodistribution

to the skeletal muscle and heart and is currently under investigation in a clinical trial for Duchenne muscular dystrophy [12, 13]. We demonstrate, for the first-time, transduction and biodistribution of AAV-SLB101 throughout the swine heart with *ex vivo* perfusion. We demonstrate that transduction during EVP can restrict tropism to the allograft donor heart, and not the representative skeletal muscle (psoas) or native heart from the recipient pig. Luciferase was expressed throughout the transplanted myocardium at all timepoints in all chambers from as early as 2 days to as late as 120 days post-transplant.

Neither the transplanted heart nor recipient organs were subject to vector-related inflammation or myocardial compromise as examined by pathology and CMR assessment. CMR-based myocardial tissue characterization has recently

emerged as a non-invasive method to clinically assess transplanted cardiac allografts in human patients for rejection, inflammation, myocarditis, and fibrosis. In particular, T1 and T2 mapping correlate well with histopathological evaluation and are highly sensitive for detecting fibrosis and edema [27]. Allograft edema (as measured by T1 enhancement) and fibrosis (measured by via delayed-enhancement CMR, DE-CMR) increased comparably between the native heart and the transplanted allograft due to post-surgical changes not related to acute rejection. Indeed, a similar phenomenon is observed clinically [28]. The ECV of the allograft both before and after vector exposure/transplantation remained within the standard expected value of $25.3 \pm 3.5\%$, once again indicating a lack of vector-mediated fibrosis or inflammation [29]. Functional cardiac assessment in this study was limited by the fact that these allografts were transplanted heterotopically. Hence in an unloaded state, the change in ejection fraction or other typical functional assessments are not reliable. However, ECV and T2 mapping correlated with functional measurements such as systolic longitudinal function, peak myocardial velocities and desynchrony [28]. Given the correlation between structural and functional CMR measurements and the lack of changes in structural assessment (e.g., ECV) in our study, it is likely that vector delivery via EVP did not cause any measurable functional changes in the allograft.

Hepatotoxicity is a well-known adverse event attributed to AAV-mediated gene therapies as viral vectors pass through hepatic circulation when provided systemically [30, 31]. Hinderer et al described rapid asymptomatic transaminitis and bilirubin elevation after administration of 2×10^{14} VGC/kg of an AAV9 variant in 2 non-human primate (NHP) animals while one NHP exhibited profound transaminitis, systemic inflammation, disseminated intravascular coagulation (DIC), and shock leading to liver necrosis and death [32]. Similarly, Hordeaux et al. showed that at a dose of 7.5×10^{13} VGC/kg, mature NHPs developed acute liver enzyme elevation, evidence of DIC, and hemorrhage requiring euthanasia [33, 34]. In the current study, there were no elevations in liver enzymes throughout the study, nor evidence of end-organ damage aside from brief tacrolimus-associated nephrotoxicity despite a high dose of 1×10^{14} VGC. By avoiding exposure of the liver to AAV vector, EVP prevented the potential for hepatotoxicity or other associated adverse events despite the high vector dose delivered to the transplanted donor heart. Though some viral DNA was seen in the liver at day one post-transplant, this was negligible compared to the allograft myocardium (liver 400 vs. > myocardium 4×10^6 VGC at day one) and decayed rapidly over the next 24 h. Importantly, levels of vector detected in the liver are several thousand-fold lower than levels anticipated to result in hepatotoxic issues [30–34].

An analysis of viral DNA within the OCS perfusate over the entire period of perfusion demonstrated that the EVP circuit remained saturated with AAV throughout 90 min of EVP at the current dosage. The dosage of AAV used in this study, 1×10^{14} vg, was based on previous AAV3b experiments. Based on these findings, it may be possible to decrease the dosage of AAV-SLB101 while still achieving the same transduction and transgene expression. This adjustment could dramatically lower

manufacturing costs and reduce the risk of AAV-associated immune responses.

We demonstrated persistent luciferase expression that increases over 120 days following heterotopic pig heart transplant. Though pigs were SLA-matched in this study, we utilized triple-regimen immunosuppression for a brief 2-week period to mitigate allograft rejection, the current standard of care for heart transplantation, followed by tacrolimus monotherapy [35]. Host innate and adaptive immune responses to the vector capsid and/or transgene have been reported in a number of preclinical and clinical studies which may negatively impact patient health, vector transduction efficiency, therapeutic durability and dosing strategies [36, 37]. These immune responses and resulting morbidity have led to the adoption of short-term systemic immunosuppressive regimens after AAV-gene therapy in clinical practice.

However, there is no standard immunosuppressive regimen for the suppression of anti-vector responses at present [29]. Corticosteroid regimens as short as 7 days to as long as 133 days have been used in 95% of clinical trials thus far, either alone or in combination with other immunosuppressive agents [38]. Tacrolimus and mycophenolate mofetil (MMF) were both used in 15% of studies. Tacrolimus and MMF are most helpful in mitigating the T-cell dependent acute cellular response that is often responsible for both reduced transgene expression and hepatotoxicity. The lack of detectable myocarditis in the donor hearts at 90 and 120 days support the conclusion that continued use of tacrolimus interfered with an anticipated inflammatory response to the foreign luciferase protein in the allograft.

The effect of pre-existing and/or *de novo* anti-vector antibodies remains unclear at this time. We found an initial increase in anti-vector antibodies postoperatively that subsequently decreased over time. This mirrors what has been seen in clinical trials where increases in anti-AAV antibodies were present regardless of AAV serotypes, administration route, and the presence of an immunosuppressant regimen [38]. The appropriate immunosuppressive regimen to target both cellular and humoral responses has yet to be defined. Despite this, our study demonstrates that transgene expression and overall safety of the recipient are unaffected by humoral responses even when present due to exogenous delivery of the vector to the allograft and a chronic single-agent immunosuppressive regimen.

While transgene expression remained robust and increased over time, the measurable vector DNA present within transduced allografts decreased over time. The inverse correlation between expression and vector DNA may be explained by cellular trafficking of AAV through the cytoplasm. Following cellular entry and transport of AAV particles through the endosomal/lysosomal pathway, vector particles may be either targeted for proteasomal degradation of the capsid in the cytoplasm or transported to the nucleus where capsid uncoating and release of the single-stranded DNA cargo occurs [39]. Ubiquitination of conserved tyrosine residues on the AAV capsid surface targets capsid particles for proteasome degradation, which may leave fewer capsids available to reach the nucleus [31]. Extrapolating proteasome degradation with concomitant nuclear entry of

AAV-SLB101 over time supports our observation that the AAV-SLB101 vector DNA decreases over time as a consequence of proteasome degradation, while vector particles are also entering the nucleus and release the vector DNA payload resulting in increased luciferase expression. Hence it is possible that at the early post-EVP and transplant stage (i.e., day 1 and 2), much of the viral DNA measured is a combination of both cytoplasmic and nuclear vector genomic cargo while at the later time points (day 90 and 120) only the extrachromosomal nuclear DNA remains, thereby supporting increased luciferase expression.

There are still questions that remain unaddressed from this study that could serve as the foundation for future work. Assessment of vector-related markers of inflammation or endothelial activation in the allograft during EVP was not possible due to the confounder of donor-derived inflammatory markers in the perfusate. Using of non-donor derived blood for EVP perfusate in subsequent experiments will facilitate evaluation of this if present. The vector utilized in this study utilized a creatine kinase promoter. Hence, we would not expect it to show transgene expression in any other cells appreciably other than muscle. As a result, we were unable to assess cell-specific transduction within the allograft that are implicated in acute and chronic rejection such as the endothelium and fibroblasts. Our earliest timepoint was 24 h after transplantation. A baseline quantification of VCNs immediately after EVP may be obtained in future experiments by performing a core needle biopsy just prior to transplantation. Whether expression would persist after 120 days or whether there would be an immune response to the transgene in the absence of tacrolimus immunosuppression remains unclear. Removal of immune suppression would also provide a clear understanding of whether transgene expression would continue to increase or plateau and if AAV vector transduction was impacted. The high vector dose used in this study ensured transduction throughout the heart, but optimal effective dose was not determined. Reducing dose can result in enhanced safety and cost savings. Lastly, given the physiological changes associated with the unloaded state of the allograft, the use of a heterotopic model precludes the ability to assess functional changes to the heart. Although out of the scope of this manuscript development of a preclinical orthotopic model would be amenable to building a functional understanding of the allograft.

Conclusion

Despite these limitations, this study demonstrates that utilizing a muscle-tropic AAV, AAV-SLB101, to modify the swine donor heart during normothermic *ex vivo* perfusion is effective, with transgene expression detectable as early as 48 h and persisting for at least 120 days without decay. Histological and MRI analyses showed no functional or structural impact on the allograft and no off-target gene expression in the major organs of the recipient. Future research should focus on refining immunosuppression protocols, exploring the long-term durability of gene expression beyond 120 days, and evaluation of dose-dependent long-term transgene expression. Overall, this approach holds significant potential for enhancing the outcomes of solid organ transplantation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was approved by Duke University Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

Conceptualization, methodology: KD, J-WC, AC, CM, and DB; data acquisition and analysis: KD, J-WC, AL, RG, KR, KT, SN, DW, CG, AE, SH, WC, MB, KP, AC, CM, and DB; figures and table draft: KD, J-WC, DW, and VJ; data interpretation: KD, J-WC, AL, RG, KR, KT, SN, DW, CG, WC, MB, KP, AC, CM, and DB; statistical analysis: KD and J-WC; drafted the final manuscript: KD, J-WC, CM, AC, and DB; reviewed and edited final manuscript: all authors; supervision and oversight: DB and CM. PL provided valuable advice and supplies. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

CM has received stock compensation for serving as a consult for TransMedics Inc. WC, MB, KP, and AC are employees and shareholders of Solid Biosciences Inc. PL is an employee of TransMedics, Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

GENERATIVE AI STATEMENT

The authors declare that no Generative AI was used in the creation of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontierspartnerships.org/articles/10.3389/ti.2025.13971/full#supplementary-material>

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Association of Inflammatory Profile During *Ex Vivo* Lung Perfusion With High-Grade Primary Graft Dysfunction: A Systematic Review and Meta-Analysis

Andrea Costamagna^{1*†}, Eleonora Balzani^{1†}, Matteo Marro², Erika Simonato², Alessandro Burello¹, Mauro Rinaldi¹, Luca Brazzi¹, Massimo Boffini¹ and Vito Fanelli¹

¹Department of Surgical Sciences, University of Turin, Turin, Italy, ²Cardiovascular and Thoracic Department, Azienda Ospedaliero Universitaria Città della Salute e della Scienza di Torino, Turin, Italy

PGD3 is the manifestation of ischemia-reperfusion injury which results from inflammation and cell death and is associated with poor outcome. This systematic-review and meta-analysis of non-randomized controlled trials on patients undergoing Ltx with reconditioned lungs via EVLP, aims to assess the association between the levels of proinflammatory biomarkers during EVLP and PGD3 development within the firsts 72 h post-Ltx. Biomarkers were categorized by timing (1-hour, T0 and 4-hours, Tend from EVLPstart) and by their biological function (adhesion molecules, chemokines, cytokines, damage-associated-molecular-patterns, growth-factors, metabolites). We employed a four-level mixed-effects model with categorical predictors for biomarker groups to identify differences between patients with PGD3 and others. The single study and individual measurements were considered random intercepts. We included 8 studies (610 measurements at T0 and 884 at Tend). The pooled effect was 0.74 ($p = 0.021$) at T0, and 0.90 ($p = 0.0015$) at Tend. The four-level model indicated a large pooled correlation between developing PGD3 at 72 h post-Ltx and inflammatory biomarkers values, $r = 0.62$ ($p = 0.009$). Chemokine group showed the strongest association with the outcome (z -value = 1.26, $p = 0.042$). Pooled panels of inflammation markers, particularly chemokines, measured at T0 or at Tend, are associated with the development of PGD3 within the first 72 h after Ltx.

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*Correspondence

Andrea Costamagna,

✉ andrea.costamagna@unito.it

[†]These authors have contributed equally to this work

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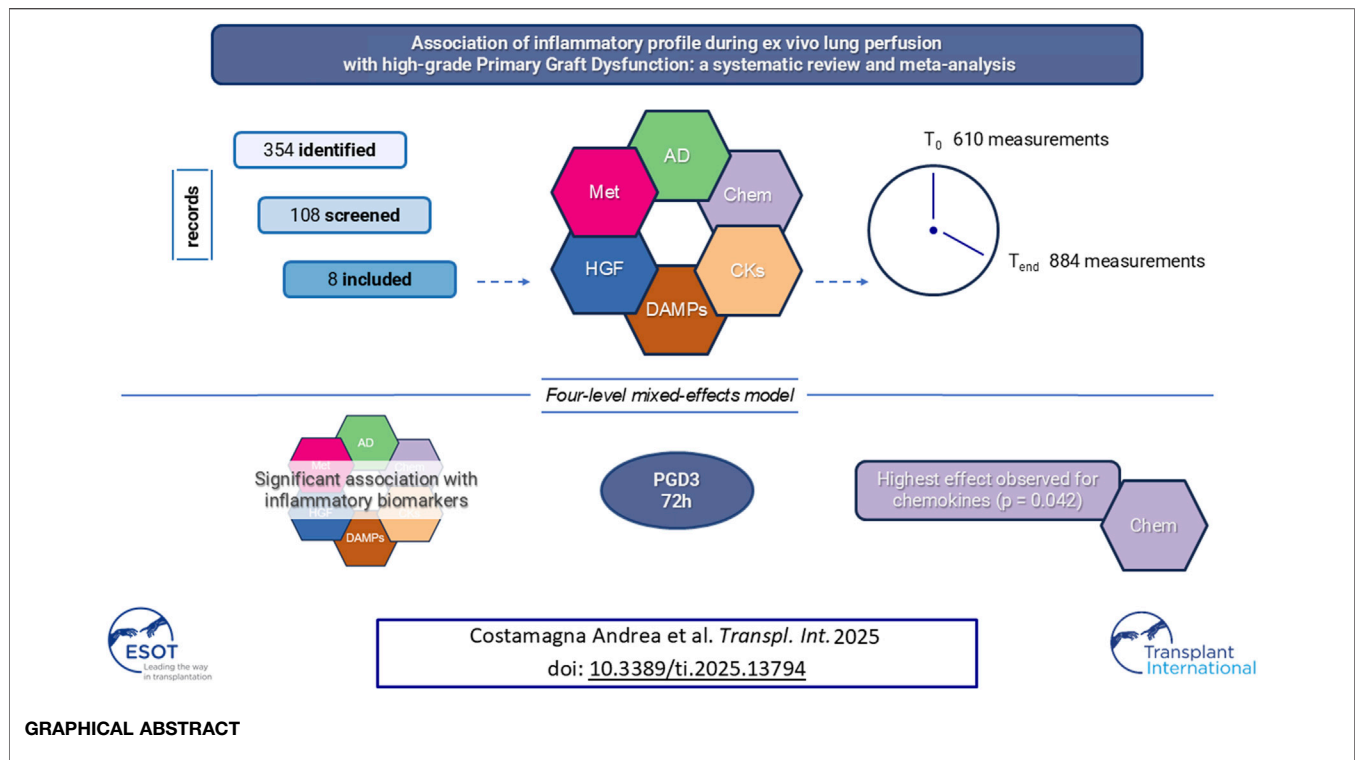
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Keywords: *ex vivo* lung perfusion, ischemia-reperfusion injury, primary graft dysfunction, inflammation, biomarkers

INTRODUCTION

Ex vivo lung perfusion (EVLP) is a well-established platform to assess and potentially treat suboptimal donor lungs to mitigate organ shortage expanding the pool of lungs suitable for transplantation [1]. During 6 h of ventilation and perfusion, conventional clinical parameters such as respiratory mechanics, gas exchange, hemodynamics and radiologic appearance are monitored to assess organ suitability for transplantation. Additional measures and techniques



have been proposed to enhance the accuracy of lung evaluation during EVLP, such as the application of machine learning models to radiographic findings [2], direct lung ultrasound assessment [3, 4] and tissue microdialysis for assessing lung tissue metabolism [5].

However, EVLP lungs may develop primary graft dysfunction (PGD), which is an early form of acute lung injury that occurs during the firsts 72 postoperative hours [6]. Severe form of PGD (namely, grade 3 PGD), characterized by $\text{PaO}_2/\text{FiO}_2 < 200$ mmHg, represents an established risk factor for poor recipients survival [7–9] and bronchiolitis obliterans development [6, 8].

PGD is the clinical manifestation of ischemia-reperfusion induced lung injury (IRI), which results from the complex interplay between inflammation, cell death and leukocyte activation in the donor lung [6, 10]. After lung procurement, damage-associated molecular patterns (DAMPs), such as HMGB1 and nuclear and mitochondrial DNA, are released as a consequence of cell death occurring during cold ischemic time and subsequent rewarming and reperfusion [11–15]. These endogenous molecules stimulate in turn a variety of immune (e.g., macrophages and lymphocytes) and non-immune cells (e.g., lung epithelial and endothelial cells and fibroblasts) to release pro-inflammatory mediators, such as chemokines and cytokines [10, 16]. Both pro-inflammatory mediators and DAMPs promote the expression of adhesion molecules, associated with endothelial activation and consequent leukocyte recruitment, leading to endothelial permeability increase with oedema formation [11, 17–19]. In addition, recent

studies show that point of care protein assay based on IL6 and 8 cytokine levels in lung perfusate has a good accuracy in predicting recipients outcome [20]. To identify lung recipients who at higher risk of PGD 3 development after EVLP and to optimize their treatment, an appreciation for the effects of different classes of biomarkers measured in lung perfusate is relevant. The aim of this systematic review and meta-analysis is to evaluate the association between the levels of pro-inflammatory biomarkers collected at the start or at the end of the EVLP procedure and the development of PGD 3 in the Ltx recipient within the firsts 72 post-operative hours. We hypothesized that different classes of soluble mediators in lung perfusate would exert different effects on PGD3 development in lung transplant recipients.

MATERIALS AND METHODS

This systematic review and meta-analysis of randomized controlled trials (RCTs) and non-randomized controlled trials (NRCTs) was prepared in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement [21]. The Cochrane Handbook for Systematic Reviews of Intervention was chosen as the methodological guidance [22]. The protocol was registered in PROSPERO (PROSPERO #CRD42022296486).

The following databases were used: PubMed, Embase, and Scopus. Searches were conducted for studies published up to 03 August 2023. RCTs and NRCTs published in English, and

Italian were considered eligible for inclusion. Other potentially relevant studies were searched in study registers (i.e., PROSPERO, ClinicalTrials.gov), and in gray literature sources. Specific search strategies were created for each database (**Supplementary Figure S1**).

Each step outlined by the PRISMA flow diagram, along with corresponding lists of included and excluded articles together with their respective justifications, can be found online.¹

Eligibility Criteria

The search was restricted exclusively to RCTs and NRCTs, encompassing participants who satisfied the subsequent inclusion criteria: adult human patients of all genders undergoing Ltx employing reconditioned lungs via EVLP.

The exclusion criteria were: investigations encompassing organ system care (OCS), and studies that reported inflammatory biomarkers at a timepoint preceding EVLP.

Patients who developed PGD with a P/F ratio < 200 mmHg along with radiographic lung infiltrates or requiring extracorporeal life support, are classified as having PGD 3 [23]. Considering the different impact on survival outcomes, we categorized our study population into those with severe PGD (PGD 3) and those with grade 1 or 2 PGD, or no PGD. The primary outcomes were biomarker levels measured in the perfusate at 1 h (T_0) and 4 h (T_{end}) from the start of EVLP. We selected all studies in which specific mediators of inflammation -e.g., Interleukin-8, Interleukin-6, etc.- were quantified in their genotypic or phenotypic expression related to the development of PGD 3 at 72 h.

Selection Process

Search results were collated and exported to EndNote V.X9 (Clarivate Analytics, PA, United States). Duplicates were automatically removed. The review process consisted of two screening levels using Rayyan QCRI online software [24]:

- (1) a title and abstract review
- (2) full-text review

For both levels, 2 authors (AC and EB) independently screened the articles, with conflicts resolved by a third author (VF).

Data were extracted in a planned standardized Excel spreadsheet (*study characteristics, year of the study, type of population, biomarkers detected, timing of measures, and main results*). When the data were not directly available, we used WebPlotDigitizer or directly contacted the authors.

To prevent biased inclusion of data based on the results, the authors decided that 1) where trialists reported both final values and changes from baseline values for the same outcome, final values were recorded; 2) where trialists reported both unadjusted and adjusted values for the same outcome, unadjusted values were extracted; 3) where trialists reported data analyzed based on

the intention-to-treat sample and another sample (e.g., per-protocol, as-treated), data from the former were extracted.

Subgroup Analysis

Biomarkers were divided into subgroups basing on their biological function and mechanism of action in specific inflammatory pathway. Six categories were identified: adhesion molecules (sE-selectin, sICAM, vCAM, ET-1, Big ET-1), chemokines (IL-8, MCP, GRO α , MIP-1a, MIP-1b), cytokines (IL1 β , IL6, TNF α), damage-associated molecular patterns (M30, HMGB, nuDNA, mtDNA), growth factors (M-CSF, G-CSF) endogenous metabolites produced during inflammatory phenomena (CO and NOx).

Study Risk of Bias Assessment

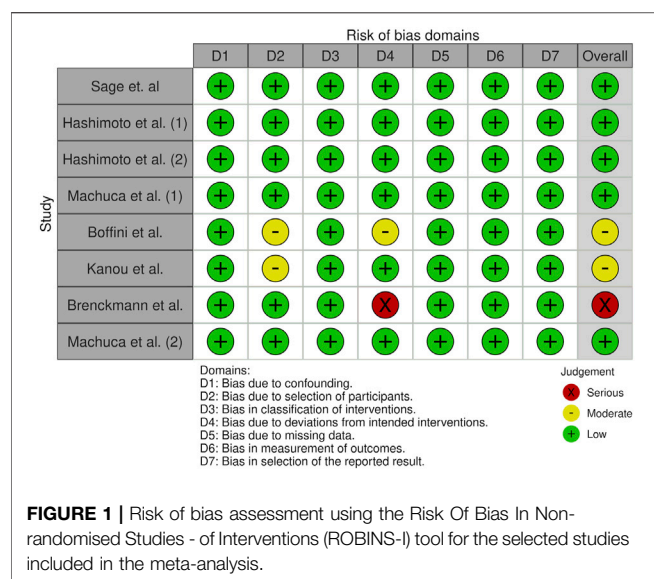
Two authors (AC and EB) independently assessed the risk of bias through the Risk Of Bias In NRCTs – of Interventions (ROBINS-i) tool [25]. RoB graph was created through RobVis visualization tool [26]. For NRCTs, an initial assessment considered potential confounders and co-interventions. Subsequently, the risk of bias was evaluated as either “low,” “uncertain,” or “high” in various domains, including confounders, participant selection, intervention classification, deviation from intended intervention, missing data, outcome measurement, and reported results. Any disagreements were resolved by a third reviewer (VF).

Statistical Analysis

Since we assessed a continuous outcome (pro-inflammatory status) measured through different variables, we computed the standardized mean difference (SMD) along with its associated 95% confidence interval (95% CI). In the process of pooling the data, we employed a random-effects model and inverse variance method. To ensure robust performance in this analysis, we opted for the restricted maximum likelihood estimator (REML) for tau². We used Q-Profile method for CI of tau² and tau. We used the Hartung-Knapp adjustment for random effects model. The prediction interval was based on t-distribution. The calculation of SMD was carried out utilizing Hedges' g method. The outcomes were represented using either forest plots or drapery plots, as suggested in the literature [27]. Prediction intervals were calculated and represented in the respective forest plots [28]. Statistical significance was set at $p < 0.05$. To evaluate the size of the effect of the SMD, we considered levels of 0.2, 0.5, 0.8 as small, medium, and large effects. Statistical heterogeneity was determined with the Q statistic and I², with values of 25%, 50%, and 75% taken to indicate low, moderate, and high levels of heterogeneity, respectively [22]. To identify potential publication bias, the Egger's regression test was performed.

Since study participants are nested within studies, we set a four-level meta-analysis to assess potential moderators of the overall effect using a four-level mixed-effects model. We included random effects for specific factors that could influence model reliability, such as the research group (level 4), individual authors (level 3 – nested within level 4), and the marker of interest (level 2 – nested within level 3). Level 1 represented individual measurements. As fixed effects, we incorporated the specific

¹<https://osf.io/gkxzh/>



group of molecules (e.g., chemokines, cytokines, etc.), categorized based on their potential molecular mechanisms contributing to PGD, and Timing, which referred to the timing of individual measurements. Timing was defined as either immediately after the lung transplant subjected to EVLP or days later, categorized as T_0 vs. T_{end} . We used the REML method to estimate model parameters. To evaluate the model's fit, we reported the Akaike Information Criterion (AIC). After establishing the suitability of the four-level model, we proceeded to assess potential moderators of the overall effect. The effect of individual biomarker categories was determined by adding the intercept value to their estimate [27].

We performed a sensitivity analysis using the same four-level meta-analysis, but restricted it to specific timepoints, namely, T_0 and T_{end} .

All the analysis were performed with R studio using the packages dmetar, meta, metafor version 2023.06.0+421 (2023.06.0+421) [29].

RESULTS

The search strategy retrieved 350 articles from databases and 4 from registers. After removing the duplicates, the remaining 108 articles were independently screened for titles and abstracts by 2 authors (AC and EB), and 96 records were excluded. A detailed list for each exclusion reason is available at link.¹ Full texts of the remaining 12 records were screened, and 5 records were excluded. One last paper was added after a careful revision of the bibliography [30].

A total of eight NRCTs were included [12, 19, 20, 30–34] in the systematic review and meta-analysis. A detailed selection process is shown in the PRISMA flowchart (Supplementary Figure S1) and in a repository online.¹ Then two authors (AC and EB) independently extracted the data following study protocol.

Risk of Bias in Studies

All the studies included were judged of low RoB arising from the randomization process with ROBINS-I tool for NRCTs (Figure 1). The overall risk of bias was moderate-low due to the well-selected population, uniform protocols for detecting inflammatory mediators, and a low number of missing data. Potential confounders, such as the cutoff values for the measured variables and the kits used to detect the biomarkers, were identified.

Description of Included Studies

A detailed description of the included studies is reported in Table 1.

Biomarkers at Initial T_0

In total, 610 measurements were conducted at the initial time. The inflammation mediators considered were sE-selectin, sICAM, vCAM, ET-1, Big ET-1, IL8, MCP, GRO α , MIP1a, MIP1b, IL1 β , IL6, TNF α , M30, HMGB, nuDNA, mtDNA, M-CSF, GCS-F, inhaled CO and NOx (Figure 2, and Supplementary Figure S2). The pooled effect according to the random-effects model was 0.75, with the 95% CI ranging from 0.19 to 1.31 (Figure 2 and Supplementary Figure S2). The effect size was 2.84 ($p = 0.012$), detecting a significantly higher probability to develop PGD grade 3 at 72 h post-transplant as the inflammatory biomarkers increase. The restricted maximum likelihood method estimated a between-study heterogeneity variance of τ^2 , which was 0.82 with a 95% CI of (0.38–2.66) (Supplementary Table S1). The statistical heterogeneity (I^2) was 78.6%, with a 95% CI of (66%–87%) and the significance test of Q ($Q = 70$, $df = 15$, $p < 0.001$) confirmed this heterogeneity (Figure 2). In the Eggers' test, the intercept of our regression model was 4.66 (95% CI –0.67–9.98). This is non-significant ($t = 1.714$, p -value = 0.11), and indicates that the data in the funnel plot are quite symmetrical (Supplementary Figure S2).

Biomarkers at T_{end}

In total, 884 distinct measurements were conducted at the final time. The inflammation mediators considered were sE-selectin, sICAM, vCAM, ET-1, Big ET-1, IL8, MCP, GRO α , MIP1a, MIP1b, IL1 β , IL6, TNF α , M30, HMGB, nuDNA, mtDNA, M-CSF, GCS-F, inhaled CO and NOx. The pooled effect according to the random-effects model was 0.90, with the 95% CI ranging from 0.38 to 1.41 (Figure 2, and Supplementary Figure S3). The effect size was 3.60 ($p = 0.0015$), detecting a significantly higher probability to develop PGD 3 at 72 h post-Ltx as the inflammatory biomarkers increase. The REML method estimated a between-study heterogeneity variance of τ^2 , which was 0.93 with a 95% CI of (0.61–3.32) (Supplementary Table S1). The I^2 was 74.9%, with a 95% CI of (62.7%–83.1%), and the significance test of Q ($Q = 92$, $df = 23$, $p < 0.001$) confirmed this heterogeneity.

In the Eggers' test, the intercept of our regression model was 4.94 (95% CI 2.10–7.77). This is significant ($t = 3.42$, p -value = 0.025), and indicates that the data in the funnel plot are asymmetrical (Supplementary Figure S3). Single molecules are reported divided based on their category and the timing in

TABLE 1 | Summary of findings of the included studies.

Author	Year	Enrollment period	Country	Population characteristics	Biomarker evaluated	Exclusion criteria of the study	PGD 3 overall incidence
Sage et al.	2021	From 2009 to 2019	Canada	Not stated	- IL6 - IL8	Bilateral transplants that were not in the ICU or on ECMO support before transplant were excluded. Lungs with hemorrhages.	PGD 3 at 72 h was 16%
Kanou et al.	2021	December 2018 to June 2019	Canada	39 % of the control group and 57 % of PGD 3 were DCD donors	- nuDNA - mtDNA		PGD 3 at 72 h was 52.6%.
Brenckmann et al.	2020	May 2010 and December 2015	France	45 % of the control group and 30 % of PGD 3 were DCD donors	- CO - IL6 - IL8 - IL1β - TNFα	Excluded 12 cases due to sample unavailability or brige to lung transplant with ECMO.	PGD 3 at 72 h was 15.1%
Hashimoto et al.	2017	September 2008 to August 2013	Canada	62 % of the control group and 83 % of PGD 3 were DCD donors	- sICAM - sVCAM - sE-selectin	ECMO recipients before transplantation or who received single or lobar lung transplantation were excluded.	PGD 3 at 72 h was 24%
Hashimoto et al.	2018	September 2008 to August 2013	Canada	38 % of the control group and 46 % of PGD 3 were DCD donors	- M30 - HMGB	ECMO recipients before transplantation or who received single or lobar lung transplantation were excluded.	PGD 3 at 72 h was 24%
Machuca et al.	2015	September 2009 to November 2012	Canada	44 % of the control group and 43 % of PGD 3 were DCD donors	- M-CSF - G-CSF - MIP-1a - MIP-1b - IL8 - GROα	Single-lung transplants, lobar transplants, and recipients bridged to transplant with extracorporeal life support were not included in this study	PGD 3 at 72 h was 15%
Machuca et al.	2015	February 2009 to January 2010	Canada	48 % of the control group and 43 % of PGD 3 were DCD donors	- ET-1 - Big ET-1 - NOx	Bridge with ECMO and unilateral or bilobar transplants	PGD 3 at 72 h was 22%
Boffini et al.	2023	July 2011 to March 2020	Italy	adsorption vs no adsorption population	- GCS-F - IL6 - MCP-1	Sample unavailability	PGD 3 at 72 h was 23%

Abbreviations: Big ET-1: Big Endothelin-1; CO: Carbon Monoxide; ECMO: Extracorporeal Membrane Oxygenation; ET-1: Endothelin-1; GCS-F: Granulocyte Colony-Stimulating Factor; G-CSF: Granulocyte Colony-Stimulating Factor; GRO α : Growth-Regulated Oncogene alpha Protein; HMGB: High Mobility Group B Protein; IL1 β : Interleukin-1 beta; IL6: Interleukin-6; IL8: Interleukin-8; M30: Apoptosis Marker MCP-1: Monocyte Chemoattractant Protein-1; M-CSF: Macrophage Colony-Stimulating Factor; MIP-1a: Macrophage Inflammatory Protein-1 alpha; MIP-1b: Macrophage Inflammatory Protein-1 beta; mtDNA: Mitochondrial DNA; NOx: Nitrogen Oxides; nuDNA: Nuclear DNA; sE-selectin: Soluble E-selectin; sICAM: Soluble Intercellular Adhesion Molecule; sVCAM: Soluble Vascular Cell Adhesion Molecule; TNF α : Tumor Necrosis Factor alpha.

Figure 3 (biomarkers at T_{end}) and **Supplementary Figure S4** (biomarkers at T₀).

Four-Level Mixed Linear Analysis for Biomarkers Subgroups

We found a large pooled correlation based on the four-level meta-analytic model ($r = 0.62$ –95% CI: 1.21–3.50; $p = 0.009$ - **Table 2**), meaning that there seems to be a substantial association between developing PGD 3 at 72 h post-Ltx and inflammatory biomarkers.

The estimated variance components were $\tau^2_{\text{Level2}} = 0.63$, $\tau^2_{\text{Level3}} = 0.30$ and $\tau^2_{\text{Level4}} = 0.00$. This means that $I^2_{\text{Level2}} = 67\%$ of the total variation can be attributed to within-markers heterogeneity, and $I^2_{\text{Level3}} = 33\%$ to within-authors heterogeneity, while the within-research group heterogeneity was approximately $I^2_{\text{Level4}} = 0\%$. Overall, this indicates that there is substantial between-study heterogeneity on the second level, or differences within studies. Yet, we also see that a large proportion of the total variance, more than one-fifth, can be explained by differences within markers (Level 3). In this model, the Akaike Information Criterion (AIC) of the four-level model was 109.7, indicating that the model fits the data well.

We checked if correlations differed depending on the biomarker group (AD, CKs, Chemokines, DAMPs, Hematop GF, Met) using a four-level moderator model ($p = 0.235$). In the model, the only group that showed a significant difference was the Chemokine group, with a z-value of 1.26 ($p = 0.042$). From our model, the timepoints were not associated with the outcome (OR 1.21 95% CI [0.89–1.65], p -value 0.213). We conducted a sensitivity analysis using data only at the timepoint T₀ or at the timepoint T_{end}, but the findings were consistent with the previous model (**Supplementary Table S1**).

DISCUSSION

In this systematic review and meta-analysis, soluble pro-inflammatory markers measured during EVLP were associated to PGD grade 3 at 72 h after transplantation. Chemokines levels exerted the highest effect on PGD development. Moreover, the timing at which these markers were collected did not affect their predictive value. In fact, patients with grade 3 PGD had higher biomarker levels measured at both early and late stages of EVLP.

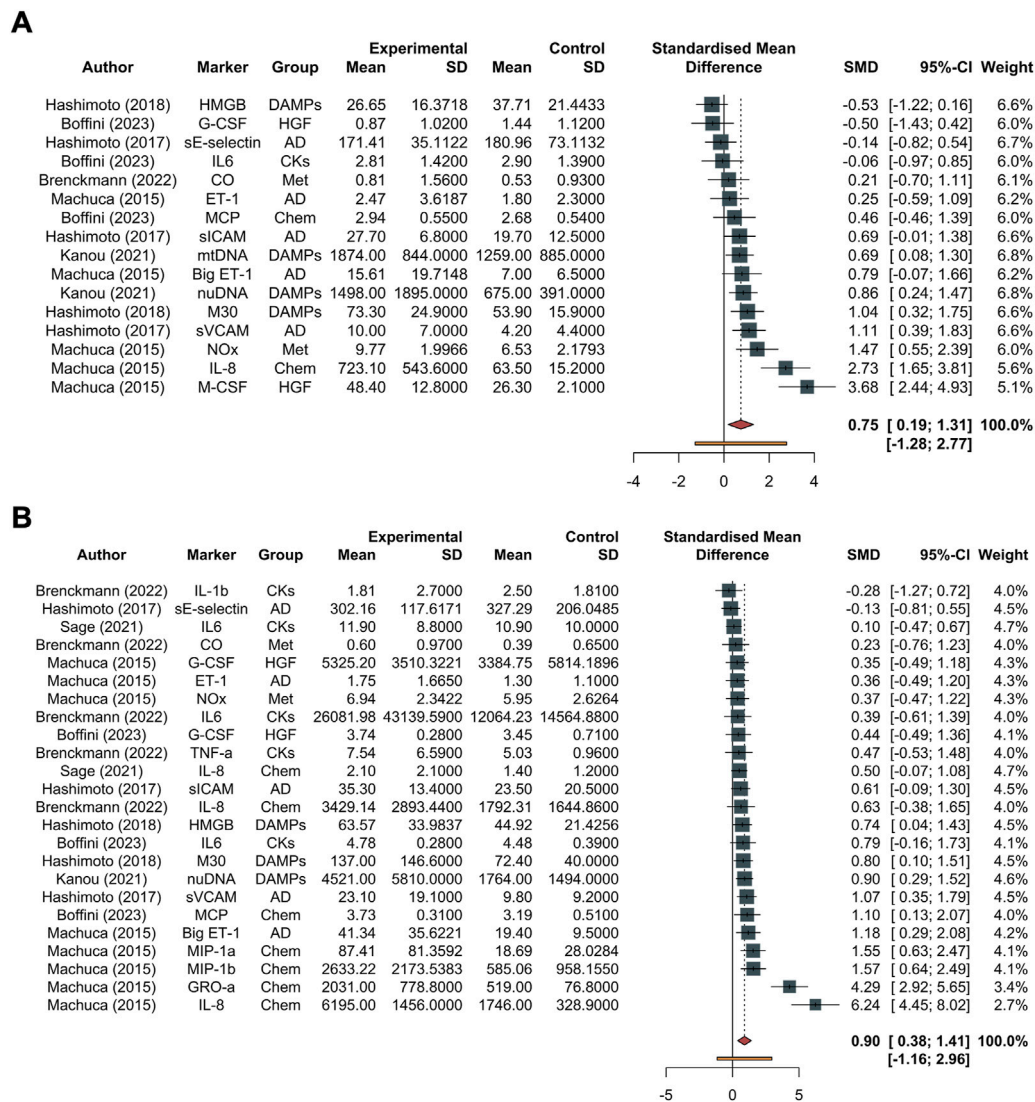


FIGURE 2 | Forest plot of studies assessing inflammatory biomarkers. These are detailed in the column labeled “Marker,” accompanied by their corresponding groups denoted as “Group.” The experimental group corresponds to the PGD grade 3 at 72 h group, while the control group represents the non-PGD grade 3 group. The standardized mean difference (SMD), along with its respective 95% confidence interval (95% CI) and the individual weight for each study, is reported on the right. In the forest plot, squares placed to the right—considering 0 as the midpoint—indicate higher marker levels in the experimental group. **(A)** Forest plot for overall studies, excluding outliers, **(B)** all the studies. Abbreviations: AD, adhesion molecules; CKs, chemokines; DAMPs, damage-associated molecular patterns; Hematop GF, growth factors; sE-selectin, endothelial selectin; sICAM, intercellular adhesion molecule; vCAM, vascular cell adhesion molecule; ET-1, endothelin-1; Big ET-1, big endothelin-1; IL-8, interleukin-8; MCP, monocyte chemoattractant protein; GROα, growth-related oncogene alpha; MIP-1α, macrophage inflammatory protein-1 alpha; MIP-1β, macrophage inflammatory protein-1 beta; IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNF-α, tumor necrosis factor alpha; M30, M30; HMGB, high mobility group box 1; nuDNA, nuclear DNA; mtDNA, mitochondrial DNA; M-CSF, macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor.

The relationship between inflammatory molecules during IRI and the development of PGD is the consequence of the warm and cold ischemia following donor organ retrieval. Pro-inflammatory mediators can be activated during CIT as a result of oxidative stress, sodium Na^+/K^+ ATPase inactivation, calcium overload, and a variety of cell death mechanisms, including apoptosis, necrosis, autophagy, pyroptosis and ferroptosis [10, 35, 36].

Cellular stress triggers the release of DAMPs, mediating acute lung injury through TLR bonding. HMGB1 and nucleic

acids were higher at T_{end} in the high-grade PGD patients [12, 33]; while the circulating amount of DNA was also higher at T_0 in the PGD3 cohort, reflecting the amount of cell injury and death following CIT, HMGB1 levels were instead lower. In fact, HMGB1 can be actively released by alveolar macrophages, lymphocytes and epithelial cells too, after reperfusion, and maybe sustaining a pro-inflammatory effect in the damaged grafts [11–15]. High CKs levels (namely, IL1β, TNFα and IL6) at T_{end} might reflect lung injury, but in the examined studies the

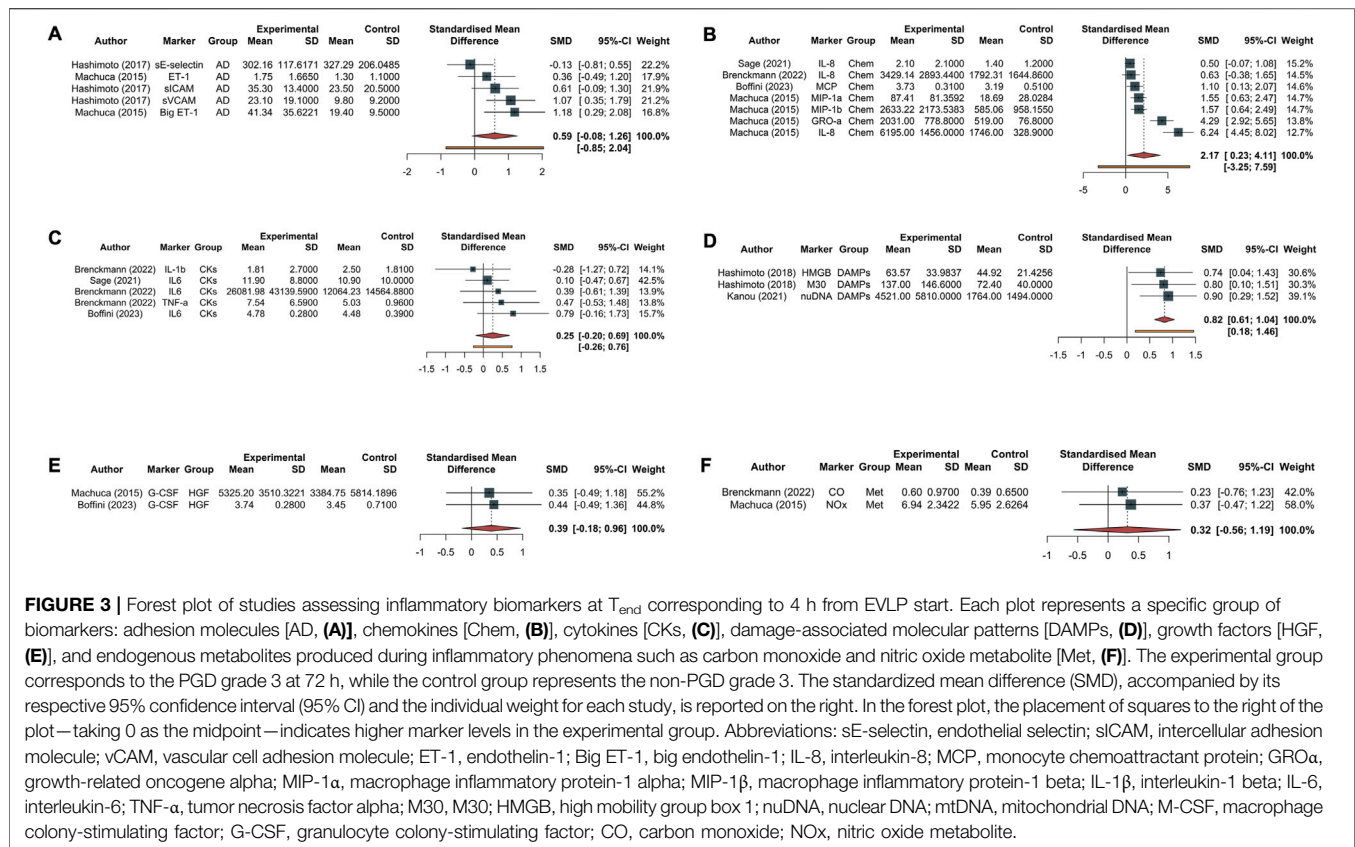


TABLE 2 | Four-level multivariate meta-analytic model.

a. Random effect

Factor	Variance	SD (sqrt)	Levels (n/lvs)	Fixed	Variance (%)
Research group	0.0	0.0003	3	no	0.0
Research group	0.304	0.5513	7	no	32.65
+ Author					
Research group	0.6271	0.7919	26	no	67.35
+ Author					
+ Marker					

b. Fixed effect

Group	Odds ratio (OR with CI)	p-value
Chemokines	3.51 (1.05–11.79)	0.042
Cytokines	1.23 (0.30–5.05)	0.764
DAMPs	1.61 (0.34–7.69)	0.542
HGF	1.85 (0.43–8.04)	0.400
GroupMet	1.15 (0.23–5.71)	0.865
timing	1.21 (0.89–1.65)	0.213

Abbreviations: SD, standard deviation.

association between the total amount of these molecules and the development of PGD3 was not unanimous [20, 31, 32]. Hoffman et al. found no differences in terms of TNF α and IL1 β in the plasma of LTx recipients with or without PGD3, and a significant increase in terms of IL-6 levels in patients with high grade PGD only 48–72 h after surgery [37]. This fact

might corroborate the hypothesis that these CKs do not reflect the amount of tissue damage right after CIT. The included studies instead showed a strong association between chemokines levels in perfusate and PGD3, in particular when measured at the end of the procedure [20, 31, 32, 34]. Chemokines are involved in the recruitment of neutrophils

(IL-8) and monocytes (MCP). IL-8 levels in particular are associated with worse graft function [10, 20, 37] and were also found to be higher at T_0 in the recipients with worse outcome after transplantation [34]. Although G-CSF and M-CSF might theoretically play a role as the signature for a higher pro-inflammatory milieu, considered globally they were not strongly associated with PGD3 development, except for M-CSF levels in perfusate at T_0 [31, 34]. In fact, M-CSF is involved in monocytes and macrophages proliferation and differentiation [38], plays a role in the pathogenesis of pulmonary fibrosis [38] and has been associated with hyperinflammatory state in COVID-19 patients [39]. Soluble adhesion molecules are released into circulation from the cell surface and their levels correlate with the degree of endothelial activation during inflammation [18, 19]. They are known to be upregulated in lung tissue samples from ARDS patients who died for Gram-negative bacteria induced septic shock [40] and in ARDS patients with worse outcome [17, 41, 42]. Adhesion molecules were increased in every graft, except from sVCAM-1, which remained stable during the procedure only in those lungs which did not develop PGD3 [19]. Our metanalysis confirms that sVCAM-1 levels are constantly higher in the PGD3 recipients both at the beginning and at the end of EVLP. Moreover, the higher level of Big ET-1 in PGD3 grafts might rather reflect an alteration in capillary permeability and recruitment of inflammatory cells [30].

Compared to lungs that are transplanted without examination, EVLP provides a unique platform for dynamically assessing the inflammatory load. The molecules that can be retrieved in the perfusate might be the expression of a previous or ongoing biological damage or themselves sustain lung injury. A dynamic picture of the biological effects of the CIT and the degree of lung injury that occurred before organ retrieval may be obtained. Implementing the standard EVLP evaluation with a panel of biomarkers specific for the different stages of cell and tissue injury, might help in defining a peculiar biological signature for the single organ. This would be possible only with the availability of faster, standardised and reliable multi-parametric point of care tools. Lung assessment would then benefit from this upgrade under two perspectives: first, identifying - and discarding - organs with an unacceptable risk for early complications, such as high grade PGD and eventually death. Second, EVLP would become a platform for targeted treatments to a molecular level, to heal and recover lungs by acting on one or more mechanisms of the IRI injury cascade.

The Toronto Lung score is the only available and validated score that takes into consideration a point-of-care evaluation of pro-inflammatory CKs levels in EVLP perfusate; however, the analysis is restricted to IL6 and IL8 and does not account for the entire cascade that underlies lung injury [10, 17, 20]. This might not be enough. In fact, gene expression profiling on lung tissue has shown that whereas the inflammatory pathways are upregulated in DBD lungs, cell death, apoptosis, and necrosis predominate in the transcriptomic signature of DCD donors' lungs [43].

This is the first study to summarise the current research about the predictive and prognostic role of IRI biomarkers measured during EVLP in a systematic and quantitative

fashion. We employed a robust statistical method (namely - multilevel meta-analysis), which accounts for effect sizes. In addition, limiting the research to EVLP reduces the confounders that would have been generated including studies considering the donor lungs before the retrieval and/or in the recipient. In the first case the levels of the measured biomarkers are the result of a complex interaction between the multi-organ derangement following brain or cardiac death and of the interplay between lungs and the other organs. Additionally, because CIT has not yet occurred, its contribution to lung injury would not be measured. Conversely, the recipient faces the donor's inflammatory load, together with the activation of the immune system and the effects of immune suppression, in a context of an end-stage pulmonary disease with potential multi-organic involvement. Another strength of this meta-analysis is that normothermic acellular EVLP is performed in few highly specialized centres in the world and even less ones collected and published their results. This guarantees that the procedures are homogenous, with similar learning curves and few deviations from the original protocols, making measurements highly comparable.

This study has some limitations. Firstly, the small number of studies limits the analysis to a pooled one and does not allow for molecule-by-molecule analysis. Therefore, the overall sample size is small and there is possible overlap of patients, because the same group might have measured different biomarkers on the same specimens. Secondly, it was not possible to compare directly across different studies due to their limited numbers. However, we mitigated this issue by using standardized mean differences to group molecules with similar biological significance, ensuring that all the biomarkers measure the same checkpoint in the inflammatory cascade but employing different scales [22]. Thirdly, the papers included comprise a long period of time and refer to specimen acquired in an even broader time span. In any case, normothermic acellular EVLP technology has not changed much over the past 15 years, and many studies refer to lung assessments performed from about the same period. Fourthly, we attempted to analyse the distribution of true effect sizes with the mean ($\theta_{i,j}$), considering all possible sources of heterogeneity. Despite conducting a meticulous analysis, we consistently observed high levels of heterogeneity, particularly between-studies. Considering the incorporation of prediction intervals in our results - providing a range to anticipate the effects of future studies based on current evidence - we posit that our findings might not attain statistical significance in the future, especially with the potential expansion of the sample or exploration of different biomarkers. This underscores the need to prioritize our focus on specific biomarkers. Fifth, most of the studies were of a retrospective nature, using prospectively collected materials, thus limiting the availability of data in terms of laboratory test results. Lastly, our prediction intervals, which can estimate between-study heterogeneity variance and the standard error of the pooled effect, suggest that our results may be subject to confirmation or revision by future studies, as the overall estimated effect is not statistically significant.

CONCLUSION

Lung perfusate concentration of inflammatory biomarkers, in particular chemokines, are associated with Grade 3 PGD development at 72 h in recipients of EVLP lungs. Future multicentre, prospective observational studies are needed to confirm the results of this meta-analysis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AC: study design, data interpretation and manuscript writing and revision. EB: study design, data interpretation and analysis and manuscript writing and revision. MM: data interpretation and manuscript revision. ES: data interpretation and manuscript revision. AB: data interpretation and manuscript revision. MR: data interpretation and manuscript revision. LB: data interpretation and manuscript revision. MB: data interpretation and manuscript revision. VF: study design, data interpretation, manuscript writing and revision. All the authors approved the final version of the manuscript to be published and agreed to be accountable for all aspects of the work.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontierspartnerships.org/articles/10.3389/ti.2025.13794/full#supplementary-material>

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GLOSSARY

PGD primary graft dysfunction

ICU intensive care unit

Ltx lung transplantation

PaO₂ partial pressure of arterial oxygen

FiO₂ fraction of inspired oxygen

IRI ischemia reperfusion injury

CIT cold ischemic time

DAMPs damage-associated molecular patterns

EVLP *Ex vivo* lung perfusion

TLS₂ Toronto Lung Score 2

RCTs randomized controlled trials

NRCTs non-randomized controlled trials

PRISMA Preferred Reporting Items for Systematic Reviews and Meta-Analyses

ROBINS-i Risk Of Bias In Non-randomized Studies-of Interventions

sE-selectin serum E selectin

sICAM soluble intercellular adhesion molecule-1

vCAM soluble adhesion molecules vascular cell adhesion molecule-1

ET-1 endothelin-1

Big ET-1 big endothelin-1

IL8 Interleukin 8

MCP monocyte chemotactic protein

GRO α growth-regulated oncogene α

MIP-1a macrophage inflammatory protein 1a

MIP-1b macrophage inflammatory protein 1b

IL1 β Interleukin 1 β

IL6 Interleukin 6

TNF α tumour necrosis factor α

HMGB High mobility group box 1

nuDNA nuclear DNA

mtDNA mitochondrial DNA

M-CSF macrophage colony-stimulating factor

G-CSF Granulocyte colony-stimulating factor

CO carbon monoxide

NOx nitric oxide metabolite

TLR Toll-like receptor



Veno-Arterial Extracorporeal Membrane Oxygenation (VA-ECMO) Support in New Era of Heart Transplant

Lorenzo Giovannico, Giuseppe Fischetti, Domenico Parigino, Luca Savino, Nicola Di Bari, Aldo Domenico Milano, Massimo Padalino and Tomaso Bottio *

Cardiac Surgery Unit, Department of Precision and Regenerative Medicine and Ionian Area (DiMePRe-J), University of Bari Aldo Moro, Bari, Italy

Heart failure is a serious and challenging medical condition characterized by the inability of the heart to pump blood effectively, leading to reduced blood flow to organs and tissues. Several underlying causes may be linked to this, including coronary artery disease, hypertension, or previous heart attacks. Therefore, it is a chronic condition that requires ongoing management and medical attention. HF affects >64 million individuals worldwide. Heart transplantation remains the gold standard of treatment for patients with end-stage cardiomyopathy. The recruitment of marginal donors may be considered an asset at the age of cardiac donor organ shortage. Primary graft dysfunction (PGD) is becoming increasingly common in the new era of heart transplantations. PGD is the most common cause of death within 30 days of cardiac transplantation. Mechanical Circulatory Support (MCS), particularly venoarterial extracorporeal membrane oxygenation (V-A ECMO), is the only effective treatment for severe PGD. VA-ECMO support ensures organ perfusion and provides the transplanted heart with adequate rest and recovery. In the new era of heart transplantation, early use allows for increased patient survival and careful management reduces complications.

OPEN ACCESS

Keywords: heart transplant, heart failure, primary graft dysfunction, ECMO, heart disease

*Correspondence

Tomaso Bottio,
✉ tomaso.bottio@uniba.it,
✉ tbottio@gmail.com

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INTRODUCTION

On the night of 3 December 1967, Dr. Christiaan Barnard performed the first human heart transplant [1]. Since then, organ transplantation has made marked progress in reducing the morbidity and mortality of patients with heart failure (HF) [2]. Heart Transplantation remains the gold-standard therapy for end-stage HF. Although the number of transplants has increased over the decades, the greatest limitation in performing heart transplants is a lack of donors. In addition, patients who undergo heart transplantation are increasingly complex: elderly people with multiple pathologies have a higher level of calculated panel reactive antibody (cPRA) antigen sensitization (>20%), some of which undergo transplantation with short-term mechanical circulatory support (ST-MCS) [3]. Due of this situation, the use of marginal donors could be considered an asset provided there is a careful match between donor and recipient, and satisfactory results can be obtained both in the short and long term [4]. Several studies have aimed to expand selection criteria for marginal donors. As we find ourselves working with marginal donors and increasingly complex recipients, short-term mechanical circulatory support (ST-MCS) and venoarterial extracorporeal membrane

TABLE 1 | Adopted from [7].

	Jan 1992–Dec 2000 (N = 37,616)	Jan 2001–Dec 2009 (N = 33,588)	Jan 2010–Jun 2018 (N = 36,830)	P-value
PRA ≥ 20%	5.2%	10.0%	17.9%	<0.0001
PRA ≥ 80%	0.9%	2.3%	3.6%	<0.0001
Hep B antibody positive	3.8% ^a	5.2%	4.9%	<0.0001
Diabetes	16.7% ^a	22.3%	27.0%	<0.0001
History of malignancy	3.8% ^a	5.8%	8.7%	<0.0001
Pre-transplant dialysis	3.0% ^a	4.1%	4.8%	<0.0001
Previous cardiac surgery	37.8%	43.6%	50.1%	<0.0001

Summary statistics included transplants with known/non-missing data.

^abased on Apr 1994 to Dec 2000.

oxygenation (V-A ECMO) will become increasingly fundamental. In 2018, in the USA, the United Network for Organ Sharing implemented a 6-tier allocation policy to replace the prior 3-tier system. The new allocation system allows stratification of candidates by mortality on the waiting list and reduces the time on the waiting list for patients with a higher priority. At the first evaluation performed by Maitra et al. in 2023, the change in the allocation system made it possible to improve the stratification of patients on the waiting list, to increase the number of patients arriving at the transplant with ST-MCS support (VA-ECMO or IABP), intubated, requiring hospitalization in intensive care, ischemia times and distance traveled for organ harvesting, and the use of marginal donors. At the same time, it reduced waiting list times, waiting list mortality, and post-heart transplant mortality in patients weaned from VA-ECMO support [5, 6].

This review aimed to analyze the use of venoarterial extracorporeal membrane oxygenation (ECMO) in the new era of cardiac transplantation.

RECIPIENT CHARACTERISTICS

Those who have experienced the evolution of heart transplantation have noticed how over the years, the population of donors and recipients has changed significantly from 30 years ago.

Many centers worldwide have expanded acceptance criteria for adult heart transplant candidates. This, combined with changes in population demographics, has led to significant changes in the recipient characteristics [7].

Notably, the proportion of listed candidates aged >70 years increased from 2.5% in 2000 to 11% in 2017, reflecting the trend of an aging population and a greater focus on biological aging and other comorbidities. Guidelines regarding HT candidacy recommend that patients >70 years of age should be carefully selected, but do not provide further guidance [8–10].

Candidacy for HT depends on the intersection of chronological and biological age, and requires close investigation of social support, cognitive function, and frailty [8].

Allosensitized patients had elevated calculated panel reactive antibody (cPRA) values. Sensitizing events included pregnancies, blood transfusions, MCS devices, or prior surgeries [9].

Therefore, women and patients with LVADs are more likely to be sensitized. It can be difficult to find suitable donors with acceptable immunogenetic profiles for these patients, which can lead to longer waiting times and higher waitlist mortality [8].

Furthermore, there has been an increase in the number of recipients with diabetes, history of malignancy, history of pre-transplant dialysis, and previous cardiac surgery (**Table 1**). Although the number of hospitalized and mechanically ventilated patients and the use of inotropes have decreased, the number of patients in ST-MCS (ECMO) and LT-MCS (L-VAD) has increased (**Table 2**) [7].

DONOR CHARACTERISTICS

Considering the increase in HF patients worldwide and the changing recipient population, the use of marginal donors can be considered an additional resource after careful matching between donor and recipient to guarantee a heart transplant to those who need it and satisfy the heart transplant waiting list [11]. Several studies have aimed to expand selection criteria for marginal donors [12–15]. Controversial results have been reported on posttransplant outcomes following an MD donation, with relatively short-term data [13]. In Italy, according to the regulations of the National Transplant Center, MDs were defined according to the following criteria: age >60 years, reduced left ventricular performance (ejection fraction 40%–50%), left ventricular hypertrophy (septal thickness >14 mm on echocardiographic evaluation), focal lesion of the coronary artery, and significant valvular heart disease. The main concerns regarding the use of these donors are related to their increased susceptibility to high perioperative mortality, coronary graft vasculopathy, and primary graft failure (PGF) [13].

PRIMARY GRAFT DYSFUNCTION

Primary graft dysfunction (PGD) is becoming increasingly common in the new era of heart transplantations. This syndrome is a dysfunction of the transplanted heart that occurs immediately following heart transplantation [16]. PGD is the most common cause of death within 30 days of cardiac

TABLE 2 | Adopted from [7].

	Jan 1992–Dec 2000 (N = 37,616)	Jan 2001–Dec 2009 (N = 33,588)	Jan 2010–Jun 2018 (N = 36,830)	P-value
Inotrope use	46.7% ^a	43.4%	35.7%	<0.0001
IABP use	6.0%	6.2%	6.6%	0.0672
ECMO use	0.2% ^b	0.7%	1.1%	<0.0001
Type of MCS use				
- None	-	77.2%	55.6%	<0.0001
- VAD	-	18.9%	40.4%	
Ventilator use	3.4%	3.2%	1.7%	<0.0001
Hospitalized	60.7%	47.6%	44.6%	<0.0001

Summary statistics included transplants with known/non-missing data.

^aBased on Apr 1994 – Dec 2000 transplants.

^bBased on Apr 1995 – Dec 2000 transplants.

TABLE 3 | Adopted from [25].

PGD left ventricle (PGD-LV):	Mild PGD-LV: one of the following criteria must be met: Moderate PGD-LV: must meet one criterion from I and another criterion from II: Severe PGD-LV	LVEF <40% by echocardiography, or Haemodynamics with RAP >15 mmHg, PCWP >20 mmHg, CI < 2.0 L/min/m2 (lasting more than 1 h) requiring low-dose inotropes I. LVEF <40%, or Hemodynamic compromise with RAP >15 mmHg, PCWP >20 mmHg, CI < 2.0 L/min/m2, hypotension with MAP <70 mmHg (lasting more than 1 h) II. High-dose inotropes—inotrope score >10a, or Newly placed IABP (regardless of inotropes) Dependence on left or biventricular mechanical support including ECMO LVAD, BiVAD, or percutaneous LVAD. Excludes requirement for IABP. i. Haemodynamics with RAP >15 mmHg, PCWP <15 mmHg, CI < 2.0 L/min/m2 ii. TPG <15 mmHg and/or pulmonary artery systolic pressure <50 mmHg iii. Need for RVAD
PGD right ventricle (PGD-RV):	Diagnosis requires either both i and ii, or iii alone:	

BiVAD, biventricular assist device; CI, cardiac index; ECMO, extracorporeal membrane oxygenation; IABP, intra-aortic balloon pump; MAP, mean arterial pressure; LVAD, left ventricular assist device; LVEF, left ventricular ejection fraction; PCWP, pulmonary capillary wedge pressure; PGD, primary graft dysfunction; RAP, right atrial pressure; RVAD, right ventricular assist device; TPG, trans pulmonary pressure gradient.
Inotrope score = dopamine (1) + dobutamine (1) + amrinone (1) + milrinone (15) + epinephrine (100) + norepinephrine (100) with each drug dosed in mg/kg/min.

transplantation [17], and the most recent report from the International Society for Heart and Lung Transplantation (ISHLT) Registry reported that 42.6% of deaths within 30 days of transplantation were due to PGD [18]. Several factors have been implicated in the development of PGD, including donor age, cause of donor cerebral death, organ preservation technique (e.g., cold storage), ischemic time >4 h, sex mismatch, preoperative presence of recipient VAD or ST-MCS, pulmonary hypertension, and elevated inotropic score [19–25]. Temporal, echocardiographic, hemodynamic and clinical criteria such as the use of high doses of inotropic drugs and/or mechanical support with ST-MCS are necessary to diagnose PGD.

(Table 3) The ISHLT recently published a consensus document with standardized criteria for PGD diagnosis following cardiac transplantation [26].

Depending on the severity of the primary graft dysfunction, treatment may be based on the use of inotropic drugs or, in the most severe forms, on the use of short-term mechanical circulatory support.

MCS is the only effective treatment for severe PGD [21–25, 27–32]. ECMO support ensures organ perfusion, giving the transplanted heart time to rest and recover. The use of ECMO allows for a reduction in the support of inotropic drugs and

vasoactive agents that stress the transplanted heart by increasing cardiac work, vasoconstriction, and afterload.

V-A ECMO

Venoarterial extracorporeal membrane oxygenation guarantees immediate and complete support for patients with cardiogenic shock and cardiac arrest. The system consists of a centrifugal pump that can completely compensate for the cardiac output, a venous drainage cannula, and an arterial return cannula. A hollow fiber membrane oxygenator was positioned in the circuit to ensure oxygenation of venous blood and to remove carbon dioxide (CO₂) via sweep gas flow.

V-A ECMO CONFIGURATIONS

In our center, the sternum is closed after full reversal of heparin with protamine sulfate. Partial ECMO flow (2,2-3 L/min/m2) was maintained in conjunction with inotropes and inhaled nitric oxide, while the patient was intubated and sildenafil was administered when extubated to maintain ejection through the

aortic and pulmonary valves. Anticoagulation with heparin (UFH) is initiated 24 h after sternal closure at a rate of 400 U/h. Activated clotting time (ACT) was measured every 4 h with a target of 180–200 s. Partial thromboplastin time was measured every 8 h, with a target of 45–60 s.

VA-ECMO can be performed surgically and percutaneously. When positioned surgically, it can be positioned with central cannulation (arterial cannula in the ascending aorta and venous drainage cannula in the right atrium) or peripherally via femoral or subclavian arterial access and the femoral vein.

The most commonly used access method at our center for patients with primary graft dysfunction is surgical or percutaneous femoral access. The venous cannula draining the deoxygenated venous blood was positioned in the superior vena cava-RA junction. After passing through the hollow fiber membrane oxygenator, oxygenated blood is returned to systemic circulation via an arterial cannula with its tip typically positioned in the iliac artery. Selecting cannulas with appropriate diameters is critical not only to reduce the risk of vascular injury, but also to avoid significant negative inflow (preferably < 50 mmHg) and high outflow pressure (<300 mmHg). At our center, to reduce the risk of distal limb ischemia, a 6Fr distal reperfusion cannula is routinely inserted into the superficial femoral artery [33, 34]. The use of peripheral and percutaneous access makes V-A ECMO support versatile and can be used in the operating room and bedside in the intensive care unit (ICU) [35, 36]. Peripheral cannulation allows for primary chest closure, diminishing the potential risks of chest infections and early respiratory weaning at the expense of a higher risk of limb ischemia, impaired unloading of the heart, and potential risk of harlequin syndrome [37].

Patients with central access had higher vasoactive-inotropic scores and more renal dysfunction without significant differences in lactate levels before MCS. Peripheral cannulation was associated with unrelated MCS infections and less MCS-related bleeding. Severe vascular complications were more frequent in patients with peripheral MCS, lymphorrhea at the access point, and venous thrombosis. No significant differences were observed in intubation time, stroke, embolism, or continuous renal replacement therapy (CRRT) [37].

UNLOADING LEFT VENTRICLE

Recently, two large, randomized trials, EURO-SHOCK and ECLS-SHOCK, have been completed and published to address the question of whether VA-ECMO therapy in cardiogenic shock improves survival [38]. Our indications for venting the left ventricle included one or more of the following criteria: a high PCWP; Dilated, hypo-contractile LVX with blood pooling in the left ventricle seen on echocardiogram with increased LVEDP, aortic valve remains closed for the entire duration of the cardiac cycle, reduced oxygenation and persistent pulmonary edema shown on chest radiography, and ventricular arrhythmias.

In such cases, it is necessary to unload the left ventricle and place the heart “at rest.” Therapeutic strategies for dealing with this complex situation are as follows:

- Inotropic drugs: These are the first-line intervention (dobutamine) to improve myocardial contractility and, therefore, left ventricular output.
- IABP: Opinions differ regarding the usefulness of IABP in this context. IABP causes a reduction in afterload due to the “vacuum” effect and some studies have suggested that it can improve coronary flow and therefore myocardial ischemia.
- reduction of congestion using diuretics and/or continuous renal replacement therapy (CRRT).
- Impella, a coaxial pump positioned retrogradely in the transvalvular aortic position, sucks blood from the left ventricle and expels it directly into the ascending aorta.
- left ventricular venting: this is the definitive treatment for left ventricular unloading.

The main venting methods are:

- Left atrial cannulation: Used especially in children, the drainage cannula is positioned directly in the left atrium through puncture of the atrial septum.
- Direct drainage of the left ventricle: Through surgical access, a drainage cannula is positioned at the apex of the left ventricle, which is connected in a Y-shape with the peripheral venous line (usually femoral), and then connected to the centrifugal pump and oxygenator.
- transaortic and axillary artery venting.
- Conversion of VA-ECMO to LVAD: A cannula is inserted into the left ventricle with an apical approach in a left anterior mini-thoracotomy to ensure optimal unloading of the left ventricle, and is then connected in a Y-shape with the venous cannula of the ECMO.

In a recent meta-analysis, Iannaccone et al. suggested that an ECPella vs. ECMO-only strategy for the management of CS may reduce 30-day mortality and increase LV recovery, despite increased bleeding rates and hemolysis [39].

A recent meta-analysis by Bathia et al. demonstrated that patients with cardiogenic shock requiring VA-ECMO support and concurrent LV unloading with Impella had a lower likelihood of short-term mortality and a higher likelihood of progression to durable LVAD or heart transplantation. However, patients supported with ECPella had higher rates of hemolysis, limb ischemia, and renal failure, requiring continuous renal replacement therapy [40].

Although the latter are the most effective venting systems, they are rarely used to unload the left ventricle during primary graft dysfunction after heart transplantation. Generally, an inotropic support and percutaneous device such as an IABP or Impella are more than sufficient for unloading the left ventricle.

VENTILATION ON V -A ECMO

The goal of mechanical ventilation in these patients is to maintain adequate gas exchange while minimizing the risk of lung damage by placing the lungs at rest.

To keep the lung at rest and avoid the risk of volotrauma and barotrauma, it is recommended to maintain “protective ventilation,” setting a respiratory rate between 10 and 15 breaths/min, a tidal volume (tidal volume, V_t) ≤ 8 mL/kg and a plateau pressure ≤ 25 cmH₂O, tolerating a certain degree of “permissive” hypercapnia.

In patients with severe cardiac impairment such as those with ECLS, high levels of positive end-expiratory pressure (PEEP) inhibit venous return and may have negative effects on hemodynamics. The PEEP values are usually set between 5 and 8 cmH₂O.

Hyperoxia appears to be associated with increased mortality in patients with ECLS, as it causes a pro-inflammatory response to reactive oxygen species and induces vasoconstriction with consequent organ hypoperfusion [38]. Therefore, it is recommended to reduce the inspired fraction of oxygen (FiO_2) to the lowest possible level (FiO_2 0.40–0.60) with a target arterial oxygen pressure between 60 and 100 mmHg. In recent years, given the emergence of eco-support as a therapy for primary graft dysfunction, we have considered ways to reduce the complications that can occur when using this support. Many of these factors are associated with mechanical ventilation [39, 40].

Awake emergency strategies have been proposed to minimize complications related to mechanical ventilation. The findings described in case reports, pediatric cohorts, and a few series [41–44] indicate a better CS prognosis, but these studies were underpowered by the small number of patients enrolled and the inclusion of non-arousable patients with likely neurological impairment. Managing awake patients with V-A ECMO support is a relatively new and challenging task in the intensive care unit (ICU) [45].

The pros are:

- *Rehabilitation:* Muscle mass loss, critical illness, myopathy, and polyneuropathy often affect patients [46, 47].
- *Reduction of delirium:* The pathogenesis of delirium in the ICU is multifactorial; however, the use of sedative drugs is one of the main causes.
- *Interaction with relatives/friends and medical staff:* The ability of an awake patient to communicate with friends and relatives can render an unfriendly ICU environment a more usual and easy-to-cope situation for both patients and visitors. Furthermore, it is possible for awake patients to communicate their symptoms and need medical staff [45].

The disadvantages include the risk factors for invasive device displacement, patient discomfort, pain, and anxiety. An awake patient required analgesics for invasive device tolerance and pain control [45]. Montero et al. analyzed 231 patients who underwent peripheral ECMO implantation. Of these 231 patients, 91 were “awake” and 140 were “not awake” After PS-matching adjustment, the “awake ECMO” group had significantly lower rates of pneumonia (35% vs. 59%, $P = 0.017$), tracheostomy, renal replacement therapy, and less antibiotic and sedative consumption. This strategy was also associated with reduced 60-day (20% vs. 41%, $P = 0.018$) and 1-year mortality rates

(31% vs. 54%, $P = 0.021$) compared to the “non-awake” group, respectively [48]. In this paper, an “awake ECMO” is feasible, safe, and associated with improved short- and long-term outcomes.

WEANING OF V -A ECMO

Attempts to wean patients from ECMO were initiated after the first 48–72 h of ECMO assistance. ECMO support should be provided early in heart transplant patients with primary graft dysfunction to avoid organ damage. Therefore, because there are no organs damaged by low perfusion that need to be recovered, the indication for ECMO-weaning is generally indicated based on echocardiography, hemodynamics, and biological parameters.

The weaning criteria included $MAP > 60$ mmHg, $PCWP < 18$ mmHg, $CI > 2.4$ L/min/m²; $SVcO_2$ value $\geq 70\%$, hematocrit of 28%–30%; no bleeding or tamponade, left ventricular ejection fraction $\geq 25\%$, absence of dilation of the left ventricle, good contraction of the right ventricle (ejection fraction $> 25\%$) with absence of moderate or severe tricuspid regurgitation, normal blood lactate levels (< 2 mmol/L), and normal diuresis (1–2 mL/kg/h) or dialysis.

Weaning should be initiated when patients are hemodynamically stable with a mean arterial pressure (MAP) > 60 mmHg in the absence or presence of low doses of inotropes and vasopressors for at least 24 h.

Weaning was gradual with reduction of the ECMO flow in steps of approximately 0.5–1.0 L/min/m² (10% of the initial flow) approximately every 8–12 h with careful analyses of transesophageal or transthoracic echocardiography (Table 4) and evaluation of the values calculated by positioning the Swan-Ganz catheter (Table 5).

ECMO flow is normally not reduced to below 1 L/min/m² because of the high risk of circuit thrombosis at these flows. Once an ECMO flow of 1 L/min/m² is reached, the pump flow is radically reduced to 0.5 L/min/m² for approximately 15–30 min. If hemodynamics in terms of systemic arterial pressure (mean pressure > 60 mmHg), left ventricular contractility (ejection fraction $> 25\%$), $RVEF > 25\%$, central venous pressure (10–15 mmHg), pulmonary capillary wedge pressure (10–15 mmHg), and SvO_2 ($> 70\%$) are preserved, the chances of weaning will be very high. Inotrope support, if present, was maintained for at least 3 days after ECMO removal.

ECMO weaning tests are fundamental for evaluating the contractile function of the right ventricle, which is normally almost completely unloaded by a venous cannula positioned in the right atrium from the inferior vena cava during emergency support.

Few studies have aimed to identify the criteria for predicting which patients can be successfully weaned from ECMO using the same methodology. All of these studies considered clinical, hemodynamic, echocardiographic, or biological parameters. All these were observational studies, and the parameters were compared between weaned and non-weaned patients. The main limitations of these studies were the observational design and number of patients [49–54]. Ortuno et al. in a recent publication, Ortuno et al. investigated the best weaning

TABLE 4 | Echocardiographic parameters to be evaluated in V-A ECMO weaning.**Echocardiographic monitoring**

distention/overload of the left ventricle
 Aortic velocity-time integral >10 cm
 LVEF >25%
 Lateral mitral annulus peak systolic > 6 cm/s
 RVEF >25%
 RV and LV strain
 Recognition of complications

- Intracavitary thrombosis
- Cannula positioning/displacement
- Pericardial effusion/cardiac tamponade

TABLE 5 | Hemodynamic parameters to be evaluated in V-A ECMO weaning.**Hemodynamic Parameters**

MAP ≥60 mmHg
 Pulmonary capillary Wedge pressure < 18 mmHg
 Cardiac Index > 2,4 L/min/m²
 Central Venous pressure < 18 mmHg
 SvO₂ e ScvO₂
 Lactate

strategy to use, and considering the data reported, proposed a strategy to optimize weaning from V-A ECMO.

ANTICOAGULATION

The Extracorporeal Life Support Organization (ELSO) guidelines for anticoagulation during ECMO recommend unfractionated heparin (UFH) as a 50–100 units/kg bolus at the time of cannulation, followed by a continuous infusion of 20–50 units/kg/h to achieve an activated clotting time (ACT) of 180–220 s [55]. Anticoagulation administered while using the V-A ECMO system should prevent the formation of clots, which could lead to embolisms or malfunction of the system, particularly of the oxygenator. In patients who have undergone a heart transplant, the use of V-A ECMO requires careful control and balancing of anticoagulation to prevent the formation of clots in the ECMO circuit and, at the same time, prevent bleeding. Desirable hemostatic balance is often difficult to achieve and may become a nearly impossible task in a high-bleeding-risk setting [56].

According to the 2021 ELSO Adult and Pediatric Anticoagulation Guidelines [57], monitoring anticoagulation using ACT is considered the gold standard for the guidance of anticoagulation therapy in patients with ECMO [58, 59]. In addition to the ACT, the aPTT dosage is certainly fundamental and can help in assessing the patient's level of anticoagulation. Furthermore, there is evidence that standard aPTT from the laboratory shows a stronger correlation with plasma UFH concentrations than ACT does [60].

The ELSO suggested repeating the test every 1–2 h in the case of ACT and every 6–12 h to monitor the anticoagulation effect

TABLE 6 | Coagulation values based on the various tests compared to the patient's ECMO flow.

	ACT	aPTT	Anti-Xa
Flow > 2,5 lt/min	160–180 s	35–40 s	0,15–0,20
Flow 2–2,5 lt/min	180–200 s	40–50 s	0,20–0,30
Flow < 2 lt/min	200–250 s	50–60 s	0,30

with aPTT. [58, 59]. Some authors suggest the use of low molecular weight heparin (LMWH) and fondaparinux for anticoagulation in patients on VA ECMO. The primary method of monitoring both LMWH and fondaparinux is to measure anti-Xa levels [61, 62]. In our center, we use heparin (UFH) in Venous-Arterial ECMO. Anticoagulation with heparin (UFH) is initiated 24 h after sternal closure, and surgical bleeding was excluded at a rate of 400 U/h. Activated clotting time (ACT) was measured every 4 h, with a target of 180–200 s. Partial thromboplastin time was measured every 8 h, with a target of 45–60 s. In Table 6, we report the coagulation values based on various tests compared with the patient's ECMO flow.

CONCLUSION

Venoarterial extracorporeal membrane oxygenation (VA-ECMO) is a key player in controlling primary graft dysfunction (PGD) in patients undergoing heart transplantation. Through this strategy, organ perfusion of indispensable life support is guaranteed, and the transplanted heart is given sufficient time to regain normal function. The new age of heart transplantation has been marked by the earlier application of VA-ECMO, which decreases the risk of complications due to delayed mechanical support and increases survival rates.

Furthermore, the increasing use of marginal donors in the context of organ shortages and the increasing complexity of candidates waiting for transplantation make the use of high-performance mechanical support solutions indispensable for successful transplantation. VA-ECMO has a central role not only in PGD treatment but also in switching to either a long-term support system or a heart transplant, thus enhancing post-transplant outcomes.

The experience gained in heart transplantation and VA-ECMO support confirms the significance of enhanced ventilation and weaning techniques to minimize complications and improve short- and long-term prognoses. Systematic studies and meta-analyses will clarify the criteria for the optimal application of VA-ECMO and PGD management and provide a more stepped framework for healthcare professionals engaged in this delicate field.

AUTHOR CONTRIBUTIONS

The authors confirm contribution to the paper as follows: study conception and design: LG; draft manuscript preparation: LG,

GF, DP, and TB. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Electrophysiological Markers of *Ex-Situ* Heart Performance in a Porcine Model of Cardiac Donation After Circulatory Death

Jorik H. Amesz^{1,2}, Mark F. A. Bierhuizen^{1,2,3†}, Sanne J. J. Langmuur^{1,4†}, Paul Knops^{2,3}, Yvar P. van Steenis¹, Dwight Dumay⁵, Mathijs S. van Schie^{2,3}, Olivier C. Manintveld^{3,4}, Natasja M. S. de Groot^{2,3,6} and Yannick J. H. J. Taverne^{1,4*}

¹Translational Cardiothoracic Surgery Research Lab, Department of Cardiothoracic Surgery, Erasmus University Medical Center, Rotterdam, Netherlands, ²Translational Electrophysiology Lab, Department of Cardiology, Erasmus University Medical Center, Rotterdam, Netherlands, ³Department of Cardiology, Erasmus University Medical Center, Rotterdam, Netherlands, ⁴Erasmus MC Transplant Institute, Erasmus University Medical Center, Rotterdam, Netherlands, ⁵Department of Clinical Perfusion, Erasmus University Medical Center, Rotterdam, Netherlands, ⁶Department of Microelectronics, Faculty of Electrical Engineering, Mathematics and Computer Sciences, Circuits and Systems, Delft University of Technology, Delft, Netherlands

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*Correspondence

Yannick J. H. J. Taverne,
✉ y.j.h.j.taverne@erasmusmc.nl

[†]These authors have contributed
equally to this work and share second
authorship

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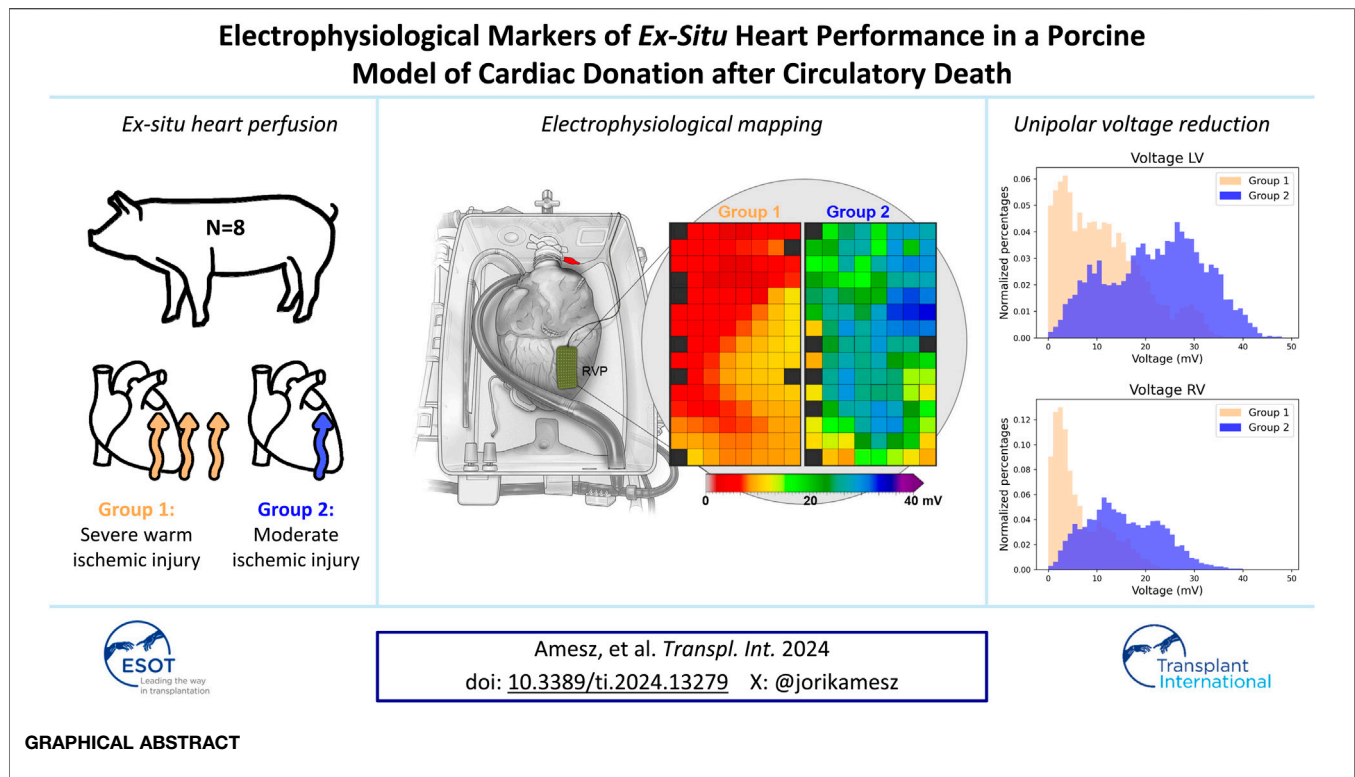
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Normothermic *ex-situ* heart perfusion (ESHP) enables assessment of hearts donated after circulatory death (DCD) prior to transplantation. However, sensitive parameters of cardiac function of DCD hearts on ESHP are needed. This study proposes a novel approach using electrophysiological (EP) parameters derived from electrical mapping as biomarkers of post-ischemic cardiac performance. Porcine slaughterhouse hearts (PSH) were divided in two groups based on the type of warm ischemia (Group 1: 10 ± 1 min with animal depilation vs. Group 2: ≤5 min without depilation). Electrical mapping of the right (RV) and left ventricle (LV) was performed on ESHP. Potential voltages, slopes and conduction velocities were computed from unipolar electrograms and compared between groups. Voltages were lower in Group 1 compared to Group 2 (RV: 3.6 vs. 15.3 mV, $p = 0.057$; LV: 10.8 vs. 23.6 mV, $p = 0.029$). In addition, the percentage of low-voltage potentials was higher and potential slopes were flatter in Group 1. Voltages and slopes strongly correlated with the visual contractile performance of PSH, but showed weaker correlation with lactate profiles. In conclusion, unipolar potential voltages and potential slopes were decreased in hearts with severe warm ischemia. As such, EP parameters could aid transplantation teams in decision-making on transplantability of DCD hearts.

Keywords: cardiac transplantation, *ex situ* heart perfusion, machine perfusion, electrophysiological mapping, graft assessment

INTRODUCTION

Normothermic *ex-situ* heart perfusion (ESHP) has been successful in enlarging the cardiac donor pool using circulatory death donors (DCD) [1–4]. Nonetheless, DCD hearts suffer from serious ischemia-reperfusion injury due to the inevitable functional warm ischemic time (WIT) prior to cardioplegic flush. For this reason, the post-ischemic cardiac quality needs to be assessed in a near-physiological beating state during ESHP.



Currently, quality assessment is based on lactate trends, together with measurements of coronary flow and aortic pressure, and visual contractile assessment of the heart by the transplantation surgeon. However, accuracy of lactate profiles for assessment of DCD hearts is subject of debate [5] and animal studies observed poor correlation between lactate levels and post-ischemic contractile function [6–8]. Additionally, lactate profiles could not reliably predict the need for mechanical circulatory support post-DCD heart transplantation in clinical practice [9]. Hence, additional, sensitive and real-time parameters of cardiac function are needed, especially in the setting of more marginal donor hearts. To date, novel sensitive biochemical markers have not been identified, and such markers need to be available as point-of-care test for eventual translation to clinical practice. Functional parameters, including ejection fraction, stroke volume and dP/dt have shown stronger correlations with myocardial performance [8], but cannot be assessed on the only clinical device that is currently available due to the Langendorff perfusion mode [10].

Electrical markers may provide rapid and real-time markers of ischemic damage. In daily clinical practice, the impact of myocardial ischemia on electrical function is detected by the surface electrocardiogram. Electrical mapping can also be used to detect effects of myocardial ischemia. This is defined as the recording of the summation of electrical activity of cardiomyocytes underlying mapping electrodes [11] and is performed directly on the epicardial surface of the donor heart on ESHP. As such, quantifiable electrophysiological (EP) parameters derived from local electrograms (EGMs) can be used to assess the electrical function of the ventricles and

detect local regions of ischemia. Ion homeostasis is disturbed during ischemia [12] and does not return to pre-ischemic levels if damage from ischemia is severe [13]. Hence, donor hearts with severe injury from ischemia and reperfusion may present with impaired electrical function. To test this hypothesis, the purpose of this study was to investigate whether, and which, EP parameters are potential markers of cardiac tissue vitality and ischemic injury. Therefore, a high resolution electrical mapping approach was performed during ESHP on beating porcine slaughterhouse hearts (PSH) with good versus poor quality. The resulting objective EP parameters may aid the transplantation team in decision-making on transplantability of (marginal) donor hearts.

MATERIALS AND METHODS

Porcine Heart Procurement

The current study was performed on PSH from animals (age: 6 months, weight: ~95 kg) sacrificed for human consumption. The protocols in the abattoir were consistent with the EC regulations 1069/2009 regarding slaughterhouse animal material for research. The procurement protocol of PSH has been described before by others [14, 15], showing good contractile function and reproducible outcomes if hearts are procured before depilation of the animal's skin [16]. However, hearts from PSH that undergo depilation in a dehairing machine are known to show poor cardiac function on ESHP [16]. To test our hypothesis, pigs were divided into two groups based on whether the heart was explanted before or after animal depilation.

The pigs were electrically stunned, hung and exsanguinated, during which 1.5–2 L of blood were collected and anticoagulation was immediately added (25,000 IU heparin, 2 mg Tirofiban). WIT was defined as the period from electrical stunning until start of cold cardioplegic flush. Group 1 had a longer WIT of approximately 10 min and animals were first washed in a dehairing machine of 60°C before excision of the thoracic organs via parasternal incision, as per standard protocol of the local abattoir. Group 2 had a shorter WIT of ≤ 5 min and the heart was procured before depilation of the pig, after which the thoracic cavity was closed and the animal continued in the standard abattoir process.

Hearts were immediately topologically cooled in 0.9% saline solution and the aorta was cannulated and clamped, in order to rapidly administer St Thomas Cardioplegic Solution (2 L with 2500 IU/L heparin) in the coronary arteries. Subsequently, hearts were transported on cold static storage to the laboratory (~50-min drive). The period between administration of cardioplegia and reperfusion on ESHP was recorded as cold ischemic time.

Ex-Situ Perfusion

Upon arrival at the lab, the ascending aorta and pulmonary artery (PA) were cannulated, caval veins ligated, and a vent was positioned in the left ventricle (LV), similar to the human ESHP transplantation protocol [10, 17]. A Langendorff perfusion system, consisting of a centrifugal pump, cardiectomy reservoir (Medtronic EL404) and oxygenator (Sorin Group Inspire 6F) was primed with a mixture of 500 mL gelofusin ((B. Braun, Melsungen, Germany) with 12.5 g mannitol, 250 mg methylprednisolone, 20 mEq sodium bicarbonate and 10,000 IU heparin) and 100 mL 20% albumin (Prothya Biosolutions, Netherlands B.V.). After priming, 1,500 mL of blood were added and the blood-priming solution mixture was heated to 35°C. The heart was reperfused by connecting the aortic cannula to the perfusion system, resulting in active coronary perfusion and spontaneous myocardial contractions. Cardioversion in case of ventricular fibrillation was accomplished by administration of 1.0 g of magnesium sulfate and electrical defibrillation. Perfusate exiting the PA cannula and LV vent was collected and recirculated. Adenosine (Adenocor, Sanofi) was infused at 0.12–0.60 mg/h to ensure perfusion with an aortic pressure of 75–85 mmHg and coronary flow of ≥ 750 mL/min. A maintenance solution (0.15 g/L calcium gluconate, 0.025 g/L magnesium sulfate, 0.11 g/L sodium chloride, 1.0 g/L glucose, 12.5 IU/L insulin (ASPART Sanofi, Paris, France)) was infused at 10–30 mL/h to compensate for the heart's uptake of electrolytes and nutrients. Biventricular electrical stimulation was performed if the heart rate was < 70 beats per minute.

Blood Analysis

Arterial and venous blood samples were taken every 20 min to monitor electrolyte (Na^+ , K^+ , Ca^{2+}), metabolite (glucose, bicarbonate, lactate) and blood gas (pH, pO_2 , pCO_2) levels, and corrected accordingly. Measurements were performed with the i-STAT 1 analyzer (Abbott Point of Care Inc., Chicago, IL, United States) using CG4+ and CG8+ cartridges. Calcium concentrations were maintained low during initial reperfusion

to reduce ischemia-reperfusion injury [18], and corrected to physiological levels afterwards. A baseline blood sample was measured before connection of the heart. Lactate trends were calculated as the slope of the difference in concentration between the first and last arterial sample after reperfusion.

Epicardial Mapping

High-resolution mapping of the heart was performed 60 min after reperfusion when the heart was stabilized on ESHP. The epicardial surface was systematically mapped by moving a custom-made 120-electrode array (electrode diameter 0.6 mm, inter-electrode distance 2 mm) over the RV and LV (**Figure 1**), comparable to our mapping approach during human cardiac surgery [19]. A steel wire was wrapped around the aorta as indifferent electrode. Five seconds were recorded at every mapping position, with a total recording time of approximately 5 min.

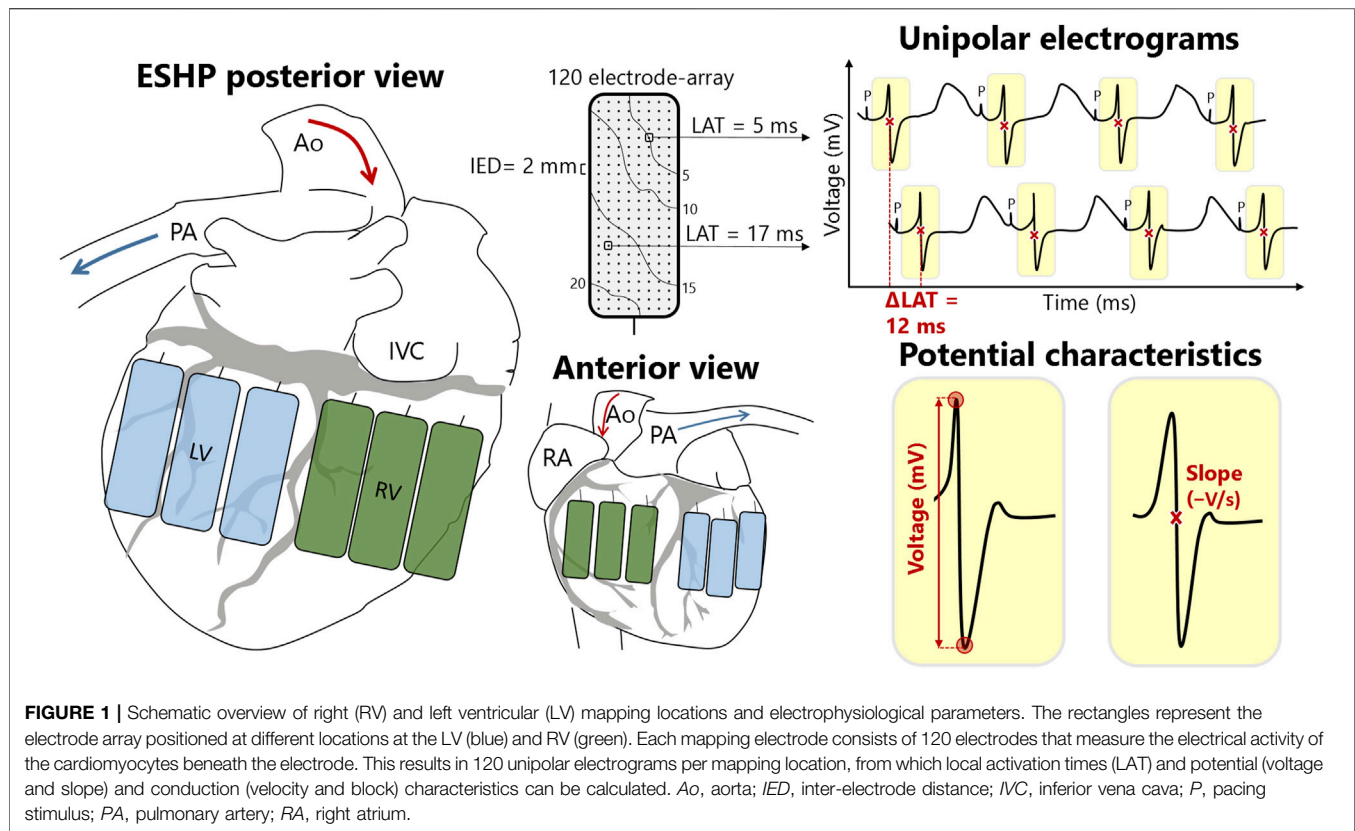
Recordings were sampled with a rate of 2 kHz, filtered with bandwidth 0–500 Hz, analog-to-digital converted (16 bits) and stored on hard disk. Color-coded local activation time (LAT) maps were created by annotating the steepest negative deflection of each unipolar potential to study abnormalities in myocardial conduction [19]. Conduction block was determined as a difference in conduction time of ≥ 12 ms between two adjacent electrodes and the prevalence of conduction block was calculated as percentage of all conduction times. Local effective conduction velocity (CV) was computed from LATs of neighboring electrodes using discrete velocity vectors [20]. In addition, color-coded maps were created visualizing unipolar extracellular potential voltages (peak-to-peak amplitudes) and slopes at each electrode [19]. Low-voltage was defined as the 5th percentile of all peak-to-peak amplitudes from the RV and LV respectively, and rounded to the nearest integer.

Contractile Assessment

Videos were recorded of all hearts and three transplant surgeons of the Erasmus MC who use ESHP in clinical practice were asked to independently assess visual contractility of the hearts. The surgeons were blinded for group assignment. They scored contractility of the whole heart, LV and RV on a scale of 1–5. In addition, they judged whether hearts were suitable for transplantation based on visual contractile performance and hearts were deemed suitable for transplantation if at least 2 surgeons agreed.

Data Analysis

Median unipolar potential voltage, potential slope, and conduction velocity and the amount of low-voltage potentials and conduction block were calculated from the EGMs of the LV and RV from each PSH. LV and RV data were separated because of intrinsic differences in electrophysiological properties between both chambers [21], and the fact that the LV is unloaded during Langendorff perfusion. For these EP parameters, a median was calculated as a summary measure per PSH. These data were then presented as median (range) per group and a Mann-Whitney U test was performed to test for differences between groups. Blood gas and contractile data were tested for normality using QQ-plots



and a Shapiro-Wilk test. Continuous variables were presented as mean $\pm$ standard deviation if normally distributed and as median (range) otherwise. Categorical variables were reported as number (percentage). An unpaired t-test or Mann-Whitney U test was performed, as appropriate. A p-value of ≤ 0.05 was considered statistically significant. A Pearson correlation test was performed to assess the degree of correlation between lactate, visual contractile assessment, and EP parameters. A correlation coefficient of < 0.3 was considered as weak correlation, $\geq 0.3 - < 0.7$ as moderate correlation, and ≥ 0.7 as strong correlation. One-way analysis of variance testing was performed to test for differences across different ventricular regions of the heart. Statistical testing was performed using SPSS software (version 28.0.1.0 (142)). Line plots were created using Graphpad Prism (version 10.2.3 (403)). Correlation plots were created using R (version 4.3.1).

RESULTS

All hearts ($n = 8$) were successfully resuscitated and cold ischemic times were similar in Group 1 ($n = 4$) and Group 2 ($n = 4$) (104 ± 5 min vs. 111 ± 6 min, $p = 0.157$). All hearts required defibrillation after reperfusion, but hearts in Group 2 were easier to restart. Biventricular electrical stimulation was performed in all hearts to compensate for low heart rates, but 2 hearts in Group 2 failed to capture LV pacing resulting in only RV pacing. The blood levels

of electrolytes, glucose and pH during 2 h of perfusion are presented in **Figure 2**. Sodium, calcium and glucose concentrations, and pH were similar between groups. Low calcium levels were corrected earlier in Group 1, but concentrations were similar after 1 h of perfusion. Potassium concentrations were significantly higher in Group 1, compared to Group 2 (**Figure 2**).

All hearts showed a decreasing lactate trend after start of ESHP (**Figure 3**) without statistically significant difference between Groups 1 and 2 ($-24 \pm 6 \mu\text{M}/\text{min}$ vs. $-36 \pm 14 \mu\text{M}/\text{min}$, $p = 0.173$). In addition, lactate concentrations (Group 1: $9.0 \pm 1.5 \text{ mM}$ vs. Group 2: $8.0 \pm 1.5 \text{ mM}$, $p = 0.371$) and arteriovenous differences in lactate concentration (**Supplementary Table S3**) were not different between groups at the time of electrical mapping.

Electrical Mapping

Electrical mapping on the RV and LV resulted in a total of $> 69,000$ unipolar potentials. The data per individual heart were provided in **Supplementary Tables S1, S2**. Unipolar potential voltage was lower in Group 1 compared to Group 2, for both the RV (3.6 mV ($2.5\text{--}12.8$) vs. 15.3 mV ($11.8\text{--}17.0$), $p = 0.057$) and LV (10.8 mV ($4.7\text{--}17.8$) vs. 23.6 mV ($19.4\text{--}24.2$), $p = 0.029$) (**Table 1**). In addition, the median percentage of low-voltage potentials (RV: 1 mV , LV: 2 mV) was higher in Group 1 (RV: 10.6% ($0.3\text{--}17.2$) vs. 0.3% ($0.1\text{--}0.5$), $p = 0.114$; LV: 7.7% ($0.6\text{--}23.0$) vs. 0.4% ($0.1\text{--}1.8$), $p = 0.200$). Also, slopes of potentials were lower in Group 1 (RV:

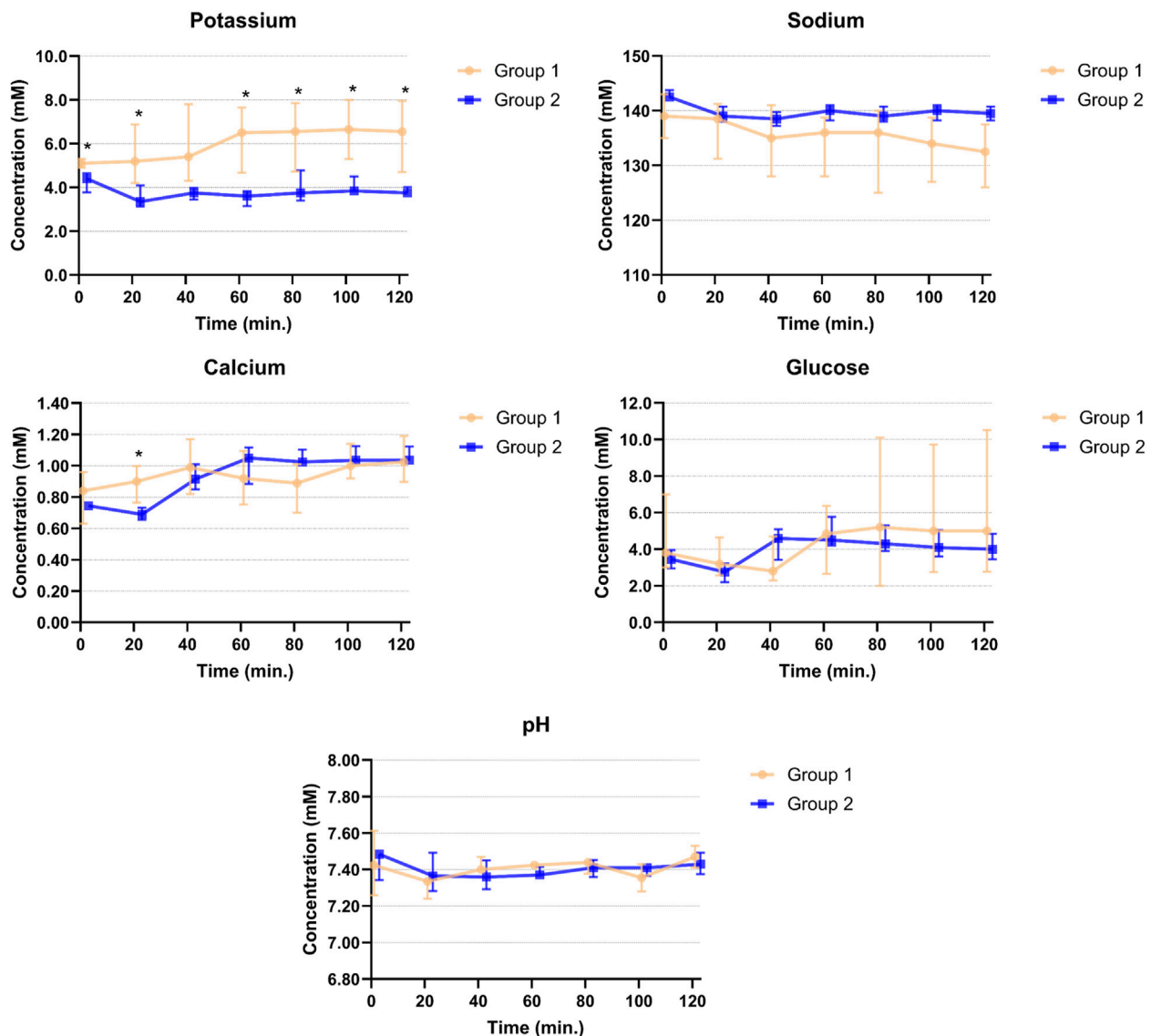


FIGURE 2 | Potassium, sodium, calcium, glucose and pH levels in venous perfusate samples of Groups 1 and 2 during 2 hours of perfusion. Points are plotted as median values with interquartile ranges and asterisks mark significant differences between the two groups.

−0.2 V/s (−0.1 to −1.0) vs. −1.2 V/s (−0.5 to −1.4), $p = 0.057$; LV: −0.3 V/s (−0.2 to −1.8) vs. −1.1 V/s (−1.1 to −1.6), $p = 0.343$) but these differences did not reach statistical significance. Median conduction velocity and amount of conduction block did not differ between groups (Table 1).

Figure 4 presents exemplary colour-coded voltage maps of the posterior wall of the RV from both groups. The red/orange colours in Group 1 indicate lower potential voltages, compared to higher potential voltages represented by green/blue colours in Group 2. In addition, histograms showed lower voltages in Group 1 (Figure 4). Differences between the anterior, lateral and posterior regions of the ventricles are represented in **Supplementary Figure S1**, without a clear preferential location for lower potential voltages.

Contractile Performance

Cardiac visual contractile performance was scored 1.5 ± 0.5 in Group 1 and 3.0 ± 1.0 in Group 2 ($p = 0.042$), thus showing worse cardiac function in the hearts procured after depilation. All hearts in Group 1 were deemed unsuitable for transplantation unanimously, and 2/4 hearts in Group 2 were considered transplantable. Exemplary videos of contractile performance of PSH in both groups are presented in the **Supplementary Material**.

Correlation

Lactate levels and visual contractile assessment were correlated to EP characteristics of the RV and LV (Figure 5; **Supplementary Figure S2**). Weak to moderate correlations

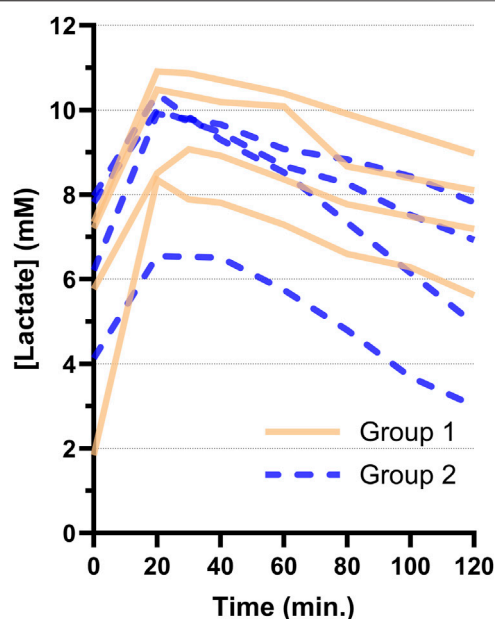


FIGURE 3 | Arterial lactate trends per heart during 2 hours of *ex-situ* perfusion for Groups 1 and 2. The first sample after connection and reperfusion of the heart was taken at 20 min. All hearts showed a decreasing lactate trend after reperfusion.

were observed between lactate and EP parameters (Figure 5). In comparison, correlations were stronger between EP parameters and contractility of the RV and LV (Figure 5). Median potential voltage of the RV significantly correlated with the contractility of the RV and median potential slopes significantly correlated with the visual contractile performance of the heart and RV. For the LV, strong significant correlations were observed between the visual contractile performance and potential voltages, slopes, low-voltage and amount of conduction block (Figure 5). **Supplementary Figure S2** also showed a significant correlation between the lactate trend and visual

contractile performance of the heart and LV, but not with the lactate levels at start and 1 hour after reperfusion.

DISCUSSION

Key Findings

This is the first study to measure and identify EP markers for the assessment of graft quality of hearts preserved on normothermic ESHP for cardiac transplantation. Unipolar potential voltages and potential slopes were decreased in hearts with extensive ischemic injury in a porcine DCD model. Voltage measured from unipolar EGMs might be an additional marker of graft viability. As such, EP parameters assessed by high-resolution epicardial mapping could serve as valuable bioelectrical markers, in addition to other metabolic and hemodynamic parameters, of the functional status of DCD hearts on ESHP.

Lactate Metabolism

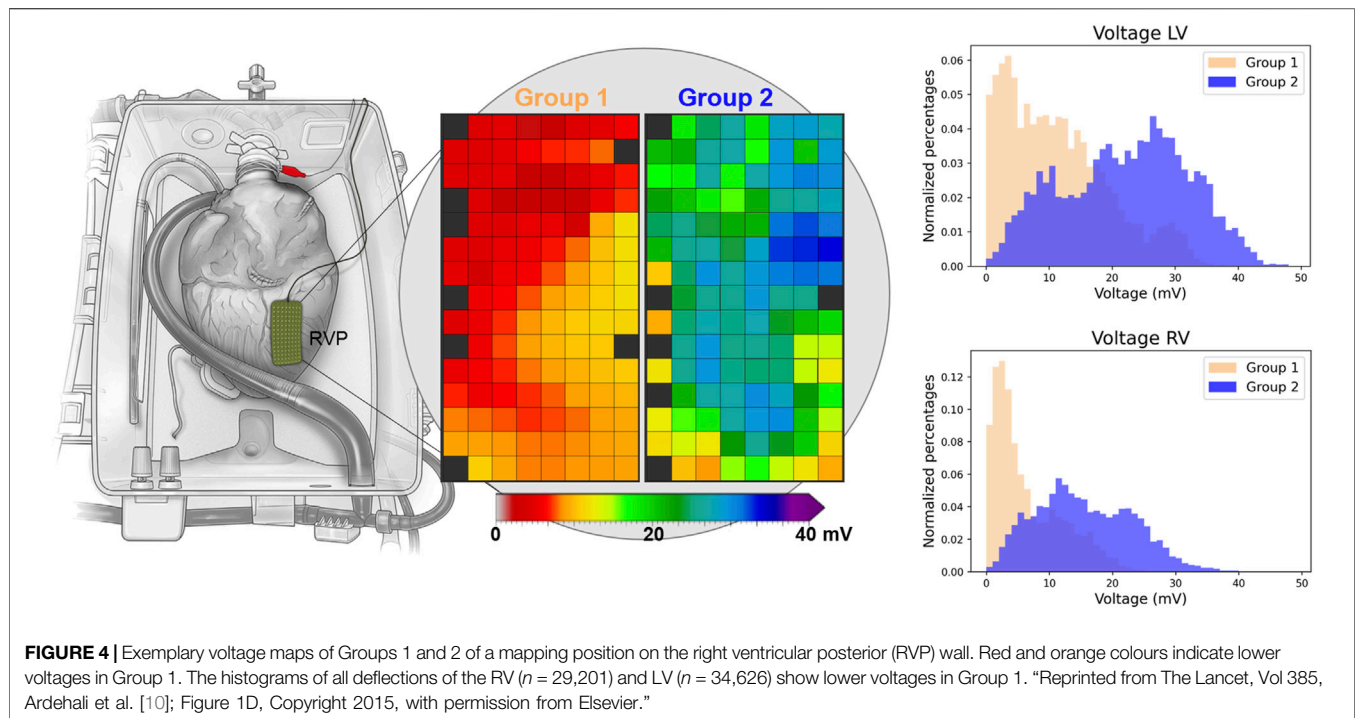
The lactate levels of PSH in the current study all showed a decreasing trend, which makes it impossible to distinguish which hearts were suitable for transplantation based on these curves (Figure 3). Furthermore, contractile performance of certain hearts was very poor (**Supplementary Video S1**) and these hearts were indisputably rejected for transplantation. As such, a decreasing lactate trend alone was not a sensitive marker of graft performance in our PSH model of DCD ESHP. This is in accordance with animal studies [6–8] and clinical outcomes [9].

Hyperkalemia

Potassium concentrations were significantly higher in Group 1 compared to Group 2. Hyperkalemia was associated with reduced cardiac function in other ESHP studies [22, 23] and is believed to be the result of prolonged ischemia [24–26] and the slaughtering and dehairing in the abattoir [14]. An acute decrease in pH due to electrical stunning during the slaughter process as previously reported [14] was not observed in this study.

TABLE 1 | Outcomes of electrophysiological parameters measured 60 min after reperfusion between two groups with different types of ischemia. Data expressed as median (range).

	Group 1 (N = 4)	Group 2 (N = 4)	P-value
Right ventricle			
Number of potentials	3,736 (3,142–5,016)	3,045 (2,628–4,854)	0.343
Voltage (mV)	3.6 (2.5–12.8)	15.3 (11.8–17.0)	0.057
Low voltage (%)	10.6 (0.3–17.2)	0.3 (0.1–0.5)	0.114
<1.0 mV			
Slope (–V/s)	0.2 (0.1–1.0)	1.2 (0.5–1.4)	0.057
Conduction velocity (cm/s)	78.7 (32.1–94.5)	82.3 (80.1–86.0)	1.000
Conduction block (%)	10.3 (2.9–15.6)	6.2 (4.3–14.9)	0.686
Left ventricle			
Number of potentials	4,923 (3,942–5,588)	5,402 (4,182–5,825)	0.486
Voltage (mV)	10.8 (4.7–17.8)	23.6 (19.4–24.2)	0.029
Low voltage (%)	7.7 (0.6–23.0)	0.4 (0.1–1.8)	0.200
<2.0 mV			
Slope (–V/s)	0.3 (0.2–1.8)	1.1 (1.1–1.6)	0.343
Conduction velocity (cm/s)	90.2 (26.4–105.3)	89.3 (74.6–93.0)	0.886
Conduction block (%)	8.9 (4.0–19.0)	4.8 (3.1–5.4)	0.343



Hyperkalemia causes electrical changes in the cardiac action potential (increased resting potential, slower upstroke, and reduced duration) [26, 27] which is reflected in the EGM morphology. An increase in resting potential due to hyperkalemia might contribute to the observed decrease in potential voltage in our study [24]. A reduced conduction velocity as expected in hyperkalemic conditions was not observed in Group 1 [27]. We contend that cardiac mapping can offer additional insights beyond perfusate potassium measurements alone, particularly since electrogram changes due to tissue damage may still be detected even after hyperkalemia is resolved.

Electrophysiological Markers of Ischemic Damage

Lower RV and LV unipolar potential voltages were observed in Group 1, compared Group 2 (Table 1). In addition, a clear difference in the number of low-voltage potentials was observed between both groups, although not reaching statistical significance due to relatively high voltages in one heart in Group 1 (Supplementary Tables S1, S2). Next, unipolar voltage and the amount of low-voltage potentials were strongly correlated to the contractility of the heart, especially the LV. As such, a lower unipolar potential voltage corresponds with more injury from ischemia and reperfusion. Likewise, low-voltages of <1.0 mV for the RV and <2.0 mV for the LV seem to be a sensitive marker of severe injury caused by ischemia and reperfusion.

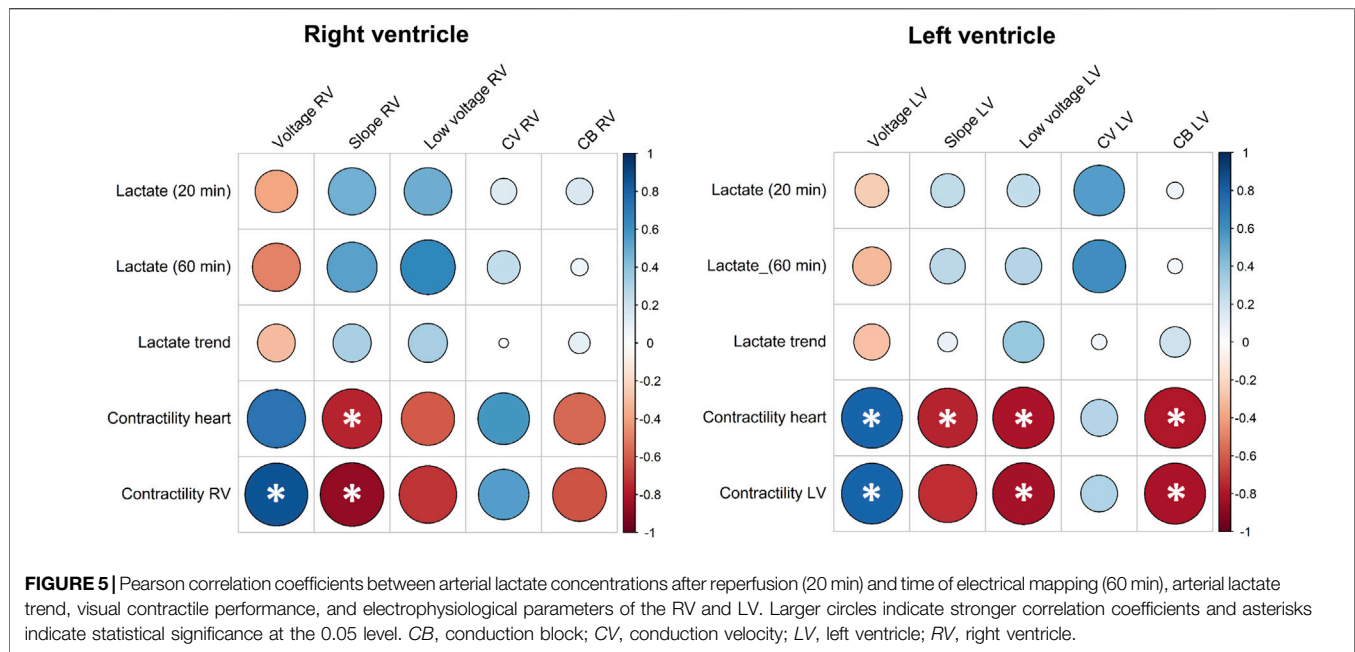
Such reduction in potential voltage following acute ischemia (and reperfusion) was previously demonstrated in animal experiments investigating the effects of coronary artery

occlusions [28, 29] and humans [30]. Additionally, potential voltage is also known to be reduced in regions affected by myocardial infarction [31, 32] where low voltage potentials may indicate scarred myocardium in which the metabolism is reduced and irreversibly damaged [32]. Hence, the current study demonstrates that measurements of unipolar potential voltages on DCD hearts are valuable to assess the amount of irreversible myocardial injury caused by warm ischemia.

Similarly, slowing of conduction following coronary occlusion is a well-known phenomenon [33], potentially leading to post-infarction ventricular tachycardias [34, 35]. However, we did not observe more conduction disorders in Group 1. Yet, conduction velocity and conduction block seem to be less sensitive markers of ischemia-reperfusion injury compared to potential voltage, at least in the acute setting of DCD donation and subsequent reperfusion with ESHP. Nevertheless, the advantage of our mapping approach is that it quickly provides multiple EP parameters which could serve as valuable adjunct in graft quality assessment. These features can be easily visualized using color-coded voltage and conduction maps (Figure 4), which makes it possible to detect local regions of ischemic damage in the DCD heart, whereas lactate levels only reflect the total performance of the donor heart.

Limitations

The cold ischemic time in our study was longer than the usual duration for ESHP machine setup in clinical practice because of the transport time from the abattoir to the laboratory. In addition, the study was performed on slaughterhouse animals, which do not allow for easy control of cardiac explantation conditions as compared to laboratory animals. Animals in Group 1 were exposed to skin depilation before cardiac explantation, which



does not resemble a clinical DCD scenario. Conversely, models using coronary ligation to produce myocardial ischemia do not reflect global ischemia from DCD procedures [36]. Depilation is known to consistently reduce cardiac function on ESHP [16] and can therefore be considered a reasonable alternative to induce global ischemia, despite being a little drastic. In our study, EP markers were isolated for functional assessment of hearts on ESHP, which should be further validated in a more clinical setting.

Another limitation is the sample size which reduces the study's power and may overstate the significance of findings. No correction for multiple testing was performed, which could have introduced the possibility of type I errors. The reported p-values are intended to provide an indication of potential differences between groups, rather than conclusive evidence of significant effects.

Furthermore, EP parameters were only measured 60 min after reperfusion and the experiment only lasted 2 h, which is shorter than the duration for which hearts are typically perfused in the clinic. Yet, measurements at multiple time points on ESHP might possibly provide more insights in the effects of ischemia-reperfusion injury on myocardial survival. Additionally, contractile performance was assessed visually, which resembles the current clinical practice, but lacks the precision and rigor of more objective methods, e.g., pressure catheters [5, 37] or ventricular balloons [38].

Future Perspectives

As a next step, evaluation of the approach of this study on human hearts transported on ESHP for cardiac transplantation is essential to identify reference values differentiating between healthy and injured tissue [39]. Consequently, electrical mapping and other novel assessment strategies might push the boundaries of ischemic time and donor criteria to further enlarge the cardiac donor pool in the future.

Conclusion

EP parameters derived from electrical mapping can aid in decision-making on transplantability of donor hearts transported on ESHP. Low-voltage potentials might be a sensitive marker of myocardial ischemic damage and can be easily combined with other EP markers including myocardial conduction characteristics. Furthermore, electrical mapping can detect local regions of ischemia and the output is visualized in a user-friendly manner to the transplantation team. As such, the presented technique introduces novel, objective EP biomarkers in addition to lactate profiles and visual contractile assessments, and may serve as novel additional diagnostic procedure for assessing graft function, especially in marginal donors.

DATA AVAILABILITY STATEMENT

The datasets presented in this article will be made available upon reasonable request to the corresponding author. Requests to access the datasets should be directed to y.j.h.j.taverne@erasmusmc.nl.

ETHICS STATEMENT

Ethical approval was not required for the study. Methods were consistent with the EC regulations 1069/2009 regarding slaughterhouse animal material for research.

AUTHOR CONTRIBUTIONS

YT, NdG, and JA conceived the idea for the presented study. JA was involved in the system set-up, together with PK, YT, DD, and SL. JA, MB, SL, PK, and YvS participated in the experimentation. PK and MvS were responsible for the data

extraction and processing. JA performed data analysis and wrote the manuscript, aided by MB and SL supervised by NdG and YT. OM and DD carefully reviewed the manuscript and provided input from their clinical experience with DCD heart transplantations. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13279/full#supplementary-material>

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The impact of the EVLP on the lung microbiome and its inflammatory reaction

Leandro Grando¹, Marc Boada¹, Rosa Faner^{2,3,4,5}, Susana Gómez-Ollés^{3,6}, Victoria Ruiz⁶, Marc Bohils⁷, Joaquim Albiol⁷, Ramses Marrero⁸, Laia Rosell⁹, Ivan Salinas⁹, Daniel Ruiz⁹, Ángel Ruiz⁷, Camino Rodríguez-Villar⁷, Anna Ureña¹, David Paredes-Zapata⁷, Ángela Guirao¹, Gerard Sánchez-Etayo⁷, Laureano Molins¹, Néstor Quiroga¹, Aroa Gómez-Brey¹⁰, Xavier Michavila¹, Alberto Sandiumenge¹⁰, Àlvar Agustí^{2,3,11}, Ricard Ramos^{1,4} and Irene Bello^{1,4*}†

¹Department of Thoracic Surgery, Respiratory Institute, Hospital Clínic, Barcelona, Spain, ²Universitat de Barcelona, Catedra Salut Respiratoria, Barcelona, Catalunya, Spain, ³CIBER Enfermedades Respiratorias (CIBERES), Madrid, Spain, ⁴Institut d'Investigacions Blomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain, ⁵Universitat de Barcelona, Biomedicine Department, Barcelona, Catalunya, Spain, ⁶Department of Pneumology, Vall d'Hebron Institut de Recerca, Barcelona, Spain, ⁷Department of Donor and Transplant Coordination, Hospital Clínic, Barcelona, Spain, ⁸Department of Anaesthesia and Perioperative Care, Hospital Clínic of Barcelona, Barcelona, Spain, ⁹Surgical Area, Hospital Clínic, Barcelona, Spain, ¹⁰Department of Donor and Transplant Coordination, Vall d'Hebron University Hospital, Barcelona, Spain, ¹¹Department of Pneumology, Respiratory Institute, Hospital Clínic, Barcelona, Spain

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*Correspondence

Irene Bello,
ibello@clinic.cat

†ORCID:

Irene Bello
orcid.org/0000-0002-8188-2361

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The pulmonary microbiome has emerged as a significant factor in respiratory health and diseases. Despite the sterile conditions maintained during *ex vivo* lung perfusion (EVLP), the use of antibiotics in the perfuse liquid can lead to dynamic changes in the lung microbiome. Here, we present the design of a study that aims to investigate the hypothesis that EVLP alters the lung microbiome and induces tissue inflammation. This pilot, prospective, controlled study will be conducted in two Spanish donor centers and will include seven organ donors after brain death or after controlled cardiac death. After standardized retrieval, the left lung will be preserved in cold storage and the right lung will be perfused with EVLP. Samples from bronchoalveolar lavage, perfusion and preservation solutions, and lung biopsies will be collected from both lungs and changes in lung microbiome and inflammatory response will be compared.

Keywords: *ex vivo* lung perfusion, EVLP, lung microbiome, primary graft dysfunction, lung donor, protocol study, inflammatory response

INTRODUCTION

Lung transplantation (LTx) is the ultimate treatment option for patients suffering from several end-stage pulmonary diseases. Despite efforts to optimize results, primary graft dysfunction (PGD) is one of the most serious and common complications of LTx [1]. PGD is an inflammatory pulmonary response that occurs due to ischemic-reperfusion damage. It is associated with both early mortality [2] and long-term outcomes, including chronic lung allograft dysfunction and late mortality.

The lungs harbor a diverse community of microbes, collectively known as the pulmonary microbiome [3]. This microbial ecosystem, intricately intertwined with the local immune landscape, exerts a profound influence on pulmonary homeostasis and inflammatory responses [4]. Alterations of the microbial composition and diversity of the pulmonary microbiome (dysbiosis)

have been associated with aberrant immune activation and sustained inflammation, contributing to the pathogenesis of acute and chronic respiratory disorders [5].

Ex vivo lung perfusion (EVLP) [6] offers a platform for pre-transplant organ assessment, optimization, and repair. It provides the opportunity to evaluate marginal donor lungs, mitigate ischemia-reperfusion injury, and even rehabilitate injured or rejected lungs deemed unsuitable for transplantation under static conditions.

METHODS

Hypothesis

We hypothesize that lung perfusion with Steen's solution, blood, and antibiotics, with the addition of temperature changes, can alter the lung microbiome and improve microbiome homeostasis. We aim to investigate this and analyze if these changes affect the inflammatory environment.

Objectives

The primary objective of this study is to understand the impact of EVLP on the lung microbiome and tissue inflammation as compared with cold ischemia.

The objectives are:

- To describe the changes in the lung microbiome with cold ischemia
- To analyze the changes in lung microbiome with ELVP
- To discover if EVLP or cold storage improve the microbiome environment
- To compare the changes in the lung microbiome between cold ischemia preservation and EVLP
- To analyze the changes in pulmonary inflammatory parameters with cold ischemia due to lung microbiome changes
- To investigate the changes in pulmonary inflammatory parameters with EVLP due to lung microbiome changes
- To contrast the changes in inflammatory parameters between cold storage and EVLP due to lung microbiome changes

Study Design and Ethics

This pilot, prospective, and controlled study will be conducted in two Spanish donor centers. The study will compare the microbiome of the right lung before and after EVLP to understand the changes that occur and the microbiome of the left lung before and after cold storage. The final microbiome of both lungs will be compared. The current clinical practice actually is cold storage; it is for this reason that we will compare the EVLP changes with the cold storage changes.

The study protocol complies with the 1975 Declaration of Helsinki and has been approved by the Human Research Committees [HCB/2022/0024 and PR(AG)106/2019]. Informed consent to participate in the study was included in the donation consent form. The protocol was registered at ClinicalTrials.gov, NCT06250517.

Study Population

We will include seven organ donors after death determined by neurologic criteria (DBD) or by circulatory criteria (DCD) Maastricht type III. The type of death will be certified in accordance with Spanish legislation.

Lungs included in the study will be older than 18yo and discarded for transplantation by all Spanish and Europe lung transplant programs; the cause of rejection will be recorded. The exclusion criterion is the presence of unilateral radiological alteration or unilateral pneumonia.

Lung Preparation Procedure Prior to the Preservation Protocol

Before bronchoscopy, in the operation room, a sample of sterile saline solution will be taken through the fiberoptic bronchoscope as a negative control of microorganisms in the bronchoscope (BAL_{TX}). A bronchoalveolar lavage (BAL) of 40 mL will be collected from the left main bronchus (BALL_{T0}). The lungs will be perfused with 60 mL/kg of Perfadex Plus (XVIVO Perfusion AB, Sweden) and they will be retrieved following clinical practice. The lungs will be split through the left bronchus using a staple.

The left lung will be preserved in cold storage and the right lung will be perfused with EVLP (Figure 1).

Cold Storage Protocol and Sample Obtention (Control Group)

The left lung will be preserved with 2L of Perfadex Plus[®] (XVIVO Perfusion AB, Sweden) in cold storage at 4°C in a Vitalpack[®] container for 3 h.

Before the cold storage of the left lung, a 50 mL sample of the transport solution (STL_{T0}) will be collected. A 3 cm lung biopsy from the lower lobe will be taken using a staple (BxL_{T0}) and preserved with RNAlater.

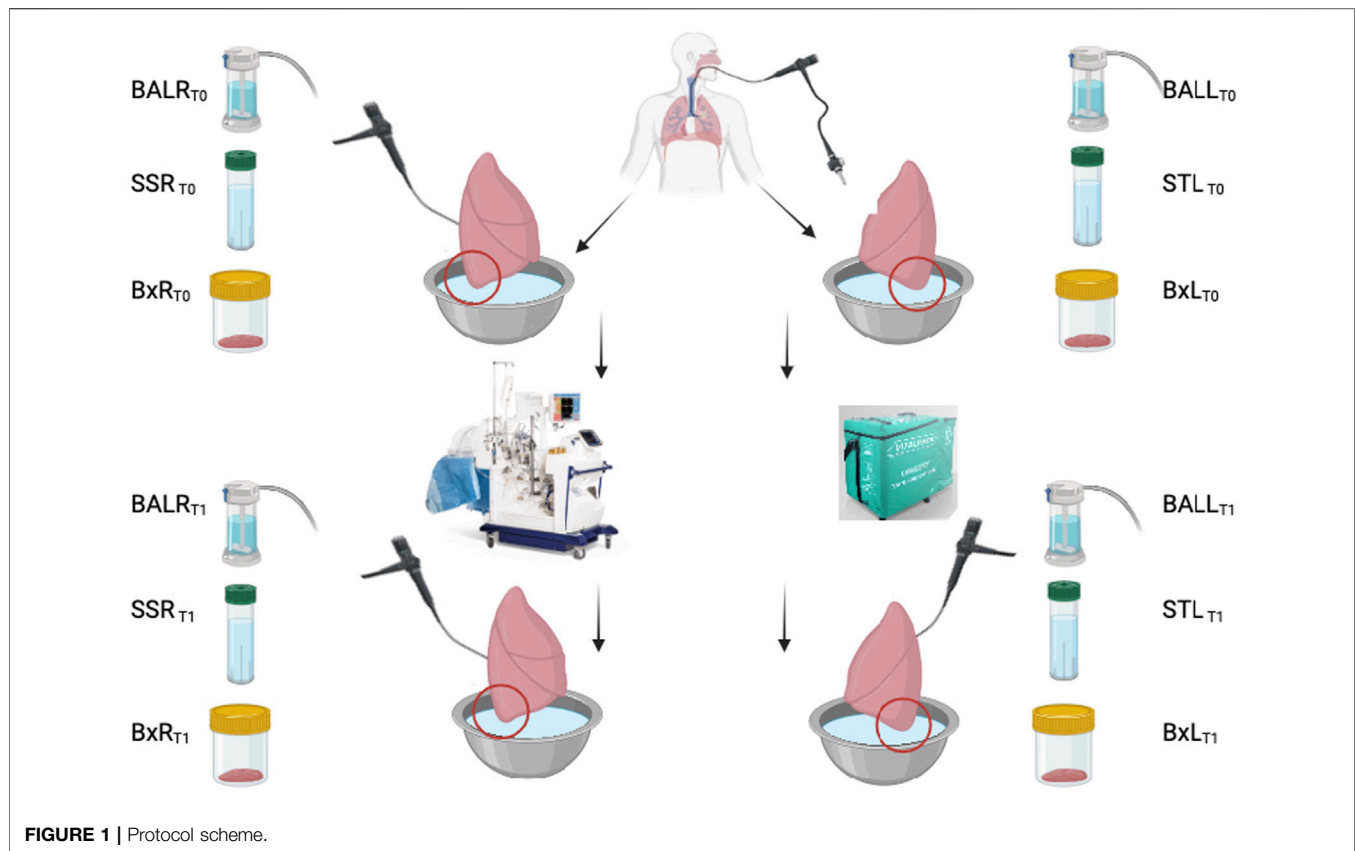
After 3 h of cold storage, the storage procedure will be considered completed. The left main bronchus will be opened, a 50 mL BAL will be collected (BALL_{T1}), and a lower lobe lung biopsy (BxL_{T1}) will be collected and preserved with RNAlater. A sample of 50 mL of the transport solution (STL_{T1}) will be recovered.

EVLP Protocol and Sample Obtention (Intervention Group)

The right lung will be perfused for 3 h with LS[™](XVIVO Perfusion AB, Sweden).

Before EVLP, 50 mL of BAL from the main right bronchus (BALR_{T0}) and 50 mL of perfusion liquid (SSR_{T0}) will be collected and one lung biopsy of 3 cm using a staple from the lower lobe will be taken (BxR_{T0}) and preserved with RNAlater.

The EVLP will be initiated 3 h after Lund's protocol [7]. The perfusion liquid will be 2L of Steen Solution[®] (XVIVO Perfusion AB, Sweden), 1 red blood cell pack of donor's isogroup, and 1g of meropenem.

**TABLE 1 |** Data collection.

Sample data	Donor data
• Microbiome before EVLP in right graft • Microbiome before cold storage in left graft • Microbiome after EVLP in right graft • Microbiome after cold storage in left graft • RNA before EVLP in right graft • RNA before cold storage in left graft • RNA after EVLP in right graft • RNA after cold storage in left graft • Cytokines after EVLP in right graft • Cytokines after cold storage in left graft	• Age • Gender • Cause of death • Length of stay in ICU • Days of intubation • Intubation site • Cause for rejection of lung donation • Respiratory cultures • Antibiotic therapy • History of bronchoaspiration • PaFi before the retrieval (FIO₂ 1 and PEEP 5)

After 3 h of EVLP, the protocol will be finalized. A BAL of 50 mL from the right main bronchus (BALR_{T1}) and a sample of 50 mL of the perfusion solution (SSR_{T1}) will be collected; one lung biopsy of the lower lobe will be taken (BxR_{T1}) and preserved with RNAlater.

Data Collection and Sample Analysis

The data collection will be from the sample and the donor characteristics (Table 1). Table 1 lists what data will be collected in both groups.

Microbiome Analysis

For the extraction of genomic DNA, the protocol that was previously used in the MetaHIT project will be applied [8]. For the analysis of microbial diversity, the V4 hypervariable region of the 16S rRNA gene will be amplified from bacterial DNA by PCR using the universal primers described in Pozuelo et al. [9]. Amplicons will be sequenced using Illumina (MiSeq) technology.

To investigate the microbial composition of each sample, raw sequence reads will be demultiplexed using the idem tool. The resulting single-end reads will be processed to obtain an amplicon sequence variant (ASV) table, to which taxonomy will be assigned by using SILVA 16s rRNA database (v. 132). Alpha diversity metrics, including Observed and Shannon diversity indices, and beta diversity metrics such as the Bray Curtis and Aitchison distance indices are going to be calculated. The Aitchison distance between samples will be calculated using the CodaSeq (v.0.99.6) and zComposition (v. 1.3.4) R packages. Data is going to be normalized by transforming the raw counts to centered log-ratios (clr).

Analysis of Genetic Expression

RNA extraction will be performed using the commercial RNeasy Mini Kit (QIAGEN). Gene expression analysis will be performed by quantitative PCR (qPCR) using the predesigned TransplantRejection panel from SignArray (AnyGenes®, Paris, France), which includes 84 genes that have been described to be

related to the immune response in transplant rejection. The genes included in the panel are CX3CR1, ICAM1, ITGA2, ITGAE, ITGAM, PECAM1, THBS1, THBS2, VCAM1, COL1A2, CCR5, CCR7, CD40, CD40LG, CD80, CD86, CTLA4, CXCR3, STAT4, TGFB1, CD44, CTGF, MMP1, MMP2, MMP7, MMP9, BMP7, CCL11, CCL2, CCL3, CCL4, CCL5, CSF2, CXCL10, IFNG, IL10, IL12A, IL13, IL16, IL1B, IL2, IL2RA, IL3, IL32, IL4, IL5, IL6, IL8, TNF, TGFB2, TGFB3, TIMP1, VEGFA, MS4A1, CXCL11, CXCL9, CXCR4, ADAM17, C3, CASP1, CASP3, CASP8, CCR2, CCR3, CD14, CD28, CD8A, FAS, FASLG, FCGR1A, GZMA, GZMB, NFKB1, NOS2, PRF1, PSMB9, STAT1, STAT6, TAP1, TLR3, TLR4, TLR9, TNFAIP3, and TNFSF10.

Cytokine Analysis

The determination of cytokines in the perfusion fluid will be carried out using immunoassays based on Luminex™ xMAP™ technology (multi-analyte profiling). We will use panels designed specifically for the gene products of interest. Cytokine levels are measured using an immunoassay based on Luminex™ xMAP™ technology, which allows multiparametric analysis of the different cytokines. The cytokines analyzed in the panel designed for this purpose are IFN gamma, IL-1 beta, IL-6, IL-8 (CXCL8), IL-18, IP-10 (CXCL10), MCP-1 (CCL2), MIP-1 alpha (CCL3), TNF alpha, and VEGF-D (Custom Procartaplex Multiplex Panel, Invitrogen, ThermoFisher Scientific, MA, United States). The immunological analysis will be carried out using the Invitrogen ProcartaPlex Analyst 1.0 software, supplied with the reagents. The trials will be carried out blind to the clinical evolution of the recipients.

Statistical Analysis

Since this is a pilot study, no formal sample size calculation was undertaken. Categorical variables are going to be expressed as number of cases and percentage and will be compared using chi-square test or Fisher's exact test. After checking for normality using the Shapiro-Wilk test, continuous variables will be expressed as median and interquartile range (IQR) or mean $\pm$ standard deviation as appropriate. To compare variables across groups, Mann-Whitney U test or Student's t-test are going to be used. To compare within the same condition before and after preservation time points, Wilcoxon signed rank test or paired t-test will be carried out. The inflammatory response will be reported by analyzing the quantitative PCR of the genetic expression of 84 genes related to immune response in lung biopsy and the concentration of cytokines in perfusion and preservation solutions before and after EVLP and cold storage.

DISCUSSION

PGD is the most common cause of 30-day mortality after lung transplantation and the second most common after the first year [8]. It is a complex syndrome characterized by severe hypoxemia and pulmonary edema occurring within the initial 72 h post-transplantation [9]. Understanding the underlying pathophysiological mechanisms, including ischemia-reperfusion injury, innate immune activation, endothelial

dysfunction, and inflammatory response [10], is essential for developing targeted therapeutic strategies that can mitigate PGD-associated complications. Furthermore, elucidating predictive biomarkers and risk factors for PGD may enable risk stratification and preventive interventions, thereby potentially improving graft and patient outcomes.

The pulmonary microbiome has emerged as a significant factor in respiratory health and disease [11]. Its role extends beyond local immune modulation within the lungs to potentially impacting systemic health.

Sharma et al. [4] observed that some microbiome signatures of distal airways promote an inflammatory reaction and contribute to the development of BOS. Prakash et al. [12] found in a murine model that inflammation generated by left lung ischemia-reperfusion dissipated after antibiotic treatment due to the depletion of intestinal microbiota. Also, alveolar macrophages can be modulated by the microbiota. Bernasconi et al. [13] showed that the bacterial dysbiosis between proinflammatory and anti-inflammatory communities were linked to inflammatory and remodeling processes. Furthermore, recent studies from McGinnins et al. [14] found a relationship between the presence of dysbiosis with an alteration *Prevotella/Streptococcus* ratio and severe PGD after lung transplantation.

EVLP is a form of isolated lung perfusion in normothermic conditions and can be achieved with a pump-driven perfusion machine that recirculates a preservation solution through the vasculature of the lung in addition to mechanical ventilation. This technology was initially developed to evaluate pulmonary donor grafts prior to transplantation and was successfully introduced in clinical practice. In addition, there is also growing evidence that EVLP could serve as a potential dynamic preservation strategy; it could also serve as a platform to actively resuscitate or recondition lungs while metabolically active. Currently, two different main protocols exist: the Toronto and Lund protocols. The question of whether the Lund or Toronto protocol for *ex vivo* lung perfusion (EVLP) is superior is complex and multifaceted, as both protocols have shown significant advantages and benefits in different aspects of lung transplantation.

The Toronto protocol, which has been widely studied and implemented, emphasizes the use of normothermic EVLP to assess and recondition marginal donor lungs, thereby increasing the pool of suitable donor organs and improving transplant outcomes. This protocol has demonstrated encouraging short- and medium-term outcomes, with no significant difference in long-term mortality, retransplantation rates, or chronic lung allograft dysfunction (CLAD) when compared to conventionally preserved lungs [15].

On the other hand, the Lund protocol has also shown promise, particularly in its ability to achieve stable multiday preservation of donor lungs through advanced technical modifications such as perfusate enrichment, low-flow, pulsatile, subnormothermic perfusion, and personalized ventilation strategies. These innovations could potentially lead to even better outcomes by allowing for more sophisticated treatment modalities during EVLP. Furthermore, the development of new perfusates, such

as the DMEM-based nutrient-rich solution, has shown potential in enhancing cell viability and reducing apoptosis, which could be integrated into either protocol to further improve donor lung quality and quantity [16]. Overall, while both protocols have their strengths, the choice between them may depend on specific clinical scenarios and the availability of resources. One of the major differences between both protocols is the use of red blood cell packets. Current studies on the use of red blood cell packets in *ex vivo* lung perfusion (EVLP) highlight both their potential benefits. Red blood cells (RBCs) are traditionally used as oxygen carriers in EVLP due to their ability to support cellular metabolism during normothermic perfusion. We chose the Lund protocol with RBCs because this is our current EVLP protocol, and it will be our protocol chosen for long-term EVLP.

The lung inflammatory micro ambient can be influenced by several factors, including the type of donor, either DBD or cDCD, or the use of EVLP. Baciú [17] demonstrated that different donor-specific gene signatures depend on the type of donor and the use of EVLP. They found that lungs from donors who died from brain death (DBD) showed higher levels of inflammatory cytokines and pathways, indicating a strong inflammatory response in these lungs. In contrast, lungs from cDCD donors exhibited gene signatures related to cell death, apoptosis, and necrosis, suggesting these lungs are more prone to cell damage. They showed that EVLP could modify the transcriptomics. In DBD lungs, the tumor necrosis factor receptor-1/2 signaling pathways and macrophage migration inhibitory factor-associated pathways were activated. The study also identified a set of genes that can differentiate between DBD lungs treated with EVLP and those that were not. In our protocol, we will analyze the inflammatory parameters and different activated genes. To understand the influence of the microbiome we will compare them with the known genes.

EVLP presents a unique opportunity to explore the interactions between the pulmonary microbiome and graft viability. Despite the sterile conditions maintained during EVLP, the perfusion with antibiotics and Steen solution and the changes in temperature may cause dynamic changes in the lung microbiome [18]. Understanding the impact of EVLP on the pulmonary microbiome is essential to optimize transplant protocols and improve patient outcomes. Elucidating the complex interplay between *ex vivo* lung perfusion and the pulmonary microbiome may uncover novel strategies to

enhance graft viability, prevent post-transplant complications, and ultimately improve the success rates of lung transplantation.

In summary, this will be the first study designed to analyze the impact of EVLP on the lung microbiome and the local inflammatory response. Understanding the composition, diversity, and functional interactions of the pulmonary microbiome in lung transplants holds promise for personalized respiratory medicine.

ETHICS STATEMENT

The studies involving humans were approved by Comité Ético de la Investigación con Medicamentos. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IB, AS, AGó, SG, and RF contributed to the design of the study. IB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Low-Volume *Ex Situ* Lung Perfusion System for Single Lung Application in a Small Animal Model Enables Optimal Compliance With “Reduction” in 3R Principles of Animal Research

K. Katsirntaki^{1,2}, S. Hagner^{1,2}, C. Werlein³, P. Braubach³, D. Jonigk^{4,5}, D. Adam^{1,2}, H. Hidaji^{1,2}, C. Kühn^{1,2}, C. S. Falk^{4,6}, A. Ruhparwar^{1,2,4} and B. Wiegmann^{1,2,4*}

¹Department for Cardiothoracic, Transplantation and Vascular Surgery, Hannover Medical School, Hannover, Germany, ²Lower Saxony Center for Biomedical Engineering, Implant Research and Development (NIFE), Hannover, Germany, ³Institute of Pathology, Hannover Medical School, Hannover, Germany, ⁴Member of the German Center for Lung Research (DZL), Biomedical Research in Endstage and Obstructive Lung Disease Hannover (BREATH), Hannover, Germany, ⁵Institute of Pathology, RWTH Aachen Medical University, Aachen, Germany, ⁶Institute of Transplant Immunology, Hannover Medical School, Hannover, Germany

OPEN ACCESS

*Correspondence

B. Wiegmann,
✉ wiegmann.bettina@mh-hannover.de

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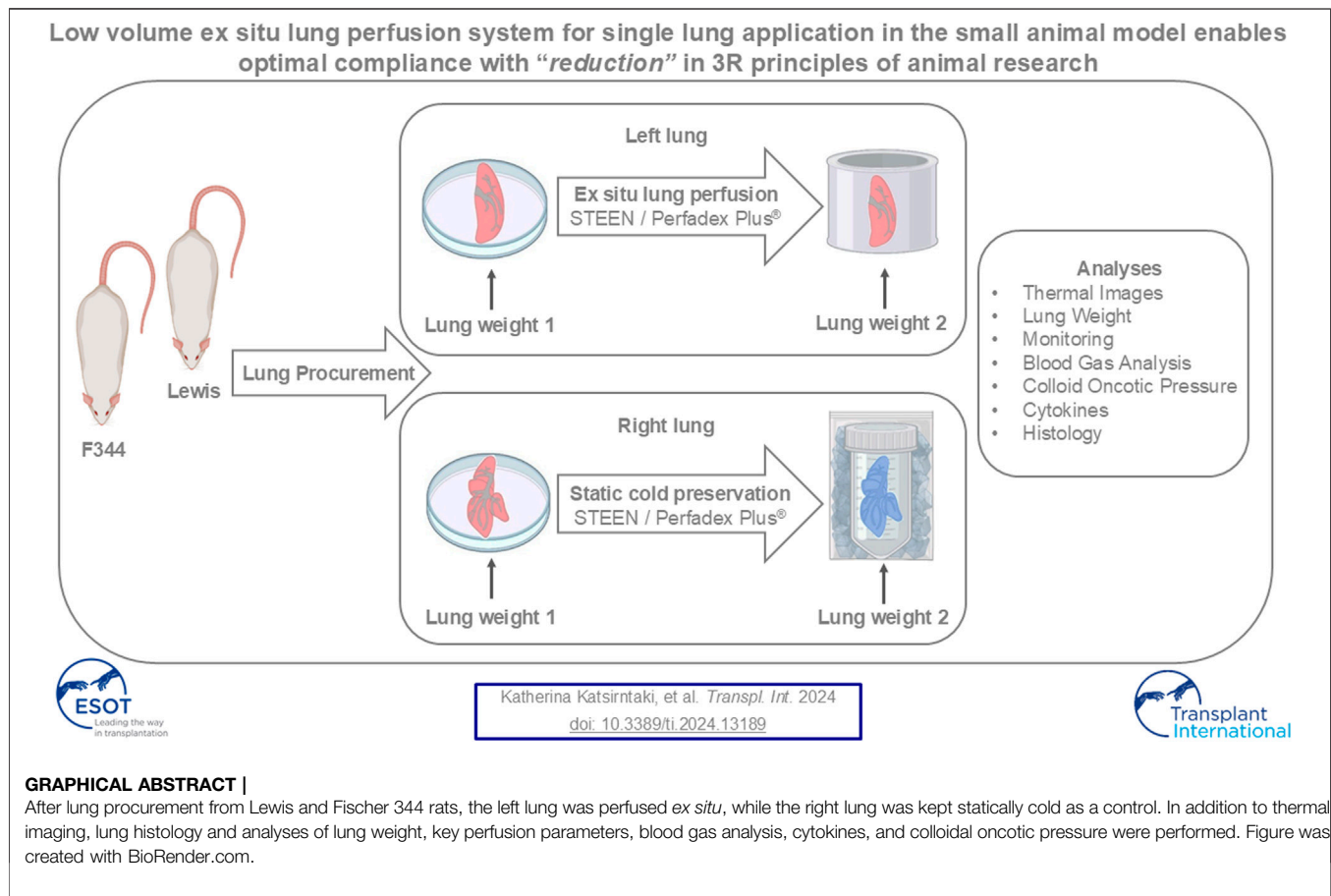
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Ex situ lung perfusion (ESLP) is used for organ reconditioning, repair, and re-evaluation prior to transplantation. Since valid preclinical animal models are required for translationally relevant studies, we developed a 17 mL low-volume ESLP for double- and single-lung application that enables cost-effective optimal compliance “reduction” of the 3R principles of animal research. In single-lung mode, ten Fischer344 and Lewis rat lungs were subjected to ESLP and static cold storage using STEEN or PerfadexPlus. Key perfusion parameters, thermal lung imaging, blood gas analysis (BGA), colloid oncotic pressure (COP), lung weight gain, histological work-up, and cytokine analysis were performed. Significant differences between perfusion solutions but not between the rat strains were detected. Most relevant perfusion parameters confirmed valid ESLP with homogeneous lung perfusion, evidenced by uniform lung surface temperature. BGA showed temperature-dependent metabolic activities with differences depending on perfusion solution composition. COP is not decisive for pulmonary oedema and associated weight gain, but possibly rather observed chemokine profile and dextran sensitivity of rats. Histological examination confirmed intact lung architecture without infarcts or hemorrhages due to optimal organ procurement and single-lung application protocol using our in-house-designed ESLP system.

Keywords: rat model, *ex vivo* lung perfusion/EVLP, *ex situ* lung perfusion/ESLP, small animal model, single lung

Abbreviations: BGA, Blood gas analysis; BW, Body weight; COP, Colloid oncotic pressure; DLTx, Double lung transplantation; ESLP, *Ex situ* lung perfusion; F344, Fischer F344 rat; HE, Hematoxylin and eosin staining; I:E, Inspiration:expiration time; IRI, Ischemia/reperfusion injury; LA, Left atrium; Lew, Lewis rat; Min, Minutes; OCS, Organ Care System Lung; PA, Pulmonary artery; PAP, Pulmonary artery pressure; PEEP, Positive end-expiratory pressure; RR, Respiratory rate; SCS, Static cold storage; THAM, Tris(hydroxymethyl)aminomethane; TV, Tidal volume.



INTRODUCTION

For patients with end-stage lung disease, lung transplantation (DLTx) currently remains the only curative treatment option and is reserved for highly selected patients, particularly due to the increasing mismatch of potential organ donors to recipients [1, 2]. However, DLTx is also associated with many challenges, specifically challenges surrounding the gold standard of static cold preservation (SCS), which contributes to limited ischemic time and ischemia/reperfusion injury (IRI), which in turn result in acute life-threatening graft failure. This is what leads to the sobering 5-year survival rate of approximately 60% [2]. To address these problems, there is a global focus on *ex situ* lung perfusion (ESLP), which allows temperature-adapted perfusion with blood-like preservation solutions in an almost physiological milieu [3–6]. This ensures *ex situ* the maintenance of organ function and, compared to SCS, shows significantly better outcomes after DLTx [3–6]. This is based in particular on the significant reduction of ischemia time, which in turn decisively minimizes IRI and allows longer organ preservation and thus greater geographic flexibility for donor pool expansion. In addition, diagnosis-dependent *ex situ* therapies and consecutive re-evaluations can be used to convert non-transplantable organs into transplantable organs [7, 8]. The two most common ESLP systems in clinical use are the portable Organ

Care System Lung (TransMedics, Inc.; OCS), which uses PerfadexPlus solution with an open left atrium (LA) strategy and allows direct implantation of the harvested lung in the donor hospital, and the XPS system (XVIVO Perfusion; XPS) [4, 5, 8], which uses STEEN™ solution with a cannulated LA as a closed circuit according to the Lund or Toronto protocol, whereby the explanted lung is initially stored statically hypothermically, which is the gold standard of organ preservation and bridges the time of transport from the donor hospital to the recipient hospital where the XPS is stationed [9]. In addition, the three protocols differ in terms of the time at which ventilation is started, the tidal volume and respiratory rate applied, and the perfusion rate and mean pulmonary artery pressure (mPAP) value to be tolerated [4, 5, 8, 9].

In the last decade, there has been a significant increase in the number of studies using these ESLP techniques, some aiming to establish its application for autologous and xenogeneic application, for example, for tumor and infection therapies, as well as for surgical, regenerative, and immunomodulatory procedures [8, 10]. This is accompanied by efforts to create preclinical animal models of ESLP for significant translational studies [11, 12]. These include both large and small animal models, especially rat models, which are characterized by relevant advantages over porcine models [12, 13]. Most researchers working with these rat models use the commercially available Harvard apparatus [12–16], which is

technically similar to the non-portable, closed XPS system and is used to mimic the Lund or Toronto protocol, whereby the double lung is subjected to cold ischemia prior to ESLP [12, 14, 15].

Summarized, this illustrates the gap in ESLP systems for rats, because to our knowledge there is currently no corresponding system that can adequately simulate the OCS technique, nor are there corresponding baseline data that initially compare the gold standards around ESLP. These data are essential to enable a detailed and reliable differentiation between ESLP- and SCS-induced as well as *ex situ* perfusion- and transplantation-associated changes in subsequent experiments [4, 5, 17–19]. In line with these requirements, we have established our ESLP, in which we have placed great emphasis on the development of a comparatively cost-effective system during technical optimization that enables optimal compliance with the increasingly relevant reduction of the 3R principles (replacement, reduction, refinement) of animal research.

MATERIALS AND METHODS

Rats, Husbandry and Care

All animal experiments were performed in strict accordance with the German Animal Welfare law and approved by the local authority. For the following experiments, adult male Lewis (Lew) (weight 269 ± 14 g) and Fischer344 (F344) (weight 236 ± 27 g) rats were housed in ventilated cage systems at $22^\circ\text{C} \pm 2^\circ\text{C}$ and $55\% \pm 10\%$ humidity with a 12 h dark/light cycle and were allowed free access to rat chow feed and water *ad libitum*.

Lung Procurement

Rats were anesthetized with 5-Vol% isoflurane-oxygen mixture, followed by subcutaneous analgesia, consisting of Butorphanol 2 mg/kg/body weight (BW) and Meloxicam 1 mg/kg/BW. Thereafter, orotracheal intubation was performed using a 14-gauge intravenous cannula so that mechanical, volume-controlled ventilation could be initiated with the following ventilatory settings: inspired oxygen fraction of 100%, respiratory rate (RR) 100/min, tidal volume (TV) 6 mL/kg/BW, inspiration:expiration (I:E) time of 1:2, and positive end-expiratory pressure (PEEP) 5 cmH₂O. After transferring the rat to the temperature-controlled heating mat, intraperitoneal anesthesia was performed with ketamine 60 mg/kg/BW and medetomidine 125 µg/kg/BW, so isoflurane-oxygen mixture was reduced to 1-Vol%. Median laparotomy was conducted to expose the abdominal aorta and inferior vena cava; 1,000 IU/kg/BW heparin sodium was administered. Baseline arterial and venous blood was collected, specifically for blood gas analysis (BGA), cytokine analysis, and colloid oncotic pressure (COP) determination, followed by exsanguination. Median sternotomy was performed and thermal baseline lung imaging was taken. In preparation for pulmonary flush, the ascending aorta and pulmonary trunk were separated so that a suture could be placed around the pulmonary trunk to secure the 16-gauge cannula, which was inserted into the right outflow tract via the right ventricle. After cutting superior and inferior vena cava and opening the LA, lungs were flushed pump-controlled with weight-adjusted 4°C cold PerfadexPlus (100 mL/kg/BW), containing 0.25 µg/kg/BW

Ventavis and 12 µL mL Tris(hydroxymethyl)aminomethane (THAM). After the second thermal lung imaging, the trachea was clamped and heart-lung block removed and placed on a Petri dish to separate the left and right lungs. Following weight determination and photo documentation, the right lung was stored in 4°C cold PerfadexPlus or STEEN, while the left lung was used for ESLP for the same period of 120 min (min). For this, a 24-gauge cannula was fixed in the pulmonary artery and a 16-gauge cannula was inserted via the left main bronchus to adapt the lung to ESLP.

Ex Situ Lung Perfusion In-House-Designed ESLP System

A schematic overview of our in-house-designed ESLP for single- or double-lung application is depicted in **Figure 1**. The system consists of the following components, whose functions are harmonized and can be monitored separately. Briefly described, the retrieved left lung was connected to the lid of the organ chamber via arterial and bronchial cannulas for single-lung perfusion. Starting from the 42°C thermostatic water bath, heated water circulated through the double-walled organ chamber, the reservoir, and the in-house-designed heat exchanger to keep both the perfusate and lungs at a constant temperature. Perfusion rate was set via the built-in pump and ventilation via the integrated respirator. By integrating three-way stopcocks, both blood sampling and monitoring were possible and supplemented by a digital pH meter in the reservoir and an integrated digital thermometer. Components were connected by silicone tubes, resulting in a priming volume of 17 mL.

ESLP

The circuit was primed with PerfadexPlus or STEEN, and samples were taken to determine baseline perfusate values. Antegrade perfusion was initiated at a flow rate of 0.675 mL, which was gradually increased to 3–4 mL/min while maintaining systolic pulmonary artery pressure (PAP) below 30 mmHg. After 5 min ESLP, the first samples were taken and documented every half or full hour, except cytokine analysis, which was only performed at the beginning and end of ESLP. After 15 min, thermal lung imaging was taken before starting ventilation at half TV using the gas mixture of O₂ (12%), CO₂ (5.5%), and N₂ (82.5%), corresponding to the preservation gas during OCS procedure [4–6]. pH was kept between 7.35 and 7.45 by continuous monitoring and adjusted addition of THAM.

In parallel, the right lung was placed under SCS and preservation solution was taken at different time points for BGA, COP, and cytokine analyses. After 120 min, the experiments were terminated, and the left and right lungs were photographed and weighed before being stored in 4% buffered formalin for histomorphological analysis.

Analyses

Thermal lung imaging was taken at the time points indicated using the Infra Tec VarioCam HDx research 645S infrared camera, offering a geometric resolution of 79,620 pixels per image. To calculate average lung surface temperature, thermal data at each lung pixel point was recorded and analyzed using IRBIS 3.1 plus software before tracing the lung structure under investigation to extract and calculate the corresponding thermal data.

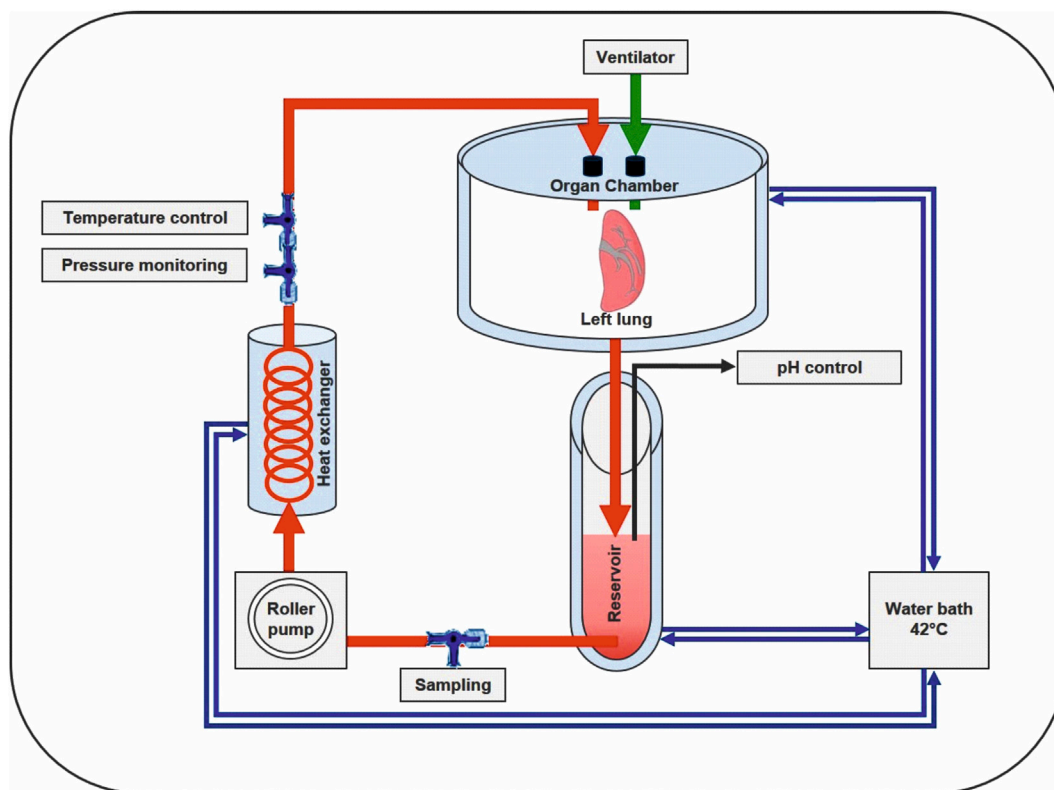


FIGURE 1 | Schematic overview of the in-house-designed rat ESLP system. The left lung of Lewis and Fischer 344 rats was treated with ESLP for 120 min, while the right lung was treated with SCS for the same period of time. The circuit comprised the double-walled organ chamber, the reservoir with the corresponding pH control unit, the roller pump, the heat exchanger, and the water bath. Temperature and pressure were controlled via integrated three-way stopcocks, which were connected to the corresponding monitoring system.

Lung weight was determined on a scale at the beginning and end of each ESLP and SCS procedure. Weight gain was calculated as: $\text{weight}_{\text{beginning}} - \text{weight}_{\text{end}}$.

Monitoring analyses associated with ESLP included the pump-generated perfusate flow, PAP using the connected pressure transducer, pH, and perfusate temperature. The perfusion solution was adjusted to a constant pH of 7.35–7.45 using THAM.

Perfusate analyses comprised BGAs, including analyses of partial pressures of oxygen (pO_2) and carbon dioxide (pCO_2), as well as potassium, sodium, glucose, and lactate using the ABL90 FLEX blood gas analyzer. The colloidal oncotic pressures were measured with the BMT 923 oncometer.

Various cytokines were collected in the perfusate and centrifuged at 1,800 rpm for 5 min at 4°C. Supernatants were then stored at -20°C until they were analyzed using the Bio-Plex Pro Rat Cytokine Group I Panel.

For morphohistological assessment, lungs were embedded in paraffin, and 2 μm thick sections were made and stained with hematoxylin and eosin (HE). Semi-quantitative assessments of atelectasis, oedema (perivascular, intra-alveolar, and interstitial), dilated alveolar spaces, and signs of intravascular perfusion solution were performed, with the experimental setup and rat strain blinded and all findings scored 0 (absent) and 1 (present).

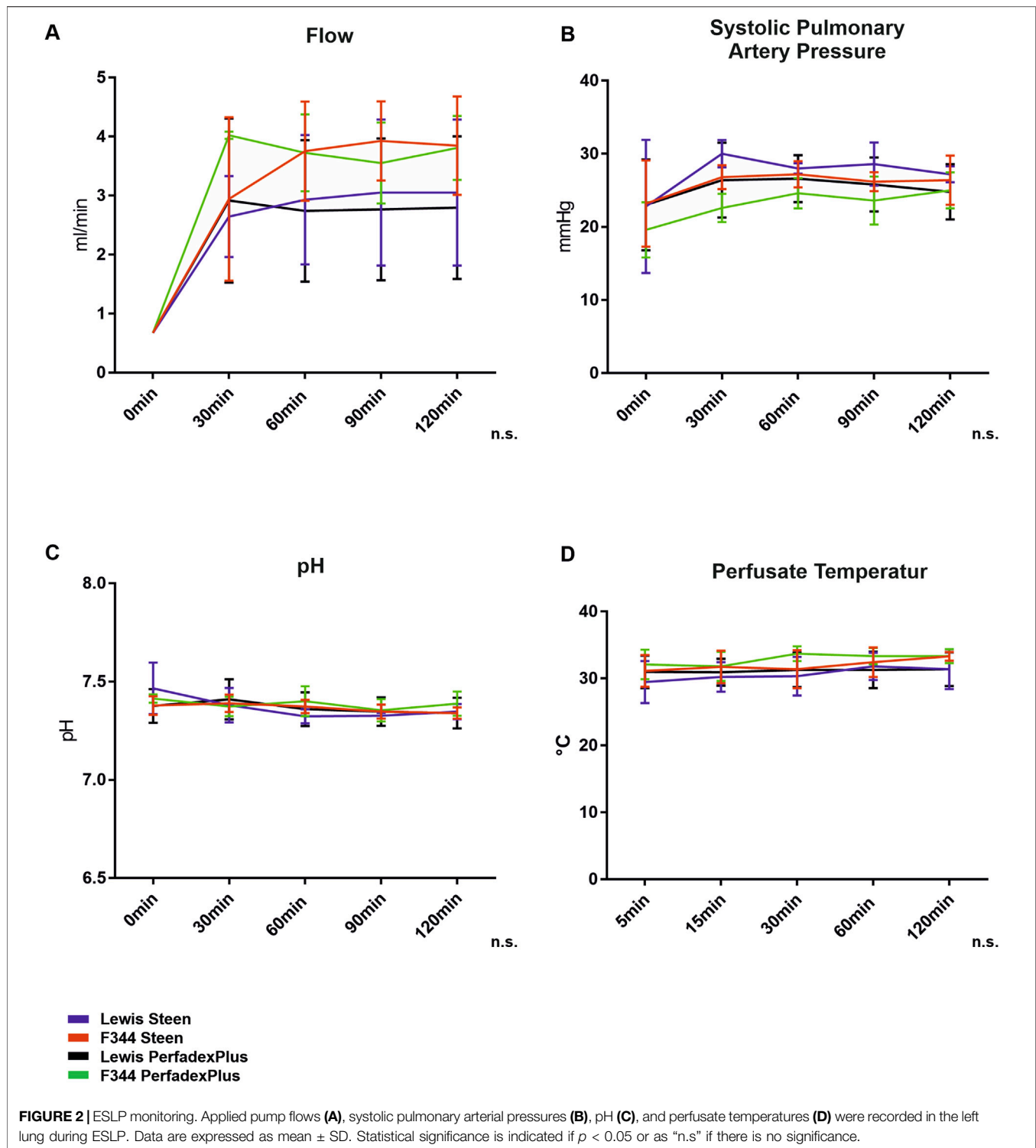
Statistical Analyses

Statistical analysis was performed using GraphPad Prism version 9 (San Diego, United States). Data sets from five independent replicates ($n = 5$) were used for each analysis. All numerical data were expressed as mean and standard deviation (SD) or standard error of the mean (SEM) and analyzed for significance using two-way ANOVA with Tukey multiple comparison test or one-way ANOVA with non-parametric Kruskal-Wallis and Dunn multiple comparison test for weight gain. $P < 0.05$ was considered statistically significant and marked with corresponding asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

RESULTS

Key Perfusion Parameters Confirm Valid ESLP for Single-Lung Application

All 20 single lungs showed stable key perfusion parameters over the entire ESLP period without significant differences (Figure 2). Perfusate flow was successively increased to the target flow within 30 min and remained constant throughout ESLP (Lew/STEEN 2.471 ± 1.018 mL/min; F344/STEEN 3.0 ± 1.4 mL/min; Lew/PerfadexPlus 2.4 ± 1.0 mL/min; F344/PerfadexPlus 3.2 ± 1.4 mL/min).



min; **Figure 2A**). Systolic PAP increased in parallel and reached its maximum perfusate flow and remained constant (Lew/STEEN 27.3 ± 2.7 mmHg; F344/STEEN 26.0 ± 1.6 mmHg; Lew/PerfadexPlus 25.3 ± 1.5 mmHg; F344/PerfadexPlus 23.1 ± 2.2 mmHg; **Figure 2B**). THAM addition secured physiological pH at all times (**Figure 2C**), whereas test groups with STEEN required significantly less THAM than

PerfadexPlus groups (Lew/STEEN 18.0 ± 11.2 μ L; F344/STEEN 10.0 ± 11.0 μ L; Lew/PerfadexPlus 57.0 ± 25.0 μ L; F344/PerfadexPlus 53.4 ± 12.8 μ L). Mean perfusate temperature remained at stable levels of $30.7^\circ\text{C} \pm 0.9^\circ\text{C}$ for Lew/STEEN, $32.0^\circ\text{C} \pm 0.9^\circ\text{C}$ for F344/STEEN, $31.2^\circ\text{C} \pm 0.2^\circ\text{C}$ for Lew/PerfadexPlus, and $32.9^\circ\text{C} \pm 0.8^\circ\text{C}$ for F344/PerfadexPlus (**Figure 2D**).

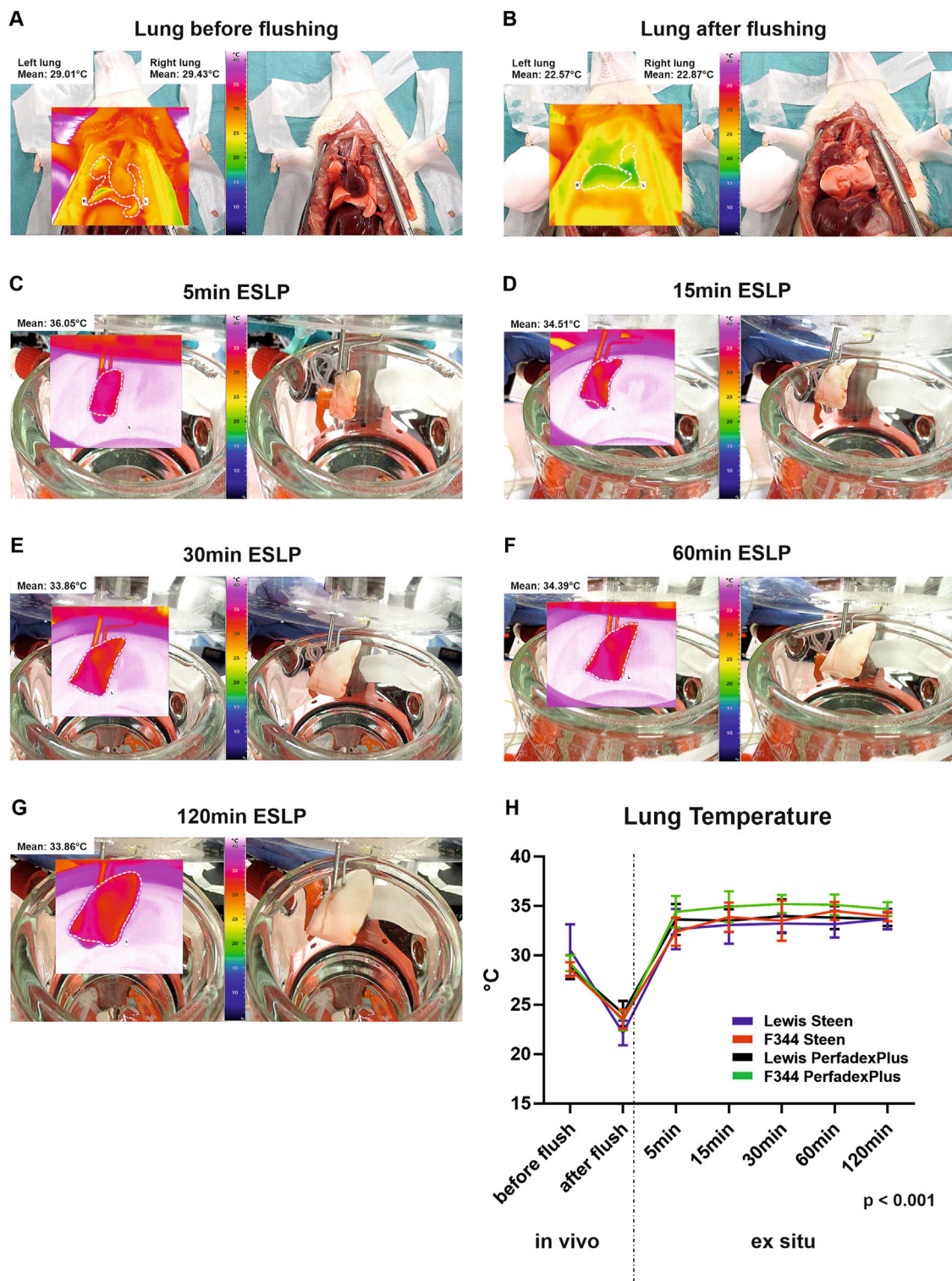


FIGURE 3 | Thermographical data acquisition and analysis of the *ex situ* perfused lungs. Representative native photos and corresponding thermal images of the lungs are shown at various time points as well as the respective average temperature of the lung surface (A–G). Graphical representation shows the surface temperatures of the different test groups over time (H). Data are expressed as mean $\pm$ SD. Statistical significance is indicated if $p < 0.05$ or as “n.s” if there is no significance.

Optimal Thermoregulation Ensures Uniform Lung Temperature as a Sign of Homogeneous Lung Perfusion

Representative thermal lung imaging is shown in **Figures 3A–G**, indicating a first temperature drop down to approximately 22°C due to cold flush (**Figures 3A, B**). Left lungs show stable surface temperatures as early as 5 min after ESLP start, which averaged 29.01°C (Lew/STEEN 31.2°C ± 4.1°C; F344/STEEN 31.5°C ± 4.0°C; Lew/PerfadexPlus 30.5°C ± 3.1°C; F344/PerfadexPlus 32.5°C ± 4.5°C; **Figure 3C**) and remained constant over the entire ESLP duration (**Figure 3H**). There was only one significant difference between F344/PerfadexPlus (32.5°C ± 4.5°C) and Lew/PerfadexPlus (30.5°C ± 3.1°C) after 15 min ESLP. Representative thermal lung imaging of the right lungs is shown in **Supplementary Figures 1A, B**. Before SCS began, mean temperature was 18.6°C, which decreased to 9.8°C during the 120 min cooling period. These values are exemplary, as there were no significant differences between the test groups (**Figure 1C**).

Blood Gas Analyses Revealed Temperature-Dependent Metabolic Activities With Significant Differences Based on Perfusion Solution Compositions

Left lung blood gas analyses revealed no significant differences for pO₂ (164.0 ± 2.5 mmHg) and pCO₂ (16.7 ± 1.6 mmHg), with constant values over the entire ESLP (**Figure 4A**). Sodium levels increased over the course of ESLP in all groups, with STEEN solution groups starting at 152.8 mmol/L (Lew/STEEN 153.0 ± 0.7 mmol/L; F344/STEEN 152.6 ± 0.6 mmol/L) and increasing to 163.4 mmol/L (Lew/STEEN 164.0 ± 4.5 mmol/L; F344/STEEN 162.8 ± 3.1 mmol/L), while PerfadexPlus groups started at 140.1 mmol/L (Lew/PerfadexPlus 140.0 ± 0 mmol/L; F344/PerfadexPlus 140.2 ± 0.5 mmol/L) and increased to 149.5 mmol/L (Lew/PerfadexPlus 149.4 ± 3.5 mmol/L; F344/PerfadexPlus 149.6 ± 4.0 mmol/L), so that significant differences were observed between different perfusion solutions but not between different rat strains. The same significant differences were found for potassium, which had significantly higher levels with 5.8 mmol/L in PerfadexPlus groups (Lew/PerfadexPlus 5.6 ± 0.1 mmol/L; F344/PerfadexPlus 5.9 ± 0.1 mmol/L), compared to 4.5 mmol/L in STEEN groups (Lew/STEEN 4.5 ± 0.2 mmol/L; F344/STEEN 4.5 ± 0.1 mmol/L), with potassium levels remaining almost constant in all groups. In terms of glucose concentration, STEEN groups had higher values at 9.6 mmol/L (Lew/STEEN 9.4 ± 1.2 mmol/L; F344/STEEN 9.8 ± 0.9 mmol/L), which decreased to 8.4 mmol/L during ESLP (Lew/STEEN 8.1 ± 0.6 mmol/L; F344/STEEN 8.6 ± 0.8 mmol/L), while PerfadexPlus groups started at 4.9 mmol/L (Lew/PerfadexPlus 4.9 ± 0.1 mmol/L; F344/PerfadexPlus 5.0 ± 0.1 mmol/L) and dropped to 4.4 mmol/L (Lew/PerfadexPlus 4.9 ± 0.1 mmol/L; F344/PerfadexPlus 3.8 ± 0.6 mmol/L), also showing a significant difference between perfusion solutions but not between rat strains.

For right lungs, no significant differences between pO₂ (Lew/STEEN 261.5 ± 19.3 mmHg; F344/STEEN 264.2 ± 13.4 mmHg; Lew/PerfadexPlus 277.6 ± 17.0 mmHg; F344/PerfadexPlus 268.8 ± 5.4 mmHg) and pCO₂ (Lew/STEEN 19.6 ± 0.7 mmHg; F344/STEEN

19.1 ± 0.6 mmHg; Lew/PerfadexPlus 12.2 ± 0 mmHg; F344/PerfadexPlus 12.0 ± 0 mmHg) were seen (**Figure 4B**). Compared to left lungs, initial values of sodium (Lew/STEEN 152.8 ± 0.8 mmol/L to 152.6 ± 0.6 mmol/L; F344/STEEN 152.8 ± 0.5 mmol/L to 155.4 ± 5.4 mmol/L; Lew/PerfadexPlus 140.0 ± 0.7 mmol/L to 140.0 ± 0 mmol/L; F344/PerfadexPlus 139.4 ± 0.6 mmol/L to 140.2 ± 0.5 mmol/L), potassium (Lew/STEEN 5.6 ± 0.04 mmol/L to 5.9 ± 0.09 mmol/L; F344/STEEN 4.7 ± 0.05 mmol/L to 4.7 ± 0.2 mmol/L; Lew/PerfadexPlus 4.6 ± 0.05 mmol/L to 4.9 ± 0.15 mmol/L; F344/PerfadexPlus 5.6 ± 0.04 mmol/L to 5.8 ± 0.09 mmol/L), and glucose (Lew/STEEN 11.4 ± 0.12 mmol/L to 10.9 ± 0.4 mmol/L; F344/STEEN 11.3 ± 0.04 mmol/L to 10.8 ± 0.8 mmol/L; Lew/PerfadexPlus 4.9 ± 0.08 mmol/L to 4.9 ± 0.1 mmol/L; F344/PerfadexPlus 4.9 ± 0 mmol/L to 4.8 ± 0.05 mmol/L) remained constant during SCS. However, there were again significant differences between perfusion solutions but not rat strains.

Lactate was in the physiological range <0.5 mmol/L in all test groups, so that graphical representation was omitted. A different basic composition of STEEN and PerfadexPlus is shown in **Supplementary Table S1**.

COP During ESLP Is Not for a Decisive Cause of Pulmonary Oedema Development

COP also differed significantly between perfusion solutions but not between rat strains in ESLP lungs. Thus, initial and final COP were significantly lower in STEEN groups (Lew/STEEN 32.6 ± 12.5 mmHg and 27.4 ± 1.5 mmHg; F344/STEEN 33.5 ± 9.7 mmHg and 26.7 ± 2.1 mmHg) than in PerfadexPlus groups (Lew/PerfadexPlus 48.4 ± 1.9 mmHg and 42.4 ± 8.3 mmHg; F344/PerfadexPlus 47.3 ± 3.4 mmHg and 38.6 ± 8.1 mmHg). In comparison, weight gain in PerfadexPlus groups (Lew/PerfadexPlus 1.0 ± 0.4g and F344/PerfadexPlus 0.9 ± 0.5g) was considerably higher than in STEEN groups (Lew/STEEN 0.4 ± 0.07g and F344/STEEN 0.3 ± 0.16g) (**Figure 5A**). Comparable significant differences between STEEN and PerfadexPlus groups (Lew/STEEN 33.2 ± 5.1 mmHg and 38.0 ± 0.4 mmHg; F344/STEEN 35.3 ± 6.3 mmHg and 34.4 ± 2.5 mmHg; Lew/PerfadexPlus 48.3 ± 1.75 mmHg and 46.5 ± 1.2 mmHg; F344/PerfadexPlus 49.9 ± 1.1 mmHg and 49.7 ± 0.7 mmHg) were observed in COP analysis of SCS lungs, with no weight gain in either group (**Figure 5B**).

Histological Examination Confirmed Optimal Constellation of Lung Procurement and ESLP Protocol Using In-House-Manufactured ESLP System

Histological evaluation showed essentially intact lung architecture with no signs of infarction or hemorrhage. There were different degrees of oedema (perivascular, intraalveolar, and interstitial), expansion of alveolar spaces, and intravascular perfusate, but these were not significant between the different perfusion groups and rat strains of ESLP and SCS. Only the frequency of atelectasis differed significantly: none of the perfused and ventilated ESLP lungs showed atelectasis, while all non-perfused, non-ventilated SCP lungs showed atelectasis (**Figure 6**).

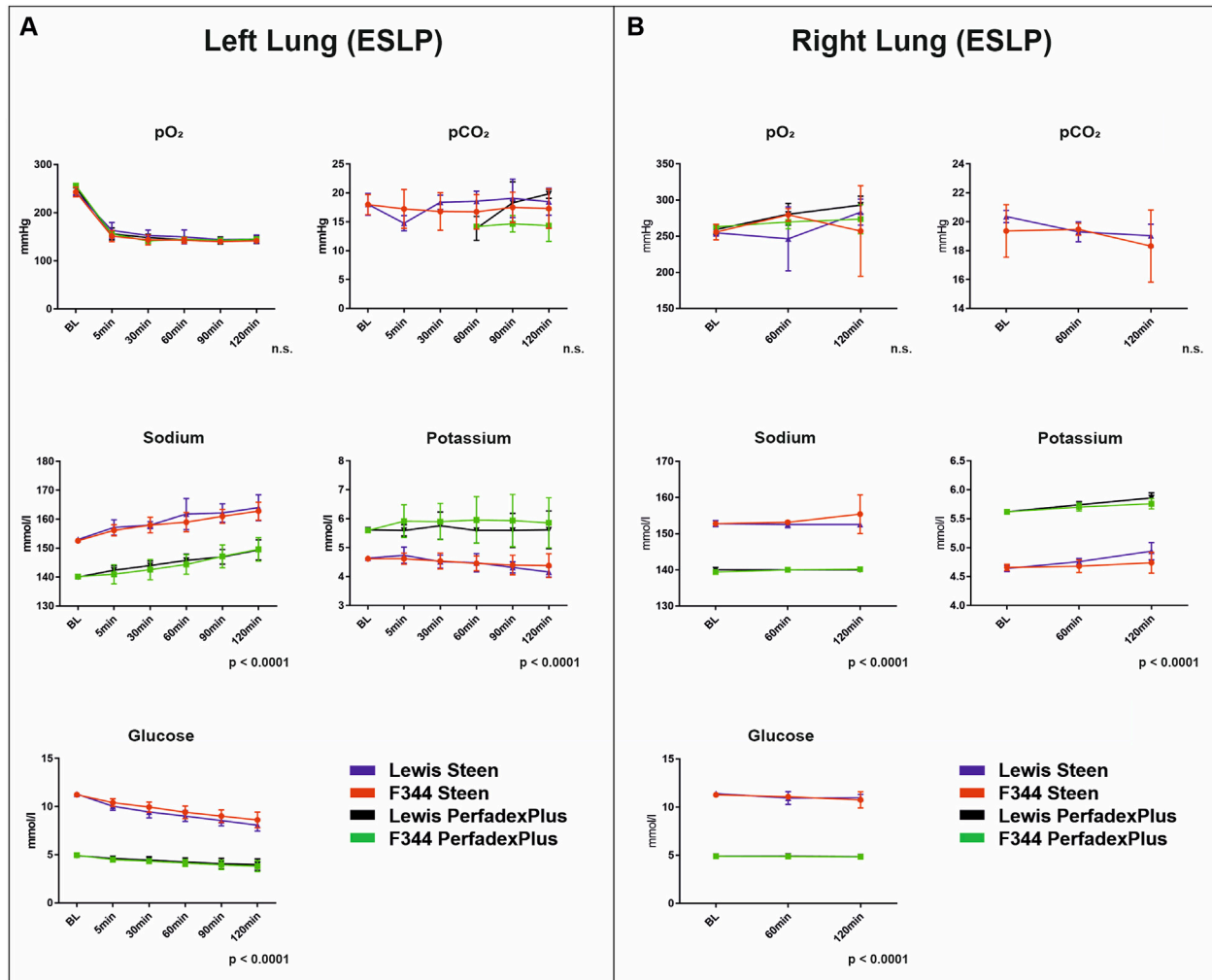


FIGURE 4 | Blood gas analyses. Time-dependent representation of pO₂ (mmHg), pCO₂ (mmHg), sodium (mmol/L), potassium (mmol/L), and glucose (mmol/L) of left lungs under ESLP (A) and right lungs under SCS (B). Data are expressed as mean ± SD. Statistical significance is indicated if $p < 0.05$ or as “n.s.” if there is no significance.

Cytokine and Chemokine Responses During 2-Hour ESLP

A broad spectrum of cytokines and chemokines was measured during ESLP (Supplementary Table S2). However, a relevant increase during 2-hour ESLP was only observed for CXCL1/KC (Lew/STEEN 57.3 ± 12.4 pg/mL; F344/STEEN 155.3 ± 48.6 pg/mL; Lew/PerfadexPlus 305.7 ± 180.4 pg/mL; F344/PerfadexPlus 186.7 ± 30.9 pg/mL), CCL2/MCP-1 (Lew/STEEN 56.3 ± 6.8 pg/mL; F344/STEEN 123.1 ± 29.7 pg/mL; Lewis/PerfadexPlus 190.1 ± 104.8 pg/mL; F344/PerfadexPlus 100.9 ± 12.0 pg/mL), CCL3/MIP-1a (Lew/STEEN 25.8 ± 9.6 pg/mL; F344/STEEN 186.4 ± 101.1 pg/mL; Lew/PerfadexPlus 61.8 ± 14.7 pg/mL; F344/PerfadexPlus 71.0 ± 18.9 pg/mL), CCL5/RANTES (Lew/STEEN 38.8 ± 9.1 pg/mL; F344/STEEN 66.0 ± 8.7 pg/mL; Lew/PerfadexPlus 44.5 ± 7.01 pg/mL; F344/PerfadexPlus 43.6 ± 2.7 pg/mL), and CCL20/MIP-3a (Lew/STEEN 10.1 ± 2.4 pg/mL; F344/STEEN 8.7 ± 1.3 pg/mL; Lew/PerfadexPlus

1.9 ± 0.9 pg/mL; F344/PerfadexPlus 1.0 ± 0.1 pg/mL). The values of CCL20/MIP-3a differed significantly between perfusion groups (Figure 7). Anti-inflammatory cytokines like IL-10 were not detectable during ESLP or SCS, in which no pro-inflammatory markers were observed either.

DISCUSSION

Originally developed to significantly reduce IRI and improve the outcome after DLTx, normothermic ESLP is now increasingly used for organ reconditioning, repair, and reassessment prior to DLTx [3–8]. In this context, valid preclinical large and small animal models are required to conduct translationally relevant studies. Small animal models, in particular rat ESLP, offer a number of advantages, especially with regard to fundamental statements on new diagnostic and therapeutic ESLP strategies

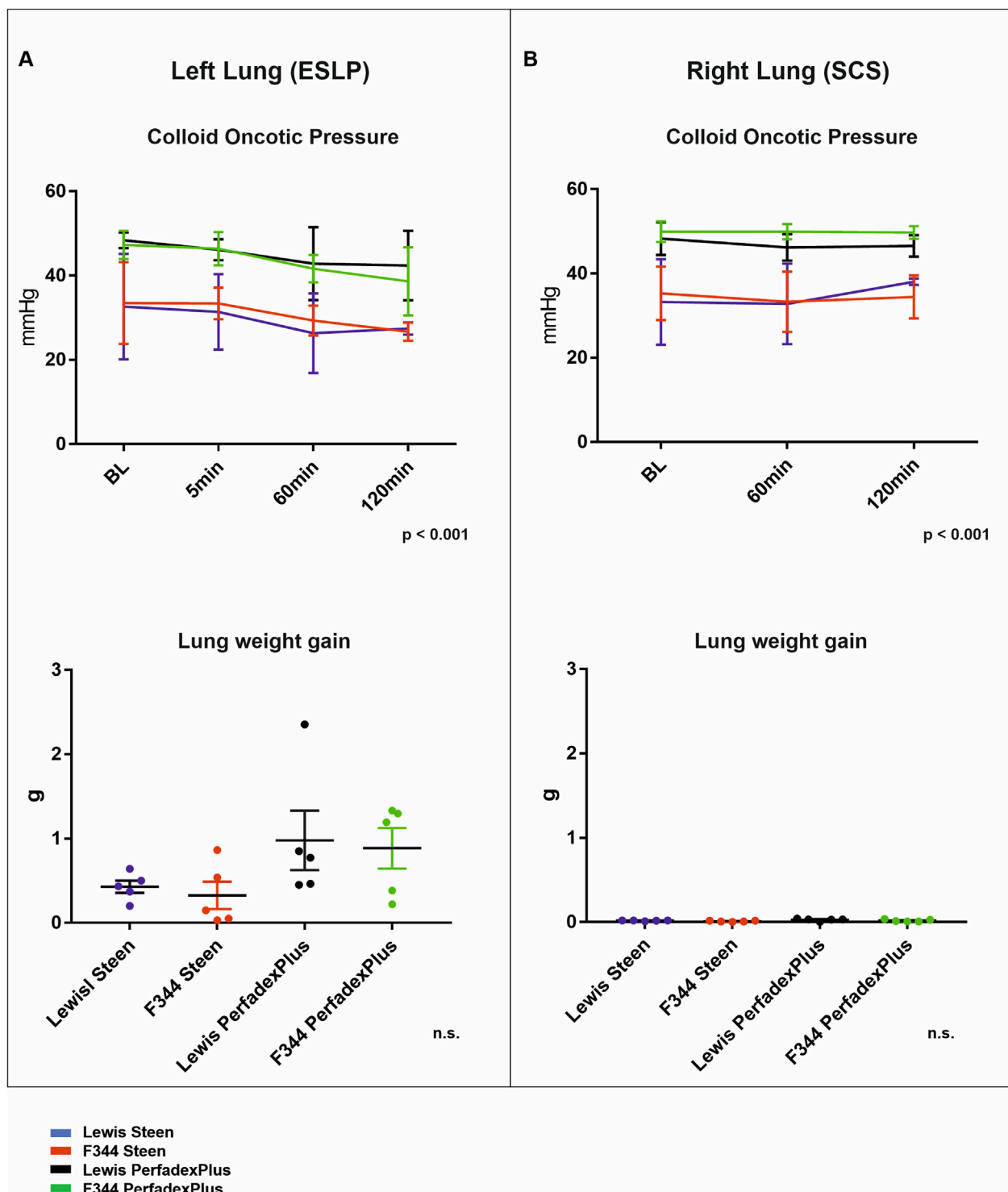


FIGURE 5 | Colloid oncotic pressure and lung weight gain. Time-dependent representation of colloid oncotic pressures (COD) and lung weight gain (g) of left lungs under ESLP (A) and right lungs under SCS (B). Data are expressed as mean $\pm$ SD for COD and mean $\pm$ SEM for lung weight gain. Statistical significance is indicated if $p < 0.05$ or as "n.s" if there is no significance.



FIGURE 6 | Overview of histological lung morphology and analysis of lung injury score. Representative images of hematoxylin-eosin-stained lungs ($\times 50$ magnification, scale bar 200 μm) and the classification of the lung injury score (0 = not present, 1 = present) by assessing the extent of atelectasis, oedema, widened alveolar spaces, and visible perfusate in the different test groups according to ESLP **(A)** and SCP **(B)**. Data are expressed as mean $\pm$ SD. Statistical significance is indicated if $p < 0.05$ or as "n.s." if there is no significance.

One of the most commonly used systems for this application is the commercially available ESLP system from Harvard Apparatus, which is designed for double-lung application by connecting the complete heart-lung block and whose initial priming volume of >150 mL is regularly exchanged in the open circuit at great expense, comparable to the Lund or Toronto protocol [3, 12, 21–23]. With the aim of having an ESLP available that is affordable both in purchase and for ongoing

experiments, we have developed our self-designed ESLP that can be used for both double- and single-lung application, working with a priming volume of 17 mL in a closed circuit. In single-lung mode, it offers the possibility to obtain both ESLP lung and negative SCS control from one animal, from which a sufficient amount of perfusate/blood mixture for ESLP can be generated according to human protocols without the need to euthanize another donor animal, in contrast to the Harvard Apparatus for ESLP in small animals, which is described and used almost exclusively in the literature and, where >3 animals must be euthanized for >150 mL priming [14]. In addition, it can only be operated in double-lung mode, so that an additional animal is required to generate the SCS control, which is then not from the same animal and can lead to a certain bias in evaluation [12, 14, 16]. Overall, the use of our ESLP enables optimal compliance with the *reduction* of the 3Rs principles of animal research. For

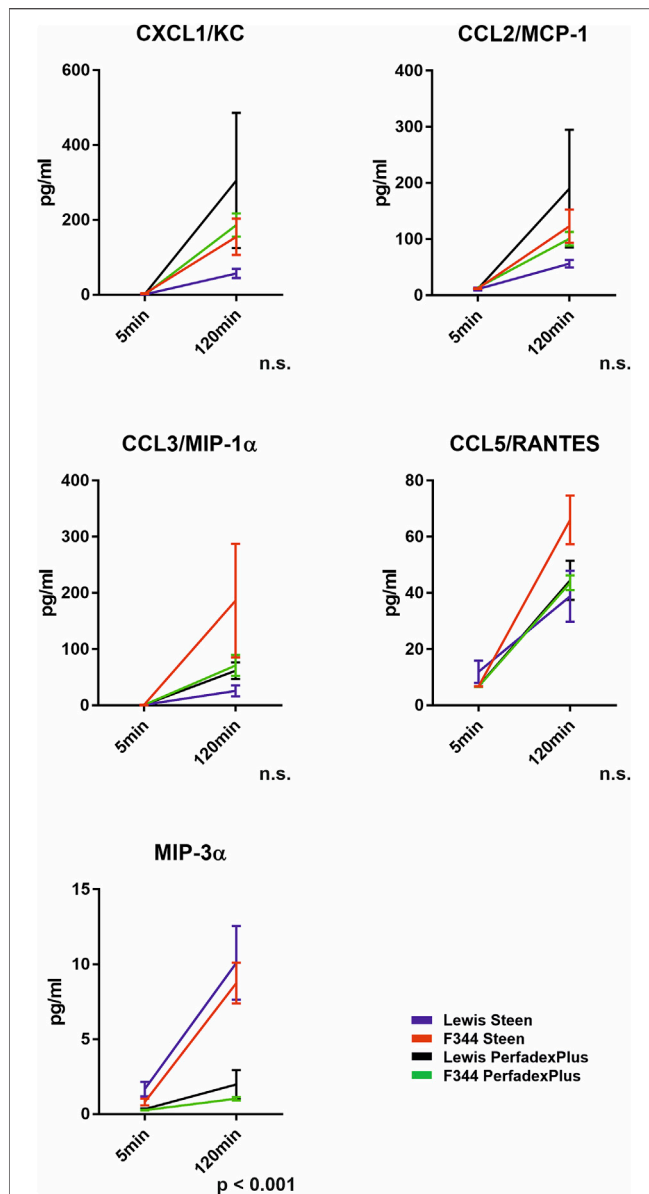


FIGURE 7 | Cytokine and chemokine analysis in the ESLP perfusate. Time-dependent representation of CXCL1/KC (pg/mL), CCL2/MCP-1 (pg/mL), CCL3/MIP-1α (pg/mL), CCL5/RANTES (pg/mL), and MIP-3α (pg/mL) of the left lung under ESLP. Data are expressed as mean ± SEM. Statistical significance is indicated if $p < 0.05$ or as “n.s” if no significance.

validation of our ESLP system and simultaneous establishment of a reliable, translation-relevant small animal model, we chose the rat strains Lew and F344, which are established for the induction of acute or chronic rejection reactions after transplantation [18, 19]. With PerfadexPlus and STEEN, we also used the gold standards and deliberately avoided further additives (e.g., Urbason) to provide relevant baseline data for future iterative experiments that will allow detailed and reliable differentiation between ESLP- and SCS-induced as well as exsitu perfusion- and transplantation-associated

changes [8]. Since our system fulfils the technical requirements and the literature almost exclusively describes the Lund or Toronto protocol with upstream warm or cold ischemia prior to ESLP, we decided to imitate the OCS protocol, supplemented by important potential improvements (e.g., systolic PAP and thermography) and standards defined in the literature for ESLP application in small animals and the adaptation of relevant parameters for the single-lung application tested here [4–6, 17, 24, 25].

Part of our study was also to establish our low-volume ESLP with an associated basic protocol, whereby we deliberately avoided the addition of potentially protective drugs in order to be able to make an unbiased statement about which constellation of transplant-relevant rat strains with which perfusion solution represents the best basic protocol. In the literature, ESLP times of 1–6 h are reported for small animal models, but ESLP must be terminated within the first hour in the event of massive oedema formation if no protective drugs such as Urbason are administered, so that an ESLP duration of 120 min was considered sufficient in our context [23]. The ESLP key parameters remained constant overall, confirming the successful establishment of a valid, reproducible system that can be used reliably in both rat strains and regardless of the perfusate used. The target flow of 20% of cardiac output defined in the literature was halved for the single-lung application in order to avoid hyperperfusion with consecutive hydrostatic oedema formation [13, 22, 23]. In addition to the physiological PAP, the barely measurable lactate values also confirmed sufficient perfusion in our ESLP for adequate cell supply.

As a non-invasive method, thermography can generally be used in ESLP in a variety of ways to not only detect but also localize areas with poor perfusion via thermal changes in surface temperature both in the rewarming and preservation phase, thus predicting lung function and differentiating between transplantable and non-transplantable lungs [26, 27]. The implementation of this technique in our protocol once again confirmed sufficient perfusion of the single lung due to the homogeneous surface temperature shown. Another advantage, especially of time-dependent thermography, was that we started ventilation when physiological lung temperature was reached, preventing temperature-dependent barotrauma, which was confirmed by histologically intact lung architecture and would otherwise have accelerated oedema formation [26, 27]. This cannot be guaranteed with the usual ESLP procedures of starting ventilation at a perfusate temperature of 32°C (XPS) or 34°C (OCS), or even independently of this at a specific time, as the systemic temperature loss can only be effectively compensated through precise technical coordination, e.g., in our case of the double-walled organ chamber, the heat exchanger and the 42-degree water bath, so that physiological lung temperature was guaranteed over the entire ESLP [3–8, 22].

In addition to effective perfusion and organ temperature, sufficient oxygen supply is the missing key component of valid ESLP to maintain cell metabolism, which was ensured by our single-lung-adapted ventilation. Thus, energy production was achieved via aerobic glycolysis, which was reflected in the constant glucose

consumption, continuous CO₂-increase within the citrate cycle, and the physiological, barely measurable lactate values [12, 23].

In ESLP, hyperoncotic perfusion solutions are used as standard to prevent oedema formation through intravascular COP increase based on albumin (STEEN) or dextran (PerfadexPlus), with the latter having significantly higher COP [28]. However, since the PerfadexPlus groups showed a significantly higher weight gain during ESLP, and our data could exclude hyperperfusion, pathologically increased intravascular pressure, and microinjury-induced barotrauma as causes, both dextran sensitivity described for rats and the time-, species-, and temperature-specific cytokine profile could be responsible for the weight gains we observed [16, 20, 22]. Analogous to the literature, we found a chemokine-heavy profile which, in addition to chemotaxis, could favorably influence oedema formation, in particular through the associated diapedesis [29]. Although weight gains of up to 30% described in the literature are comparable to our data, the omission of oedema-protective drugs (e.g., Urbason) led to the termination of the experiment within the first hour of ESLP in these studies due to massive oedema formation with fluid shift from intravascular to intraalveolar and interstitial and a resulting increase in COP [21–25]. In contrast, our study revealed a decrease in COP during the 2-h ESLP, confirming a stable and valid ESLP with associated standard protocol even without protective medication. As part of our ESLP-oriented protocol optimization, we prospectively opted for the systolic PAP as the perfusion-limiting factor (<30 mmHg), which is also within the physiological range of the mean pulmonary artery pressure (mPAP) <20 mmHg frequently used in the literature. However, the sPAP as the peak pressure acting in the vascular system has a greater influence on oedema formation, i.e., the fluid shift from intravascular to interstitial, than the mPAP, so that the positive influence of the sPAP could have come into play here and could be of greater importance for ESLP than the mPAP [30]. However, it should ultimately be noted that ESLP-related oedema is absorbed to a certain extent post-transplant and is therefore irrelevant for PGD score and outcome after DLTx [11, 21].

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was approved by Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg, Germany. The study was conducted in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

KK performed data collection, statistical analysis, and literature research and wrote the manuscript. SH performed data analysis and literature research and wrote the manuscript. CW performed data analysis and wrote the manuscript. PB, DA, and HH were responsible for data collection. CF performed data analysis and performed, comparable to DJ, CK, and AR, critical revision of the manuscript for important intellectual content and to the final approval of the version to be published in accordance with ICMJE guidelines. BW was responsible for fundraising, conception and design of the study, drafting and critical revision of the manuscript for important intellectual content, final approval of the version to be published according to ICMJE guidelines, and is corresponding author. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13189/full#supplementary-material>

Supplementary Figure S1 | Thermographical data acquisition and analysis of SCS lungs. Representative native photos and corresponding thermal images of the lungs at the different time points as well as the respective average temperature are shown (A,B). Graphical representation shows the surface temperatures of the different test groups over time (C). Data are expressed as mean ± SD. Statistical significance is indicated if $p < 0.05$, or as “n.s.” if there is no significance.

Supplementary Figure S2 | Detailed histological findings of the lung injury score. Analyzed lung samples showed the following changes to varying degrees: Perivascular oedema (A), especially around the pulmonary arteries (arrowheads); intra-alveolar oedema [(B), arrowhead] and thickened alveolar septa as signs of interstitial oedema [(C), arrowhead]; further widening of the alveolar spaces, as a sign of over ventilation [(D), arrowhead]; atelectasis (E); and, in rare cases, the presence of perfusion solution [(F), arrowhead] were noted in HE staining. Magnification: (A, D) = ×50, (B, C, E) = ×100, and (F) = ×200. Scale bars: (A, D) 200 µm, (B, C, E) 100 µm, and (F) 20 µm.

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Ex-Vivo Human-Sized Organ Machine Perfusion: A Systematic Review on the Added Value of Medical Imaging for Organ Condition Assessment

Jan L. Van Der Hoek¹, Marleen E. Krommendijk¹, Srirang Manohar¹, Jutta Arens² and Erik Groot Jebbink^{1*}

¹Multi-Modality Medical Imaging Group, TechMed Centre, University of Twente, Enschede, Netherlands, ²Engineering Organ Support Technologies Group, Department of Biomechanical Engineering, University of Twente, Enschede, Netherlands

Machine perfused ex-vivo organs offer an excellent experimental platform, e.g., for studying organ physiology and for conducting pre-clinical trials for drug delivery. One main challenge in machine perfusion is the accurate assessment of organ condition. Assessment is often performed using viability markers, i.e., lactate concentrations and blood gas analysis. Nonetheless, existing markers for condition assessment can be inconclusive, and novel assessment methods remain of interest. Over the last decades, several imaging modalities have given unique insights into the assessment of organ condition. A systematic review was conducted according to accepted guidelines to evaluate these medical imaging methods, focussed on literature that use machine perfused human-sized organs, that determine organ condition with medical imaging. A total of 18 out of 1,465 studies were included that reported organ condition results in perfused hearts, kidneys, and livers, using both conventional viability markers and medical imaging. Laser speckle imaging, ultrasound, computed tomography, and magnetic resonance imaging were used to identify local ischemic regions and quantify intra-organ perfusion. A detailed investigation of metabolic activity was achieved using ³¹P magnetic resonance imaging and near-infrared spectroscopy. The current review shows that medical imaging is a powerful tool to assess organ condition.

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*Correspondence

Erik Groot Jebbink,

✉ e.grootjebbink@utwente.nl

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Keywords: machine perfusion, heart, kidney, liver, organ condition, medical imaging, human, large animal

INTRODUCTION

Ex-vivo machine perfused organs are a widely used model for experimental research, for example, on drug delivery and transplantation [1, 2]. By (re)perfusing organs ex-vivo, an environment can be created that allows for the study of organs in a simulated in vivo situation. One reason why ex-vivo machine perfused organs are chosen over in vitro phantoms is the biological complexity, which is difficult to replicate in the latter. Moreover, the experimental flexibility that ex-vivo machine perfused organs offer is generally not attainable in in vivo research in technical, operational, and ethical sense. While the technique has already been used and improved for over 150 years, interest has spiked in recent decades due to an increase in clinical relevance and the desire to reduce animal experiments [3, 4]. The main challenge in the research field is to increase the preservation time of organs, which requires a better

Ex-vivo human-sized organ machine perfusion: a systematic review on the added value of medical imaging for organ condition assessment

CHALLENGE

Improve organ condition assessment, which can for example increase the donor pool size by the inclusion of marginal organs.

RESEARCH QUESTION

What is the added value of **medical imaging** in the assessment of **organ condition**?

INCLUSION

18 original articles:

- A** 8 magnetic resonance imaging
- B** 4 ultrasound imaging
- C** 3 ionizing radiation-based imaging
- D** 3 optical imaging

RESULTS

A



- ↑ Detail
- ↑ Global assessment
- ↓ Adaption for machine perfusion
- ↓ Costs & availability

B



- ↑ Availability
- ↑ Contrast by microbubbles
- ↓ Local assessment
- ↓ Metabolic information

C



- ↑ Global assessment
- ↓ Limited results
- ↓ Translation from in-vivo to ex-vivo

D



- ↑ Contactless
- ↑ Fast
- ↓ Imaging depth
- ↓ Lesion detection

CLINICAL IMPLEMENTATION

Several adaptations are required for clinical implementation of MRI for organ assessment, especially for combining the modality with machine perfusion. Protocols should first be devised for a thorough organ assessment using either ultrasound or optical imaging.

CONCLUSIONS

Magnetic resonance imaging, ultrasound and optical imaging have shown to be powerful tools to assess organ condition. Medical imaging derived **biomarkers** are currently **not available**.



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GRAPHICAL ABSTRACT

understanding of the preservation parameters and a more reliable assessment of organ condition [5, 6].

Preservation parameters have been studied extensively, however, the influence of these parameters on organ condition is complex and not fully understood, which is further confounded by the variations in the used perfusion systems, like system components, perfusate types, and hemodynamics [7]. If the organ condition is assessed in these studies, it is often done by measuring viability markers such as perfusate lactate levels, oxygen consumption or organ-specific parameters like bile production in the liver. Often, multiple markers are used, and results are combined to offer information on the condition of the organ, but reliability of existing markers remains questionable [8]. There is, thus, no consensus on the usage of methods to assess organ condition [9].

Novel non-invasive biomarkers are needed to increase reliability of organ condition assessment, to improve and standardize the use of *ex-vivo* perfused organs in research. Several promising biomarkers have been reported that derive from the field of medical imaging [10–12]. These markers could give unique insights into organ condition assessment on both a macroscopic as well as a microscopic scale. Medical imaging can for example, be used to investigate tissue perfusion for the early identification of ischemic regions, but a direct metabolic assessment has also been reported [13, 14].

The goal of this systematic literature review is to give an overview of the added value of medical imaging in the assessment of organ condition in *ex-vivo* perfusion setups. Various imaging biomarkers that have been used for assessment of human-sized

machine perfused hearts, kidneys, and livers will be described and compared.

MATERIALS AND METHODS

The literature review was conducted using the preferred reporting items for systematic review and metaanalyses (PRISMA) guidelines [15].

Search Strategy

Two authors (J.H. and M.K.) conducted the literature search independently after reaching consensus on a search query. The same query was used in three databases: Scopus (Elsevier, Amsterdam, Netherlands), PubMed (National Center for Biotechnology Information, Bethesda, United States), and Web of Science (Clarivate Analytics PLC, London, UK) on April 3rd, 2023. The following search headings were used to find eligible studies: (“isolated” OR “*ex vivo*” OR “*ex situ*”) AND (“organ” OR “kidney” OR “liver” OR “heart”) AND (“perfus*” OR “machine perfus*” OR “NMP”) AND (“MRI” OR “magnetic resonance imaging” OR “photoacoustic*” OR “computed tomography” OR “ultrasound” OR “medical imaging” OR “diagnostic imaging”). A resurgence in the use of machine perfusion took place in the early 2000s with the arrival of hollow fibre oxygenators, which increases the possibilities and accuracy for organ condition assessment [4, 16]. Therefore, papers published between 2000 and 2023 were included to ensure coverage of relevant articles after this resurgence. Duplicates were removed using

EndNote (version 20.3, Clarivate, London, UK) based on their title, authors, the year of publication, and the journal of publication. Articles were then imported in Rayyan Intelligent Systematic Reviewer (Rayyan Systems Inc., Cambridge, Massachusetts, United States) for title and abstract screening [17].

Selection Criteria

Studies were included for full-text assessment based on the following criteria: articles that reported (i) isolated organ *ex-vivo* perfusion results using whole (ii) kidneys, hearts, and livers from (iii) pigs, cows, sheep, dogs, and humans, where during perfusion (iv) condition was assessed by histology, blood gas parameters or organ specific viability markers, and where (v) any form of medical imaging, was applied. Studies that used hypothermic machine perfusion were excluded, as the reduced metabolic activity of hypothermically perfused organs makes the setting less suitable for organ condition assessment [4, 18]. Studies that did not present viability results or that did not report ischemia times were excluded, as the undisclosed status of the perfused organ restricts the comparison and evaluation of the different imaging modalities and methods. Studies that only used medical imaging for therapeutic reasons were excluded for the same reason. Case studies and reviews were excluded. Disagreements were discussed and resolved by consulting a third author (E.G.J.).

Data Extraction

The full texts were reviewed independently by J.H and M.K and a final selection was made by comparing the data extractions. Publication details, including the authors and year of publication, were collected. Animal and organ characteristics including type and weight were extracted, together with information on the condition of the organs based on the assessment by both conventional biomarkers as well as medical imaging modalities.

RESULTS

Literature Search

A total of 2,505 abstracts were identified. **Figure 1** depicts the flow chart of the study selection, which resulted in 18 included studies. These studies are grouped according to the used imaging modalities, which includes 8 studies with magnetic resonance imaging (MRI) [10, 11, 19–24], 4 studies with ultrasound imaging [13, 25–27], 3 studies with ionizing radiation based imaging [28–30] and 3 studies with optical based imaging [12, 14, 31].

Study Characteristics

An overview of the study designs and hemodynamics is given in **Table 1**. The variation in machine perfusion parameters that was mentioned in the introduction is highlighted by the variation in hemodynamics. Additional details can be found in the **Supplementary Material** for all tables in this review.

Study Assessment

Conventional Organ Condition Assessment

When evaluating medical imaging for organ condition assessment, several conventional biomarkers were reported in the included literature for reference. These conventional biomarkers can be categorized in four groups: hemodynamics, histology, blood (gas) parameters, and organ specific parameters. The biomarker overview is given in the context of medical imaging method evaluation, a thorough analysis of conventional biomarkers can be found elsewhere [9, 32, 33].

Hemodynamic parameters are measured in 17 of the included studies. The flow, pressure, resistance, and temperature can be analysed based on their trends after organ reperfusion. Resistance should decrease after reperfusion, where a deviation can indicate organ damage or oedema formation [14, 29]. Hemodynamic-based assessment can become more elaborate in beating heart models by for example, a contractility assessment [26].

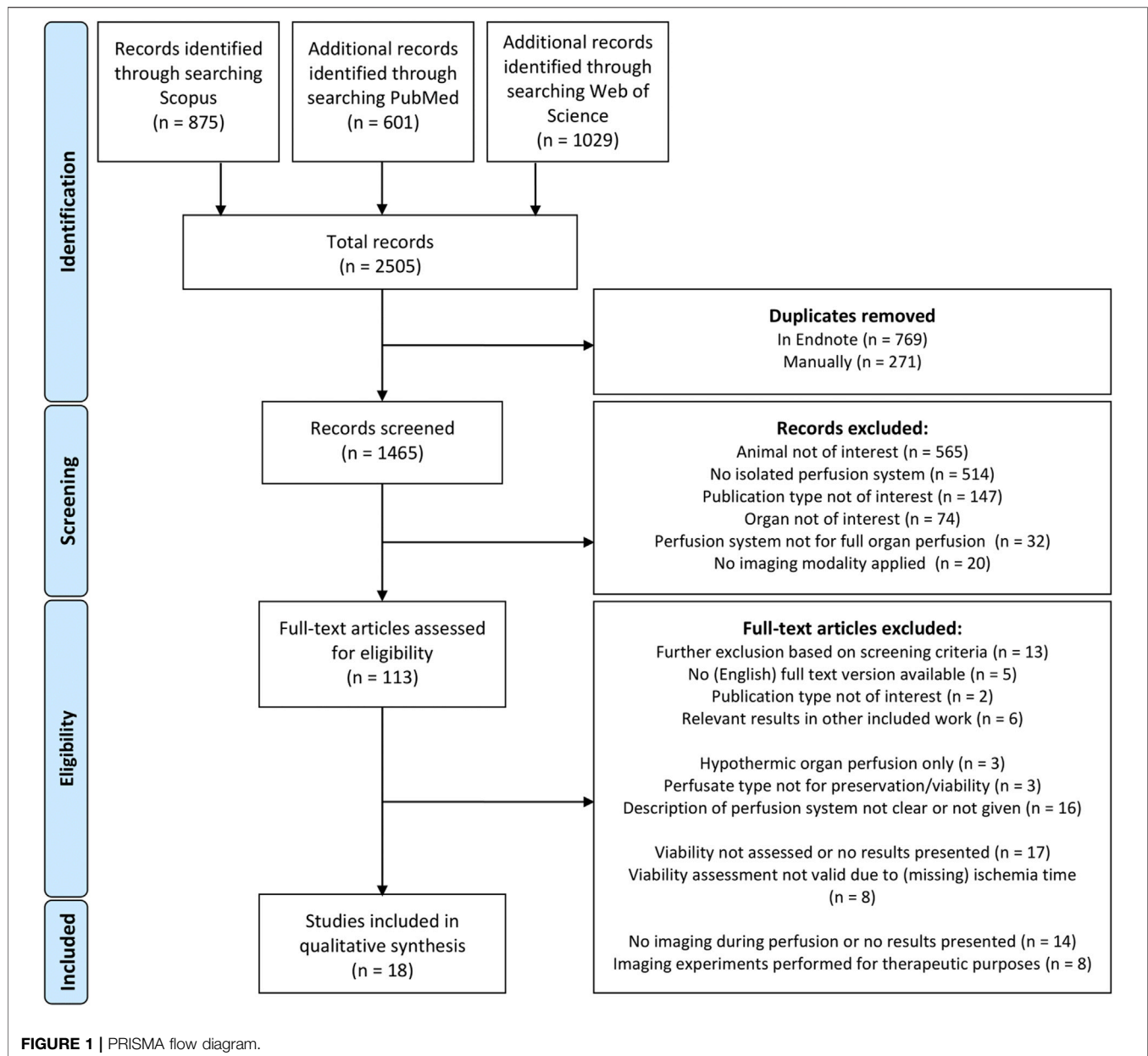
Histological assessment with biopsies is used in 10 studies for a microscopic analysis of organ tissue. Biopsies are often taken at multiple locations to approach a more global evaluation, and most studies also take biopsies at multiple time instances to analyse the effect of reperfusion. Histological assessment gives mostly qualitative information on a cellular level, however, these results are also quantified in some studies, expressed by injury scores [19]. Biopsies were analysed for organ specific structures, like glomerular integrity and tubular dilatation in kidney cortical biopsies [19], and sinusoid and hepatocyte structures in liver biopsies [13]. Several types of staining were also used that are organ agnostic, like periodic acid-schiff staining [22] and hematoxylin and eosin staining [24, 25].

A variety of blood gas parameters are reported in 16 of the included studies. Primarily oxygen (consumption), lactate, glucose, and pH were measured in these studies, which can indicate perfusion deficits and the onset of ischemia, e.g., with a built-up of lactate. Decision-making for liver transplantation is often based on such blood gas parameters, for example, pH stability (7.3–7.45) and the lactate concentration (≤ 18 mg/dL) for the transplantation of human livers as described by Fodor et al. [12].

Some parameters can also be used that are specific to the organ used in the study, which is described in 17 of the included studies. These include the bile production of the liver and urine production of a kidney, which can be evaluated based on the production quantity and its composition (e.g., pH). In beating heart perfusion studies, the heart beat is generally monitored over the duration of perfusion, where a stable sinus rhythm and ejection fraction are indicative of a healthy heart condition [26, 29].

Magnetic Resonance Imaging

A total of 8 studies used MRI during perfusion in the assessment of organs, reporting a wide range of MR techniques (**Table 2**). A distinction in these techniques can be made based on the detection of abnormalities, e.g., lesions and perfusion defects, or the direct assessment of organ metabolism and function.



Several studies have shown the capability of MRI to detect lesions and other abnormalities within perfused organs, which would otherwise remain undiscovered. More conventional MR methods are widely used in clinical practice, and several included studies show their suitability for lesion detection. For example, Mariager et al. used a balanced steady state free precession (bSSFP) gradient echo cine imaging sequence on perfused hearts for anatomical assessment, which showed oedema swelling in all hearts [21]. Schutter et al. used a SPACE sequence for T2-weighted anatomical imaging of kidneys to scan for abnormalities [10]. This resulted in the discovery of multiple abnormalities, including a benign cystic structure not visible on the exterior of the kidney, and an ischemic region that

was missed by surgical organ inspection. Gadolinium contrast can also be used to highlight lesions by contrast accumulation, which is for example, done with late gadolinium enhanced (LGE) MRI. Vaillant et al. used LGE T1-mapping to identify a myocardial lesion in one heart, which was missed by native T1mapping [23].

Assessment of the entire organ with MRI is also reported in several studies, which can be performed without contrast administration. In a kidney perfusion study by Schutter et al., intrarenal flows were analysed using a method known as arterial spin labelling (ASL) [22]. This method could capture the redistribution mechanism of the kidney, by relative measurements of the cortico-medullary ratio. In the case of

TABLE 1 | Study design and input parameters.

Publication details	Study design				Hemodynamics	
	Animal of study	Number of organs	Organ type and size	Perfusion time	Flow	Pressure
Agius 2022 [19]	Pigs: 45 kg	32	Kidney	4 h	-	40 mmHg systolic 20 mmHg diastolic
Alzaraa 2013 [25]	Pigs: 45–60 kg	10	Liver 1,659 ± 372 g	6 h	Hepatic artery: 210–300 mL/min Portal vein: 730–1,100 mL/min	Hepatic artery: 83–89 mmHg Portal vein: 22–28 mmHg
Bendjelid 2003 [28]	Pigs: 20–25 kg	15	Heart	1.5 h	1 mL/g/min, constant	3 mmHg preload 70 mmHg afterload
Fodor 2022 [12]	Human: BMI avg. 26	21	Liver	Average 20 h (17–27 h)	Hepatic artery: >150 mL/min Portal vein: >500 mL/min	-
Heeman 2021 [14]	Pigs: 130 kg	6	Kidney 338.1 ± 24 g	4 h	Sinusoidal, 60 BPM 150–200 mL/min	85 mmHg
Izamis 2014 [13]	Pigs	22	Liver 1,200–1800 g	3 h	Hepatic artery: 200–300 mL/min Portal vein: 600–900 mL/min	Hepatic artery: 100–112 cm H ₂ O Portal vein: <10 cm H ₂ O
Liu 2007 [20]	Pigs: 25–30 kg	3	Liver	-	-	-
Mariager 2020 [11]		4	Kidney 131 ± 5 g	2 h	170 mL/min	-
Mariager 2021 [21]	Pigs: 40 ± 3 kg	3	Heart 277 ± 11 g	2 h	300–400 mL/min	75–85 mmHg
Pelgrim 2017 [29]	Pigs: 110 kg	10	Heart	-	Non-stenotic: 1,400 mL/min	Non-stenotic: 54 mmHg
Ribeiro 2020 [26]	Pigs: 40 ± 4 kg	17	Heart	4 h	Working mode 1.8 L/min/m ²	Langendorff: 40 mmHg Working mode: 25–30 mmHg (diastole) 85 mmHg
Schutter 2021 [22]	Pigs: 130 kg Human	Pigs: 9 Human: 4	Kidney Pigs: 291 ± 42 g Human: 224 ± 69 g	4.5 h	Pig: mean 83–129 mL/min/100g Human: mean 143–244 mL/min/100g	
Schutter 2023 [10]	Pigs: 130 kg or 40 ± 2 kg Human	Pigs: 26 Human: 4	Kidney Pigs: 291 ± 42 g or 131 ± 5 g Human: 224 ± 69 g	3–4.5 h	Pig: 83–129 mL/min/100g, or 170 mL/min Human: mean 143–244 mL/min/100g	85 mmHg
Singh 2018 [31]	Pigs	8	Liver ±2,170 g	30 min	Hepatic artery: 150 mL/min Portal vein: 150 mL/min	-
Thompson 2020 [27]	Human	10 (5 pairs)	Kidney 199 g–331 g	7 h	50–120 mL/min/100g	Mean arterial pressure of 75 mmHg
Vaillant 2016 [23]	Pigs: 40 kg Sheep: 65 kg	Pigs: 20 Sheep: 1	Heart	±100 min	Working mode 1,000–1,100 mL/min (unloaded RV) 750–900 mL/min (loaded RV) 1 L/min for both kidneys	Langendorff: 60 mmHg Working mode: 60–70 mmHg (LV afterload)
Valenzuela 2023 [30]	Pigs: 87.40 ± 8 kg	32 (16 pairs)	Kidney	3 h	1.5 mL/g/min	Peak systolic pressure of 120 mmHg
Yang 2008 [24]	Pigs: 25–35 kg	37	Heart	±80 min		70–90 mmHg (LV systole) 0–5 mmHg (LV diastole)

heart perfusion, assessment is also possible with MRI based on the functionality of a working heart model with e.g., cine MRI. Hemodynamic values were determined with this method in two studies, which were comparable to *in vivo* values found in literature for the working heart model [23], but different for Langendorff perfused hearts [21].

Administration of contrast is used to better distinguish areas of interest, which is done in dynamic contrast enhanced (DCE) MRI. In a study by Mariager et al., the cortical perfusion in kidneys was measured with this method [11]. The function of the kidney was also assessed with this method by calculating the glomerular filtration

rate, which was comparable to values found in literature and could serve as a biomarker. Gadolinium contrast is also used in diffusion weighted MRI, which is predominantly used for diagnosis of acute cerebral ischemia [34]. However, Liu et al. found that the apparent diffusion coefficient (ADC) value derived using diffusion weighted MRI could not discriminate between ischemic and non-ischemic regions in a perfused porcine liver, contradicting *in vivo* result in the same study [20]. In the condition assessment of hearts, Yang et al. introduce manganese as an alternative to gadolinium contrast [24]. Manganese has a fairly rapid uptake in healthy heart cells through the calcium channels, which directly gives a readout of heart

TABLE 2 | Organ assessment with magnetic resonance imaging.

Publication details	Study design		Parameters measured for viability testing				Imaging
Authors	Animal	Organ	Hemodynamics	Histology	Blood parameters/ gas contents	Organ-specific	Imaging method
Agius 2022 [19]	Pig	Kidney	-	Cortical biopsies Silver Jones and Periodic AcidSchiff staining	-	Urine output	T2-weighted imaging, dynamic contrast enhanced (DCE) MRI and ³¹ P magnetic resonance spectroscopic imaging (pMRSI)
Liu 2007 [20]	Pig	Liver	-	Hemotoxylineosin stained biopsies	Aspartate Aminotransferase (AST)	-	Diffusion weighted MRI with contrast enhancement
Mariager 2020 [11]	Pig	Kidney	Flow, pressure and temperature	-	Blood gas, metabolite and electrolyte values	Urine production	T1 and T2 imaging, hyperpolarized spectroscopy and/or spectral spatial imaging and dynamic contrast enhanced MRI
Mariager 2021 [21]			Flow and pressure	-	Blood gas, metabolite and electrolyte values	-	CINE MR imaging, hyperpolarized spectroscopy and/or spectral spatial imaging and dynamic contrast enhanced MRI
Schutter 2021 [22]	Pig and Human	Kidney	Flow and pressure	Cortical punch biopsies Periodic acid– Schiff staining	Arterial blood gas values	Urine composition and production	Arterial spin labelling (ASL)
Schutter 2023 [10]	Pig and Human	Kidney	Flow, pressure and temperature	-	-	Urine production	T2- and T2*-weighted imaging, arterial spin labelling and hyperpolarized spectroscopy and/or spectral spatial imaging
Vaillant 2016 [23]	Pig and sheep	Heart	Left ventricle pressure and flow	-	pH	Heart rate and action potential	Cine imaging, phase contrast for velocity-encoded cine flow imaging, T1 mapping with late gadolinium enhanced mapping and ³¹ P MR spectroscopy.
Yang 2008 [24]	Pig	Heart	Pressure and resistance	Heart slices analysed with near-infrared and various types of staining.	Blood gas values	Heart rate	T1-weighted and contrast (Mn) enhanced MRI

condition by serving as a calcium analogue [35]. The sensitivity of manganese enhanced cardiac MRI is highlighted in this study, as ischemic regions were detected that did stain positive for triphenyl tetrazolium chloride, indicating healthy tissue.

The metabolic state of organs can also be analysed directly, by for example, mapping the oxygen consumption in entire organs. Schutter et al. analysed this in perfused kidneys with the functional MR method T2*-mapping, which measures deoxygenated haemoglobin. The T2* signal dropped especially in the functionally important renal cortex when oxygen delivery was halted, thus indicating the onset of ischemia. The myocardial energetic status of perfused hearts was measured by Vaillant et al. with ³¹P MR spectroscopy, using a high-field MR scanner and a coil tuned for both ¹H and ³¹P [23]. ATP values can be derived by the resulting spectra from this method, which showed that the myocardial energetic status was preserved. Alternatively, energetic status of perfused organs can be assessed by hyperpolarized [1-¹³C] pyruvate MRI. Injected hyperpolarized pyruvate is converted in the tissue into bicarbonate for aerobic metabolism and into lactate for an anaerobic conversion, which can be assessed directly by hyperpolarized spectroscopy in combination with spectral spatial (SPSP) imaging. Schutter et al. used this method to assess perfused kidneys, whereas Mariager et al. used this method to assess both

perfused hearts and kidneys in subsequent studies. All studies found a very high lactate conversion rate that was inconsistent with *in vivo* values, which is possibly caused by the altered metabolic profile of *ex-vivo* perfused organs [10, 11, 21].

Ultrasound

The assessment of organs was investigated with ultrasound in 4 studies (Table 3). The assessment with ultrasound was mostly based on perfusion, but two other methods have also been reported. For the detection of anatomic abnormalities, conventional B-mode ultrasound imaging was applied by Izamis et al. to scan all 30 included livers, however, no abnormalities were found [13]. The functional analysis of beating hearts was conducted by Ribeiro et al. using surface echocardiography [26]. Significant differences in the left ventricle circumferential strain and right ventricle fractional area change were found in a beating heart donor group and a cardiac death group, which could not be related to post-transplant outcomes.

Assessment of perfusion with ultrasound can be performed with and without a contrast agent. Thompson et al. used MicroFlow imaging Doppler ultrasound for subjective assessment of perfusion. Microflow imaging is a high resolution ultrasound technique that allows for mapping of

TABLE 3 | Organ assessment with ultrasound imaging.

Publication details	Study design		Parameters measured for viability testing				Imaging
	Authors	Animal	Organ	Hemodynamics	Histology	Blood parameters/gas contents	Imaging method
Alzaraa 2013 [25]	Pig	Liver	Flow and pressure	Tru-Cut biopsies Hematoxylineosin staining	Oxygen consumption	Bile production	Contrast Enhanced Ultrasound (CEUS)
Izamis 2014 [13]	Pig	Liver	Flow, pressure and resistance	Hematoxylineosin staining	Oxygen consumption and pH Blood gas and oxygen consumption	Bile production	B-mode ultrasound and dynamic contrast enhanced ultrasound (DCEUS)
Ribeiro 2020 [26]	Pig	Heart	Flow, pressure and resistance	-	Blood gas and oxygen consumption	Biventricular function	Surface echocardiography
Thompson 2020 [27]	Human	Kidney	Flow, temperature, pressure and resistance	Histopathological assessment analysis	Blood gas and biochemical	Urine production	Contrast Enhanced Ultrasound (CEUS) MicroFlow Imaging (MFI) and Doppler ultrasound

smaller vessels with more accurate flow measurements in these vessels, compared to conventional ultrasound methods [27, 36]. In the same study, Sonovue (Bracco, Milan, Italy) contrast microbubbles were injected for contrast enhanced ultrasound (CEUS) measurements, which could capture the redistribution mechanism of the kidney. Perfusion was investigated in more detail in two liver perfusion studies, where dynamic contrast enhanced ultrasound (DCEUS) was applied with Sonovue contrast microbubbles to generate time intensity curves. Alzaraa et al. used DCEUS and found a delayed and reduced wash-in in poorly perfused areas and flat curves in non-perfused areas, which were identified as ischemic regions by hematoxylineosin staining. Izamis et al. used DCEUS to monitor the perfusion, where microbubbles were injected hourly in both the portal vein and the hepatic artery [13]. Blood clots were identified that were not removed by the initial flush, and the gradual removal of these clots was visualised.

Ionizing Radiation Based Imaging Modalities

Only a few ionizing radiation based imaging modalities were found in the included literature (Table 4). Fluoroscopy was performed by Valenzuela et al. in perfused porcine kidney pairs to measure characteristic lengths and angles in the main renal arteries [30]. Pelgrim et al. applied dynamic dual source computed tomography (CT) imaging for blood flow quantification in Langendorff perfused porcine hearts [29]. The mean myocardial blood flow per segment was measured using CT for different stenosis grades of the circumflex artery. A quantitative dynamic CT perfusion analysis could distinguish between ischemic and non-ischemic myocardial segments. A fluorescent microsphere method that is accurate for the quantification of myocardial blood flow was also used and compared to the flow values quantified by CT. While similar trends were visible, the myocardial blood flow was found to be consistently lower when quantified by CT. Bjendelid et al. applied PET for imaging the myocardial status of a perfused porcine heart by monitoring the uptake

of injected ^{18}F fluorodeoxyglucose (FDG) [28]. This marker for glucose uptake increased linearly over time and a homogeneous distribution was observed over the myocardium. However, FDG could not be correlated with the metabolic increase expected from dobutamine stimulation.

Optical Imaging Modalities

Optical modalities have also been reported in the assessment of organs, which are mostly focused on the superficial microcirculation (Table 5). Singh et al. used Laser Doppler Flowmetry (LDF) to measure relative changes in the microcirculation in a small field of view (FOV) ($<1\text{ mm}^2$) [31, 37]. LDF showed that the microcirculation improved after reperfusion. Laser speckle contrast imaging (LSCI) is another optical method and allows relative measurement of the microcirculation in a larger FOV compared to LDF. Heeman et al. compared renal blood flows in a porcine model to normalized laser speckle perfusion units (FOV = $14 \times 19\text{ cm}$) [14]. The onset of ischemia was visible using LSCI, whereas the tissue discoloration due to ischemia took some time. LSCI demonstrated a high correlation with side-stream dark-field (SDF) imaging, which can detect subtle changes in the microcirculation on a red blood cell level [38]. Only a moderately correlation between LSCI and the externally measured renal blood flow was found in a flow ramping experiment, due to the redistribution effect of the kidney. For the analysis of human livers for transplantation, hyperspectral imaging is applied to acquire information on tissue oxygenation, perfusion, and haemoglobin and water concentration [12]. The oxygen saturation, tissue haemoglobin level, and microperfusion increased significantly after the start of perfusion. Livers that were transplanted, according to guidelines for e.g., lactate levels and pH, showed an enhanced microperfusion, measured by the near-infrared perfusion index. Especially the near-infrared perfusion index and the relative distribution of water in the tissue (TWI) show a good correlation with the conventional biomarkers, lactate, and pH respectively.

TABLE 4 | Organ assessment with radiation based imaging modalities.

Publication details	Study design		Parameters measured for viability testing				Imaging
	Authors	Animal	Organ	Hemodynamics	Histology	Blood parameters/ gas contents	Imaging method
Bendjelid 2003 [28]	Pig	Heart	Flow and pressure	-		Blood gas values	Heart rate
Pelgrim 2017 [29]	Pig	Heart	Flow and pressure	Fluorescent microspheres with alcoholic-KOH dissolving		Glucose	Heart rate
Valenzuela 2023 [30]	Pigs	Kidney (pairs)	Flow, pressure and temperature	None taken	-		Urine production and composition

TABLE 5 | Organ assessment with optical imaging.

Publication details	Study design		Parameters measured for viability testing				Imaging
	Authors	Animal	Organ	Hemodynamics	Histology	Blood parameters/ gas contents	Imaging method
Fodor 2022 [12]	Human	Liver	Flow	-		Blood gas values	Bile production and bile pH
Heeman 2021 [14]	Pig	Kidney	Flow, pressure, temperature and resistance	-		-	Urine production
Singh 2018 [31]	Pig	Liver	Temperature	Hematoxylin-eosin staining	pH		Bile production

DISCUSSION

A wide variety of imaging modalities have been found in the reviewed literature. Depending on the goal of the viability assessment, several imaging modalities and methods could be considered. The most comprehensive investigation of organ condition can be conducted with magnetic resonance imaging (MRI), which offers a vast range of specific methods for analysis of morphology, perfusion, heart function, and metabolism. The main downsides of MRI are its costs and availability, which is especially problematic in clinical practice for monitoring organs for extended duration. Ultrasound imaging can also be used for analysis of morphology, perfusion, and heart function, where especially the introduction of contrast microbubbles showed promising results for the analysis of perfusion. While ultrasound is a relatively inexpensive imaging option that is more widely available, full organ assessment is limited both by the field of view as well as the reduced anatomical information that ultrasound can provide compared to MRI. Ionizing radiation-based techniques showed that perfusion analysis can only be performed qualitatively, where the reported method for metabolic assessment was limited in the *ex-vivo* setting. The optical based modalities showed promising results for the analysis of both perfusion and metabolism, although these are limited by the penetration depth.

Lesion Detection

For the detection of lesions, multiple MRI sequences have been reported in the included literature. Next to the more conventional

T1 and T2 weighted imaging, late gadolinium enhanced (LGE) imaging with contrast gadolinium was also applied, which could identify lesions missed by native T1-weighted imaging [23]. Multiple studies have shown that LGE is a suitable method for the detection of cardiac lesions, like cardiomyopathies, ischemic heart disease, and fibrosis [39, 40]. However, LGE is limited for diseases that are spread out over the heart, like diffuse fibrosis [41].

While no lesions were detected with ultrasound in the included studies, the imaging modality has shown its capability to detect lesions of specific nature, like cystic structures [42]. Moreover, the introduction of contrast microbubbles with contrast enhanced ultrasound (CEUS) has shown that benign and malignant lesions can be identified and distinguished [43].

Contrary to the findings in this review, multiple *in vivo* studies have shown that computed tomography (CT) has a high sensitivity for lesion detection [42]. MRI and CT have shown comparable overall sensitivity for lesion detection, with specific strengths for each modality (e.g., CT for calcified lesions and MRI for tumours smaller than 20 mm in diameter) [44, 45]. While ultrasound is more widely available, it is often only used for initial screening for lesions, due to a relatively lower sensitivity for lesion detection compared to MRI and CT.

Perfusion Analysis

Dynamic contrast enhanced (DCE) MRI is the most used method for the perfusion analysis with MRI in the reviewed literature, which showed adequate perfusion in all studies. DCE MRI is mostly used with gadolinium contrast, but manganese is another

contrast option that is mostly used in the assessment of hearts. However, several studies have also addressed a downside of manganese contrast, which is induced cardiovascular dysfunction [46, 47]. MRI ASL is a non-invasive alternative to the invasive DCE MRI, showing a moderate to high correlation in a comparison study [48]. Interestingly, the non-invasive diffusion-weighted MRI, which is widely used in clinic for the detection of brain ischemia and tumours, did not translate to the *ex-vivo* situation in a study by Liu et al., although this pilot study based this conclusion on only one blood-perfused liver [20, 34].

For the perfusion analysis of organs, CEUS was mostly used to monitor the tissue perfusion over the duration of the experiment. While the method shows that small details like the removal of blood clots after reperfusion and the onset of ischemia can be mapped, the method does require contrast microbubble injections for each measurement. Micro-flow imaging is an alternative ultrasound method that does not require contrast enhancement. This method shows a higher sensitivity to detecting the vascularity of hepatocellular carcinoma compared to other non-contrast enhanced ultrasound methods, albeit lower than the sensitivity found with CEUS [49].

Perfusion was analysed with CT in one included study, which also indicated that perfusion analysis can only be done qualitatively as CT underestimated the myocardial blood flow [29]. Other studies do describe the quantitative evaluation of tissue perfusion with perfusion CT, which is for example, used in the diagnosis of abdominal cancer [50].

Superficial perfusion can be analysed in great detail by optical modalities like side-stream dark field (SDF) imaging and laser doppler flowmetry (LDF), although these modalities are very limited by their field of view for the analysis of entire organs. Laser speckle imaging and hyperspectral imaging could both analyse the microcirculation of perfused organs on a larger scale, which were also good indicators for transplantation outcomes as described by Fodor et al. [12].

Metabolic Analysis

In the metabolic assessment of organs, T2*-weighted imaging can be used to measure the tissue oxygenation. The T2* signal changed significantly when the oxygen supply to a perfused kidney was interrupted, although the authors mention that this method cannot be used to quantify organ dysfunction at this moment [10]. The method is used *in vivo* in the metabolic analysis of transplanted kidneys, specifically for the detection of early allograft dysfunction [51]. A metabolic analysis of perfused hearts was performed with ³¹P MR spectroscopy, which showed comparable values to *in vivo* studies for healthy hearts. Hyperpolarized spectroscopy and spectral spatial (SPSP) imaging provided insight into the metabolic distribution over perfused organs, however, the high lactate values that were found in all three studies compared to *in vivo* results from literature suggest results should be interpreted with caution. Considering the hypotheses that Mariager et al. describe in both the heart perfusion and the kidney perfusion studies, the technique requires *ex-vivo* validation to develop it as a biomarker in this setting.

The metabolic analysis with ¹⁸F fluorodeoxyglucose (FDG) positron emission tomography (PET) imaging did not correlate

with the expected metabolic increase that was induced in perfused hearts [28]. A large retrospective study by Sprinz et al. analysed FDG uptake in the liver, lungs and brain, where only in the brain FDG was a significant marker for glucose uptake [52].

Hyperspectral imaging showed its ability to assess human liver condition prior to transplantation in a proof-of-concept study [12]. However, the superficial relative blood oxygenation StO₂ could not discriminate between transplanted and non-transplanted livers, whereas the near-infrared perfusion index and the relative distribution of water over the organ could be used to this end.

Functional (Cardiac) Analysis

Cine cardiac imaging was used for the functional assessment of both Langendorff-perfused hearts, as well as hearts in working mode. Results showed that a complete replication of all parameters of the *in vivo* situation is difficult. Both included studies that performed the functional assessment found a decreased left ventricular ejection fraction and aortic flow compared to *in vivo* data with cardiac MRI. However, for the functional assessment of hearts, studies have indicated that this might be sufficient as only specific parameters are important in the functional assessment of hearts for e.g., transplantation outcomes [26, 53].

Echocardiography could not be used to predict post-transplant outcomes [26]. In this study as well, the *ex-vivo* working heart setting was difficult to replicate and the poor echocardiography post-transplant outcome correlation was ascribed to the evaluation method where only a steady pre-load was used in the assessment.

Clinical Implementation

This review has shown that several medical imaging derived parameters correlate with established biomarkers, while also providing detailed and locoregional information on the condition of organs that current biomarkers cannot provide. However, several adaptations are required for clinical implementation of medical imaging based organ assessment, particularly for pre-transplant organ assessment.

The most comprehensive analysis of organ condition is provided by MRI, where several methods provided detailed feedback on the condition of the entire perfused organ. However, the modality does also require significant adaptations in order to be implemented in the current workflow. Firstly, the Faraday cage limits placement of the machine perfusion system to the control room. For perfusion of an organ in the MRI, special MRI-compatible organ chambers should be used, as well as elongated tubing to connect the organ across the Faraday cage. The latter has a severe impact on the heat loss through the tubing and the damping of flow pulsatility over the tubing, requiring major adaptations to the workflow. Secondly, usage of the MRI prior to transplantation may not be feasible in all institutions. While studies have shown that machine perfusion can preserve a heart for over 10 h for successful transplantation [54], this timeframe together with the limited availability and priorities within MRI scheduling might impede the adoption of MRI for organ assessment.

Ultrasound is a more flexible alternative concerning costs, availability and repeated measurements over the preservation

time, where especially contrast microbubbles offer detailed perfusion information. Contrast microbubbles have shown an excellent safety profile in patients [55], and the *ex-vivo* machine perfusion setting also facilitates non-invasive administration of microbubbles. Nevertheless, ultrasound is a 2D modality that in general provides less information than 3D MRI. To enhance ultrasound assessment, protocols should be devised for the assessment of entire organs. Alternatively, 3D ultrasound might be an option for such an assessment, although accurate real-time image reconstruction remains challenging [56].

The optical imaging method hyperspectral imaging could be a suitable option for organ assessment, where the study by Fodor et al. found that hyperspectral imaging derived parameters aligned with viability markers commonly used during liver normothermic perfusion [57]. The contactless method can offer real-time information on both perfusion and the metabolic state of the organ, and permanent integration with the perfusion setup is the most straightforward compared to the other modalities. The main limitation of optical based organ assessment is the penetration depth, which limits the extent of organ assessment to the surface. Moreover, lesions are poorly detected by optical modalities, where a combination with ultrasound might be a solution.

Limitations

In this review, the imaging methods and modalities were grouped and compared on several functionalities, e.g., perfusion analysis and lesions detection. A large variation in these studies was found for the used perfusion system characteristics and parameters, where standardization is necessary. The heterogeneity of the studies complicates the comparison of the imaging methods and results, requiring cautious interpretation.

Conclusion

Magnetic resonance imaging (MRI) offers a wide range of methods for accurate assessment of organ condition, where especially functional MRI offers unique insights. Ultrasound is a more flexible alternative that becomes especially significant with the introduction of contrast microbubbles. The results for computed tomography (CT) and other ionizing radiation based imaging modalities are limited for *ex-vivo* machine perfused organs, however, literature does show its potential in the organ condition assessment. While optical modalities are slightly more experimental compared to the other modalities, analysis of the superficial microperfusion has shown to be a quick and good method to assess organ condition. Although a detailed

overview of the different imaging methods for organ condition analysis could be given, results were not conclusive on the suitability of medical imaging features as biomarkers to report on organ condition.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors made a significant contribution to this manuscript, both in the design of the study, and critical revision of the manuscript. Both JH and MK worked on the whole inclusion process, from abstract scanning to the final selection. Disagreements were discussed and resolved by consulting EG. JH wrote the manuscript, which was read, reviewed and approved by all authors. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontierspartnerships.org/articles/10.3389/ti.2024.12827/full#supplementary-material>

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Machine Perfusion and Bioengineering Strategies in Transplantation—Beyond the Emerging Concepts

Anna Niroomand^{1,2,3}, George Emilian Nita^{4,5,6} and Sandra Lindstedt^{1,2,3,7*}

¹Department of Clinical Sciences, Lund University, Lund, Sweden, ²Lund Stem Cell Center, Lund University, Lund, Sweden, ³Department of Cardiothoracic Surgery and Transplantation, Skåne University Hospital, Lund, Sweden, ⁴Department of Transplantation Surgery, Liverpool University Hospitals NHS Foundation Trust, Liverpool, United Kingdom, ⁵Institute of Systems, Molecular and Integrative Biology, University of Liverpool, Liverpool, United Kingdom, ⁶Division of Transplantation Surgery, Karolinska University Hospital, Stockholm, Sweden, ⁷Wallenberg Center for Molecular Medicine, Lund University, Lund, Sweden

Solid organ transplantation has progressed rapidly over the decades from the first experimental procedures to its role in the modern era as an established treatment for end-stage organ disease. Solid organ transplantation including liver, kidney, pancreas, heart, and lung transplantation, is the definitive option for many patients, but despite the advances that have been made, there are still significant challenges in meeting the demand for viable donor grafts. Furthermore, post-operatively, the recipient faces several hurdles, including poor early outcomes like primary graft dysfunction and acute and chronic forms of graft rejection. In an effort to address these issues, innovations in organ engineering and treatment have been developed. This review covers efforts made to expand the donor pool including bioengineering techniques and the use of *ex vivo* graft perfusion. It also covers modifications and treatments that have been trialed, in addition to research efforts in both abdominal organs and thoracic organs. Overall, this article discusses recent innovations in machine perfusion and organ bioengineering with the aim of improving and increasing the quality of donor organs.

Keywords: transplantation, machine perfusion, bioengineering, gene therapy, cross circulation

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*Correspondence

Sandra Lindstedt,
✉ sandra.lindstedt_ingemansson@
med.lu.se

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INTRODUCTION

Since the start of successful kidney transplantation in the 1950s followed by other solid organs, transplantation has taken on a steadfast role in medicine as the definitive treatment to improve the quality of life and prolong the survival of patients with end-stage organ disease [1–6]. In recent decades, major advances in surgical technique, and pre- and post-operative management have allowed for the prolonged survival of recipients. As the number and availability of transplants have increased, the imbalance between organ supply and demand has grown to unprecedented levels [7]. In 2024, the Organ Procurement and Transplantation Data showed over 113,000 patients waiting for a transplant on the US all-organ waiting list [8], while the NHS Blood and Transplant reported over 7,000 [9] potential recipients in the UK alone and Eurotransplant listed over 13,000 patients currently active in eight European countries [10].

To address this disparity, an increase in the number of available donor organs is needed, which could theoretically be accomplished through a variety of means, including increased organ donation,

procurement, and recuperation of available grafts. Restoration and regeneration of damaged donor organs is a particularly promising path for recovering a greater number of donor grafts, considering the high percentage that are currently discarded.

Grafts considered for transplantation may be rejected for several reasons. Factors such as the age of the donor, the cause of death, and the resulting sequelae of damage to the organ are often the primary reasons for rejecting the organ for transplantation. In the evaluation of donor lungs, for example, injury from trauma, infection, and aspiration may lead to acute lung injury (ALI) and the resulting functional impairment may preclude the organ from being chosen for transplantation [11, 12].

Many of these once-rejected organs are now being reconsidered in an effort to expand the donor pool. For example, the limits of what constitutes an acceptable organ have been raised with the introduction of expanded criteria for donors, donors at increased risk, and donors with acute kidney injury [13–17]. Despite these efforts, there are still organs being discarded that could theoretically be recovered for use via advanced techniques. The utilization of bioengineered organs is an expanding and promising approach and the creation of functional bioengineered organs could contribute to an immunosuppression-free state [18]. This article reviews the use of machine perfusion in several organs, exploring both the clinical application of this technology and the bioengineering techniques leveraged to increase the quality of donor organs.

MACHINE PERFUSION

Circulatory support of donor organs following organ retrieval has been developed for several solid organs, with the advantage of such *ex vivo* platforms being the ability to evaluate the functionality of organs, prolong the preservation time between explant and implant, and apply recovery therapies to the target organ.

Since Alexis Carrel and Charles Lindbergh first described *ex vivo* machine perfusion in 1935, reporting the successful perfusion of the feline thyroid gland [19], significant efforts have been made to assess organ function and prolong preservation. In the 1970s animal models of the lung [20] and Hardesty and Griffith's publication on autoperfusion of combined heart and lung transplants were highlighted [21]. Various liver-assisting therapies were researched during the 1960s and 1970s (cross-circulation, hemodilution and plasmapheresis), but they were abandoned with the development of orthotopic liver transplantation and understanding of the possible immunologic implications [22]. Animal models utilizing cold perfusion, oxygenated blood or plasma for kidney [23–25] and pancreas preservation continued to develop in the early 1980s [26, 27]. In Lund, Sweden, *ex vivo* lung perfusion (EVLP) was brought back by Steen et al. with the first marginally viable lungs transplanted following reconditioning [28–30]. This EVLP model was further developed with the establishment of protocols for prolonged

evaluation by the Toronto group [31], which led to increasing and expanding clinical application.

At their core, machine perfusion platforms consist of an organ chamber in which perfusate flows through the graft using a pump (either centrifugal or roller) via a circuit equipped with a temperature control system and an oxygenation method. In the case of lung machine perfusion called EVLP, the organ is additionally ventilated with mechanical ventilation at settings determined by the underlying protocol. In general, the flow of perfusate allows for the delivery of oxygen and nutrients as well as the removal of waste and toxins, while also providing the opportunity to deliver therapeutic agents directly to the target. Leukocyte filters may also be incorporated into the circuit, although the efficacy of such an addition is under debate [32].

Lung Perfusion

In EVLP, the organ is both perfused and ventilated, which allows for additional graft monitoring both through analysis of the perfusate as well as through bronchoscopy and bronchoalveolar lavage fluid collection. An important note about the circuit specific to the lung is the need to deoxygenate the perfusate to assess the organ's functionality, with the ratio of arterial oxygenation to a fraction of inspired air ($\text{PaO}_2/\text{FiO}_2$) being an important clinical measure.

The two most commonly used commercially available platforms for performing EVLP are the XPS™ system made by XVIVO [33] and the portable Organ Care System™ (OCS) produced by Transmedics [34]. Among these platforms, there are also three commonly referenced protocols, including the Lund protocol [28], the Toronto protocol [35], and the OCS protocol [36]. These differ in several parameters including the target perfusate flow (often calculated based on cardiac output), whether the left atrium of the donor lung graft is open or closed, and the composition of the perfusate. Regarding the perfusate, the differences lie in the underlying base solution (STEEN vs. OCS), and the use of red blood cells vs. acellular perfusate (Lund vs. Toronto). The debate over the use of cellular versus acellular perfusate continues, with some reports finding that cellular perfusate achieves results approaching clinical standards and superior lung preservation as well as a reduced incidence of lung edema and improved compliance in a porcine model. Despite these preclinical findings, human studies using EVLP have focused on acellular perfusates with favorable results.

Pioneering investigations have established the efficacy and utility of these systems in clinical applications. Starting in 2011, the Toronto group demonstrated in the HELP trial that transplantation with high-risk donor lungs maintained on 4 h of EVLP could produce comparable results to conventional lungs using their EVLP protocol [31]. The NOVEL trial which followed a few years later in 2014 extended these findings to a multicenter trial in 6 US centers with expanded criteria donors compared to conventional lungs and again encouraged the use of EVLP [37]. In the 2016 DEVELOP-UK trial, the EVLP observational cohort was terminated early, with lower survival rates, higher rates of ECMO and early grade 3 primary graft dysfunction (PGD) in the EVLP arm which utilized lungs not suitable for standard transplantation [38]. Other reports subsequently have emerged

that provide contrasting support for the continued use of EVLP, including the 2018 INSPIRE and 2019 EXPAND trials using the OCS system [36, 39]. The INSPIRE trial compared standard criteria lung transplants maintained with EVLP versus cold storage, with findings of reduced PGD grade 3 at 72 h in the EVLP arm and meeting the primary safety endpoints. The EXPAND trial was then expanded to include extended criteria donors with primary endpoints of 30-day survival and absence of grade 3 PGD. Although the performance goals of the study were not met, the study group concluded that the use of EVLP allowed the transplantation of organs that would otherwise have been rejected. Similarly, supportive data for EVLP from a single-center analysis in Vienna was published in 2017 reporting that in their use of EVLP in standard donor lungs, EVLP use provided evidence of functional results and operative outcomes comparable to standard preservation, with the cited benefit of safely extending preservation time [40].

Clinical EVLP systems have since evolved to be executed not only by a transplant service's own team (referred to as local in-house EVLP) but also to be performed at an outside but centralized institution (sometimes called a centralized lung evaluation system). This centralized version has recently gained traction due to the development of Lung Bioengineering's dedicated facility in which remote EVLP was performed for seven different United States-based lung centers [41]. Such centralized EVLP may grow in popularity with the advent of the first European centralized facility coordinating EVLP between Sweden and Denmark [42].

Within these applications, the details of optimal parameters are still being discussed and guidelines for optimal execution need to be established. Ventilation methods are an additional point of interest, particularly because of the known effects of harmful ventilation causing ventilator-induced lung injury. Because excessive pressure (barotrauma), alveolar collapse (atelectrauma), and volume (volutrauma) can induce iatrogenic damage to the graft, attention to the method of ventilation is critical. The stress index which represents the rate of change in compliance during tidal inflation has been proposed as a means of assessing ventilation in EVLP, with high indices corresponding to hyperinflation and low indices corresponding to recruitment-derecruitment [43, 44]. Ventilation outside of the stress index window has been reported to correlate with cytokine-driven inflammation and longer time on mechanical ventilation, intensive care unit (ICU) stays, and hospital stays [45]. Using an auxiliary device, exhaled breath particles can also be collected from the mechanical ventilation setup on EVLP, which has shown that greater numbers of particles are correlated with higher tidal volumes and volume-controlled ventilation [46]. Higher particle flow rates using this methodology have been correlated with lung injury [47, 48] and with primary graft dysfunction in the transplant setting [49]. Other studies have investigated airway pressure release ventilation compared to conventional volume-controlled ventilation in a porcine model to try an open lung approach to ventilation, which reduced edema during EVLP and improved $\text{PaO}_2/\text{FiO}_2$ ratios following 4 h of reperfusion after left lung transplantation [50]. Negative pressure ventilation was trialed in

another porcine experiment with 12 h of *ex vivo* perfusion and found to have lower levels of pro-inflammatory $\text{TNF-}\alpha$, IL-6 and IL-8 along with less edema during EVLP [51].

Heart Perfusion

Similar to the lung, the perfusion circuit of the *ex vivo* machine can be altered to accommodate the specific needs of the heart, including wires that allow for defibrillation and pacing. Currently, the two systems in place for *ex vivo* heart perfusion (EVHP) are the Heart Box™ (XVIVO) and the portable Organ Care System (OCS) Heart™ (TransMedics). The main difference between these systems is the use of normothermic perfusion in the OCS Heart versus hypothermic preservation at 8°C in the Heart Box. The reported benefits of EVHP in these systems are notable not only for the clinical results obtained, but also for the system's ability to assess hidden cardiac pathologies [52] and to find specific and sensitive markers of outcome [53].

In the 2014 trial of normothermic heart perfusion, standard criteria donors were compared against conventional preservation techniques, looking at the survival of the recipients over a 2-year period [54]. During this time, survival rates were comparable in the early 30-day post-transplant period, and surpassed cold static storage at one and 2 years along with a reduction (although not to a statistically significant degree) in primary graft dysfunction. In the European-based PROTECT trial [55], EVHP was evaluated for safety and was followed several years later by the PROCEED II trial, a prospective, randomized non-inferiority trial in 10 different transplant centers [56]. Short-term clinical outcomes were again comparable between groups at 30 days and in a one-center follow-up to the 2-year mark, there was again no significant difference in survival despite significantly longer ischemic times in the EVHP group [57]. Separately from the EXPAND lung trial, an EXPAND heart preservation trial examined the utility of the system for extended criteria donors, with primary endpoints of survival at 30 days and absence of PGD at 24-h post-transplant [58]. In this study, the perfusion was not compared to standard preservation techniques, but did show high utilization of donor grafts, low rates of PGD, and high survival rates. To further characterize its use in unfavorable donors, Sáez et al. placed extended criteria donor hearts on machine perfusion, describing their single-center experience using the system and reporting improved short-term outcomes, although this was not directly compared to standard criteria [59]. In the same group, patients who were bridged to transplantation using left ventricular assist devices were compared to those whose donor organ was preserved with EVHP versus standard cold storage [60]. Recipients whose grafts had received machine perfusion had lower rates of mechanical circulatory support and decreased need for blood transfusion with longer out-of-body graft time, although ultimately there was no significant difference in overall survival [60]. Another examination of extended criteria transplants supported by EVHP compared to conventional storage showed that there was an effective increase in the number of transplants possible given the recovery of organs using the system, and that the survival of the donor hearts was comparable in terms of both hospital length of stay and survival [61]. A long-term follow-up

report of normothermic EVHP from the Berlin group followed recipients 4 years post-transplantation and showed high survival rates, supporting the use of the platform [62]. Other notable smaller studies include the case report of prolonged out-of-body time of 10 h using machine perfusion [63], as well as a case series from St. Vincent's in Australia demonstrating distant procurements on donation after cardiac death (DCD) with favorable results [64]. This group also demonstrated favorable lactate profiles during EVHP in their porcine DCD model, in which they also reported that following orthotopic transplantation, only those previously placed on EVHP were able to be weaned from cardiopulmonary bypass support [65].

The hypothermic alternative to EVHP has been trialed in both porcine and human cardiac transplantation. In a study of porcine DCD procurement, following normothermic regional perfusion, cold storage was compared to hypothermic machine perfusion, finding that contractility was only found with hypothermic EVHP along with a lower need for inotropic support and signs of myocardial damage [66]. A trial of hypothermic EVHP was run by the Australian St. Vincent's group, with a demonstration of 13 transplants, in which one patient required post-operative VA-ECMO and no postoperative mortality was observed at 30 days [67]. In the first prospective trial of hypothermic EVHP by the Lund group, standard criteria donors were compared in both modalities, showing improved mortality and cardiac-related adverse events in the machine preservation arm [68]. The HOPE trial followed with a multicenter investigation of the system in donor hearts with both short and long preservation times, up to 8 h and 47 min. When compared to comparator data retrieved from the International Society of Heart and Lung Transplantation (ISHLT), the EVHP group demonstrated improved 30-day survival and low rates of PGD development [69].

Liver, Kidney, and Pancreas Perfusion

The machine perfusion landscape in liver, kidney, and pancreas transplantation has been similar to that of thoracic organ transplantation. Attempts to improve donor organ viability, functional assessment, and overall graft and patient outcomes led to the rapid expansion of *ex vivo* hypo- and normothermic machine perfusion (HMP, NMP) and *in vivo* normothermic regional perfusion (NRP).

The concept of NMP was developed in the 1960s when it was initially studied for kidney preservation. Its development over the years has expanded its application to the liver [70], significantly improving outcomes by lowering graft injury, and discarded organs and increasing the mean preservation time compared to conventional static cold storage (SCS) techniques. Similarly, HMP was conceptualized in the 1960s. Significant advances in HMP technology were made in the 1990s through the pioneering work of Belzer and Southard [71], who developed the University of Wisconsin (UW) solution for the preservation of the liver, kidney, and pancreas.

A systematic review and meta-analysis by Jakubauskas et al. compared outcomes between NMP and HMP in liver transplantation versus static cold storage (SCS). Their study demonstrated the role of machine perfusion in reducing graft

injury and decreasing the rate of liver graft rejection, early allograft dysfunction (EAD) and non-anastomotic biliary strictures compared to SCS [72].

Machine perfusion in kidney transplantation showed a similar significant improvement in graft outcomes. The international randomized controlled trial by Moers et al. demonstrated a decrease in delayed graft function and an improvement in graft survival rates when utilizing HMP as opposed to SCS alone [73].

Ex vivo machine perfusion has also been utilized in the field of solid pancreas transplantation for quality assessment of discarded organs [74] and to improve preservation capacity, both the ability to resuscitate organs and to reduce delayed graft function [75].

ORGAN ENGINEERING ON MACHINE PERFUSION

As previously mentioned, the discrepancy between the availability of donor organs and the number of potential recipients waiting for a graft demands the development of novel therapies and treatments to bridge this gap. While *ex vivo* perfusion itself can help to evaluate marginal organs that would otherwise be discarded or facilitate the transplantation of more distantly procured grafts, there are many rejected damaged donor organs that could be retrieved. The addition of different therapies to the machine perfusion circuit presents an opportunity to recover a greater number of grafts. Advantages include highly specific temporal and spatial control of treatment delivery along with the avoidance of potential systemic effects.

Gene Therapy

Following the adoption of viral-based vectors, gene therapy has been pursued for its potential in immunomodulation and mitigation of the inflammatory response often seen in the events surrounding transplantation.

In cardiac transplantation, early rat models using adenovirus vectors carrying LacZ demonstrated that transfection could occur with minimal effect on graft survival, but the results were tempered by short gene expression lasting only weeks [76, 77]. Subsequent efforts have demonstrated that the hypothermic *ex vivo* platform can be a reliable conduit for vector delivery, with further development of adenovirus vectors of LacZ in both porcine and rat models and a need for continuous perfusion beyond single injections [78–80]. Further testing in rabbit models of normothermic EVHP demonstrated transfection using similar vector-gene combinations [81, 82]. Notably, the Duke group produced a porcine model in which luciferase was transfected and investigated the components of the platform that promoted or hindered successful transfection [83] in a 5 day post-transplantation model. This group additionally reported on a recombinant adeno-associated virus carrying luciferase that showed high efficiency and expression for 30 days without off-target effects or signs of rejection [84].

Within the heart, targets mainly aimed at immunomodulation have been identified and successfully delivered in animal models, including constructions of interleukin-10 or transforming growth factor-beta 1 (TGF- β 1), in which transplanted grafts have shown higher vector concentrations as well as prolonged graft survival

and inhibition of allograft rejection [85, 86]. Other vectors have included the delivery of a CTLA4 gene with a demonstration of localized immunosuppression in rats [87] and liposome-based delivery of IL-4 and IL-10 to rabbits with significantly increased survival and reduced rejection [88].

In the lung, gene therapy has also gained momentum for its potential for immune regulation. An important decision point is the route of administration: intravenous delivery is theoretically better at targeting the endothelium while the endotracheal route can target the epithelium. Several studies have investigated these routes of delivery, with the endotracheal route often being preferred [89, 90]. In earlier studies performed on rat transplantation by the Toronto group, the feasibility of gene transfection was explored using six and 12 hour *ex vivo* transfusions, although importantly this was done in cold preservation prior to the advent of modern EVLP [91]. In further developments of the model, human IL-10 within this adenovirus vector was studied, again with six or 12 h of *ex vivo* incubation with a reported improvement in post-transplant organ function [92]. The IL-10-containing vector was further examined in porcine and human lungs, now placed on modern EVLP systems, with endotracheal administration of treatment [93]. In the porcine model, following transplantation with a 4 h follow-up, cytokines IL-6 and IL-1 β were significantly decreased in the transfected group and the rejected human organs, there was no observed deterioration in function on EVLP. The group compared delivery of the viral vector using an *ex vivo* model with an *in vivo* model, and in both cases transplanted the lung to assess post-transplant function. The *ex vivo* group showed improved lung function compared to the *in vivo* group, with the authors hypothesizing that this methodology led to less inflammation associated with vector delivery to explain the improved function [94]. In a prolonged 7-day survival model, the same therapy was applied, demonstrating continued IL-10 expression through day 7 in addition to a lack of systemic toxicity, and improved lung function on day 7 in the treated group compared to the control conditions [95]. Of note, transfection with an IL-10-carrying vector has been shown in rodent models of transplantation without the use of EVLP and has shown decreased rates of histologically scored acute rejection [96], lower levels of inducible nitric oxide synthase [97], as well as improved gas exchange and decreased IL-2 expression [98].

Gene therapy in the lung has also been pursued with other targets, notably with the delivery of small interfering double-stranded RNA (siRNA) via lentiviral vectors targeting the class-II-trans-activator and β 2-microglobulin, which ultimately reduced MHC-I and II transcription in lung endothelial cells in a porcine model [99]. This mechanism leveraged by Figueiredo et al. allows for the evasion of the transfected cells from immune system activation, a valuable attribute when transplanting a donor organ into a recipient.

Gene therapy in kidney transplantation offers a novel approach to addressing common post-transplant complications such as rejection, ischemia-reperfusion injury (IRI), and the nephrotoxic effects of immunosuppressive drugs. By targeting the genetic foundations of these issues, gene therapy has the potential to significantly improve transplant outcomes. Bogacz et al. investigated the correlation between tacrolimus dose and genetic variation for IL-10 and its effect on therapeutic outcomes

in kidney transplantation patients, revealing the potential influence of IL-10 polymorphism on immunosuppressive drug dosage and the risk of acute graft rejection [100]. In cold-IRI mouse models, the anti-IL-2 immune complex was found to attenuate ischemia-reperfusion injury (IRI) after kidney transplantation by increasing renal regulatory T cells [101].

In the pancreas, gene therapy can introduce encoding for immunomodulatory proteins (e.g., interleukin-10, TGF- β) to create a local immunosuppressive environment. An example is the role of the VEGF gene in modifying isolated pancreatic islets for transplantation. VEGF has anti-apoptotic and angiogenic effects that are critical for islets under hypoxic stress during transplantation. Studies reveal that islets modified with the VEGF gene display have improved viability and more effective glucose regulation post-transplantation compared to non-modified islets [102].

Gene therapy in liver models in the context of transplantation remains limited [103–105]. Lorvellec et al. and the UCL group designed an *in vitro* whole liver [106]. The “Bioreactor grown Artificial Liver Model” (BALM) concept is a custom-designed 3D culture of human induced pluripotent stem cell-derived hepatocyte-like cells (hiHEPs) on a decellularized mouse liver scaffold. Adenoviral and lentiviral vectors introduced by intravascular injection have demonstrated that BALM has the potential to serve as a substitute for some *in vitro* and *in vivo* therapeutic testing methods.

It is of course important to consider the effects of the therapy delivered to the target organ and whether there are any unintended consequences. Potential risks of gene therapy include the possibility of certain types of cancer in the long term, including the suspension of Bluebird Bio’s clinical trial of LentiGlobin gene therapy for sickle cell disease due to cancer diagnoses in two recipients 5 years after the treatment [107]. Although the lentiviral vector may not have been the direct cause of cancer, there are still concerns about how well we really understand the long-term sequelae of the treatments we deliver. This highlights the need for studies that not only look at the immediate consequences of gene therapy in transplantation, but also at what might occur to treated organs years down the line. Other hypothetical concerns include how gene therapy could potentially activate the immune system, which would be of great concern in immunosuppressed transplant recipients, and whether there are off-target effects, which would ideally be mitigated by the use of therapies in an *ex vivo* setting where the therapy was applied directly to the target organ. Of course, it is critical to note that gene therapy is not a panacea for all transplantation troubles. Often, the disease processes that emerge are complicated and multifactorial and it is unlikely that any one target identified by gene therapy will provide a universal answer to transplantation-related complications. It is likely that multimodal therapies will be needed in order to truly optimize grafts for transplantation.

Mesenchymal Stromal Cells (MSCs)

Cell therapy has emerged as a form of treatment for damage owing to ischemia-reperfusion injury (IRI) and acute lung injury due to the properties exhibited by mesenchymal stromal cells (MSCs). This cell population has been shown to act on inflammatory pathways and play a role in immunomodulation, with multi-targeted effects and has been

studied for its paracrine effects leading to tissue regeneration. Bone marrow-derived MSCs have been linked to increased levels of IL-10 to promote anti-inflammation [108] as well as the secretion of immunomodulatory growth factors [109]. A further advantage is the lack of co-stimulatory CD40, CD40L, CD80, and CD86 which confer a lack of immunogenicity to MSCs [110], making them a suitable therapy for immunosuppressed and sensitive recipients.

In lung transplantation, EVLP has provided a platform for MSC delivery. At the University of California, San Francisco, cells applied during *ex vivo* perfusion were shown to restore endothelial permeability in damaged lobes of human lungs and improve alveolar fluid clearance [111]. Improved alveolar fluid clearance was also observed with the treatment of cell culture medium conditioned with MSCs without the cells themselves. Further studies of rejected human lungs within this group also showed improvement in alveolar fluid clearance with 5×10^6 cells instilled into the lungs on EVLP [112]. In studies of cells isolated from human umbilical cord tissue by the Toronto group, administration of MSCs aimed at ameliorating prolonged cold ischemia in previously healthy donor lungs was performed first with the intent of determining ideal cell concentrations following a comparison of intrabronchial vs. intravascular routes of administration [113]. As a result, intravascular administration was found to be superior given the higher airway pressures associated with intrabronchial doses, as well as the identification of a suitable dose equivalent to 5×10^6 cells per kg in the porcine model. In a longer cold storage experiment with 24 h of cold ischemia followed by 12 h of EVLP, MSCs showed decreased apoptosis and reduced pulmonary edema after transplantation consisting of 4 h of reperfusion [114]. In an effort to leverage the immunomodulatory effects of MSCs, cells were engineered to produce anti-inflammatory IL-10 and were given to human lungs rejected for transplantation under 12 h of EVLP [115]. In this study, there were no significant differences in pulmonary vascular resistance, oxygenation capacity, and compliance compared to control lungs, which was attributed to an acidic lung microenvironment and heterogeneity of injury across human organs. Umbilical cord-derived MSCs were also examined during rat EVLP by Pacienza et al., indicating that intravascular infusion showed better lung compliance in addition to a reduction in neutrophil-related markers, supporting an anti-inflammatory role of the cell treatment [116].

In the heart, *ex vivo* perfusion has only been studied with the application of MSC-conditioned medium, rather than the stromal cells themselves. In an investigation of hypothermic perfusion in rats, a conditioned medium was associated with the downregulation of proinflammatory cytokines following transplantation and was hypothesized to protect against myocardial IRI [117]. In a later study by the same group, the conditioned medium was tested in brain-dead donors, showing improved systolic function after preservation and reduced cell apoptosis [118]. In normothermic EVHP, the conditioned medium as studied by the Guangzhou group showed that oxidative stress, inflammation, and apoptosis were mediated by the treatment and that warm ischemic injury was improved [119]. When the same group transplanted the conditioned hearts, spontaneous contraction and left ventricular systolic diameter improved in the treated group and lower levels of plasma cytokines

were detected [120]. Compared to whole-cell treatment, these studies offer an opportunity to explore the conditioned medium as a therapy in and of itself, which may have advantages over MSC administration in terms of both time and cost savings. Interestingly, MSCs have been studied in the treatment of ischemic and non-ischemic cardiomyopathy and have even been labeled as cardioprotective, implying an avenue for further investigation in the transplantation setting [121–127].

In the kidney, MSC therapy has also been studied for its potential to repair damage from ischemia, which was tested in a human kidney model of *ex vivo* perfusion. MSC treatment showed reduced inflammatory cytokines and increased adenosine triphosphate and growth factors [128]. In a rat model, kidney grafts were treated with MSCs or with extracellular vesicles released by the MSCs, both of which were associated with improved metabolism and ion transport and Gregorini et al. concluded that both forms of treatment protected the kidneys from reperfusion damage [129]. In later work by Pool et al, porcine kidneys were treated with MSCs during normothermic machine perfusion and labeling of bone marrow-derived MSCs showed that the cells rapidly decreased in the circulating perfusate over time and that a small portion was intact and appeared within the glomeruli [130]. The authors noted that the study demonstrated that the cells can reach and reside in the kidney, but that the infusion rate must be high enough to visualize within the capillaries. The group then also reported that with normothermic perfusion, MSC treatment at these sufficiently high cell infusion rates reduced injury markers and induced the release of immunomodulatory cytokines during ischemia-reperfusion injury [131]. MSC treatment was further followed in a transplantation model by Lohmann et al. with the finding that while MSC treatment was safe and well tolerated within their autotransplant cohort, there were no significant effects of the therapy within the 14 days post-transplantation [132]. An important question within this line of work is the dose tolerance of the kidneys to large amounts of MSCs, which was investigated by the same group through their porcine model comparing doses of ten million and one hundred million MSCs injected into the renal artery [133]. The lower dose was well tolerated, while the high dose resulted in tissue inflammation and glomerular and tubular damage. A balance must be struck between administering doses of cell therapy that are both effective and not so high as to cause damage themselves.

Extending the investigation of MSC therapy to the liver, several studies have examined the efficacy of the therapy within this solid organ. In a rat model, MSCs were administered during machine perfusion following donation after cardiac death, with treatment correlating with improved bile production and histologic findings [134]. MSC treatment was shown by another group to ameliorate injury due to oxidative stress and increase mitochondrial function within their rat liver cohort [135], which was further extended in other studies to demonstrate improved histopathology, hepatocyte ultrastructure, and microcirculation-related indices [135, 136]. In a porcine model, MSCs were shown to be retained in a machine-perfused liver with the reported continuation of their paracrine activity via increased human-specific IL-6 and IL-8 levels in porcine blood [137].

Cross-Circulation

Although the system does not employ a machine, the perfusion method of cross-circulation is noteworthy for its novelty in the development of methods to regenerate damaged donor lungs and livers. Reported advantages of cross-circulation over machine perfusion include longer recovery times and an increased duration of extracorporeal support [138]. An early report of cross-circulation in the lung appeared under the work of Dr. Joel Cooper who reported cross-circulating aspirin-treated sheep with non-treated sheep to examine the mixing of the circulating blood volumes and to study the circulating elements [139]. More recent work has emerged to establish a cross-circulation platform as a means to rehabilitate damaged donor lungs. The Bacchetta group first reported the use of cross-circulation to achieve prolonged perfusion times in excess of 36 h, followed by a porcine study in which lungs damaged by the aspiration of gastric contents were then reconditioned using the platform [138, 140]. To demonstrate the system's ability to provide prolonged support, porcine lungs were additionally maintained in cross-circulation for 4 days, with preserved lung function and decreased levels of several cytokines, including IL-1 β , IL-8, and IL-12 among others [141]. Gastric aspiration-damaged recipients were also shown to help recover donor lungs subjected to prolonged cold storage in a model that leveraged injury in both the donor and recipient within cross-circulation [142]. In a step closer to clinical application, rejected human lungs were added to the xenogeneic circuit to show functional and histological recovery under the platform [143, 144] with 24 h of support. There are of course concerns about the immunogenic response that might follow when exposing human lungs to a xenogeneic recipient. Wu et al reported that in their established cross-circulation model, porcine immune cells and immunoglobulins can be found infiltrating human donor lung tissue after 24 h of xenogeneic perfusion [145]. Similarly, in an investigation by Glorion et al., immune cells from the porcine host were found in lung cell suspensions, in bronchoalveolar lavage fluid, and in histological examination of lung tissue [146]. The group also posited that cross-circulation could act as a platform to investigate the immune processes that follow transplantation without carrying out full transplantation and could serve as a means to assess anti-inflammatory and immunomodulatory drugs based on the response of the porcine host.

Cross-circulation has also been investigated in porcine livers, as early as 2001 when a group from Kyoto studied porcine liver perfusion with baboon cross-circulation [147]. The group found microthrombi formation in the perfused porcine livers and macroscopic hemolysis in 3 out of 5 cases with a maximum duration of 6 h of cross-circulation. In a publication from the same year, Nara et al. in Hirosaki, Japan cross-circulated hepatectomized dogs with donor pigs to test a semipermeable membrane in their model of double filtration plasmapheresis cross-circulation [148]. This model was further explored by the group in their later work with a canine whole liver supporting a porcine model of hepatic failure induced by lipopolysaccharide and alpha-amanitin, which demonstrated improvements compared to the porcine group not supported by the liver cross-circulation [149].

The group reported that the lack of immune-mediated reactions was the result of a semipermeable membrane blocking the movement of IgM, which they investigated in later work [150].

Bacchetta et al, who had previously investigated lung cross-circulation also followed up with work on liver cross-circulation in 2022, with a publication using a porcine host for an extracorporeal porcine liver to show improvements in synthetic function, metabolic activity, and histopathological examination [151]. This effort was then replicated using explanted human donor livers that were supported by xenogeneic cross-circulation, which also demonstrated improved histopathologic assessment at 24 h of support [152].

While other organs have not been investigated as extensively, there are reports of cross-circulation being studied in heart transplantation. In one model, standard *ex vivo* cardiac perfusion in a porcine graft was compared to EVHP supplemented with cross-circulation from a paracorporeal support pig [153]. This was then further compared to a third setup in which blood from the support animal was filtered into plasma during cross-circulation. Cross-circulation was reported in both set-ups to have high oxygen consumption and vascular resistance, with the conclusion that it improved preservation compared to standard EVHP. Cross-circulation was also used to support EVHP for 72 h in a study by the Extracorporeal Life Support Laboratory at the University of Michigan, which supported ovine hearts with paracorporeal sheep. All cross-circulated hearts were deemed suitable for transplantation by the end of 72 h, while the control group, which was not cross-circulated, all failed after 6–10 h of EVHP [154]. The group then further explored porcine EVHP support in a publication showing that EVHP could be maintained for 24 h with plasma exchange, which they reported eliminated the need for a paracorporeal animal [155].

FUTURE DIRECTIONS IN BIOENGINEERING STRATEGIES

The development of bioengineered organs that could be produced in the laboratory would be the ultimate step in alleviating the problems caused by the shortage of donor organs. In the quest to develop such a product, techniques have emerged to grow or build organs. One such method uses organoids made by three-dimensional cell cultures formed from patient-derived cells designed to replicate *in vitro* the microarchitectural and functional characteristics of their *in vivo* counterparts. One advantage of building such models is the ability to further study diseases and potential drugs and therapies on a platform that closely resembles human physiology while reducing the need for animal-based research. In the lung, for example, the advances made in microfluidic organ-on-a-chip devices have been driven by the study of specific disease processes such as those arising from particulate exposure, pulmonary fibrosis, and acute respiratory distress syndrome [156–158].

The study of human organ physiology and disease occurrence through microfluidic devices can be extrapolated for use in transplantation surgery in general. In transplantation, these

could represent an avenue for patient-specific study of response to immunosuppression in an effort to optimize regimens, and minimize toxicity, adverse drug reactions, and side effects, balancing these against the risk of rejection [159].

Another potential application is the detection of transplant rejection, which remains a clinical challenge given the high rates of both acute and chronic forms of immune-mediated rejection. Madhvapathy et al. used rat models in which a wireless electronics interface was connected to transplanted kidneys, allowing for real-time monitoring of ultradian rhythms, disruption of the circadian cycle, and organ temperature. These patterns change once immunosuppression is discontinued before creatinine elevation [160].

On top of leveraging advanced techniques to further study transplant-related complications, bioengineering principles could potentially be used to develop artificial organs. In decellularization-recellularization experiments, cellular material is removed during a decellularization process to then be replenished with a new repopulation of cells during the recellularization phase. Decellularization of living tissue can be accomplished by chemical, enzymatic, physical, or combined approaches to generate an extracellular matrix scaffold that provides structural support for the future regenerated organ. The most common materials used for decellularization are a combination of detergents such as sodium dodecyl sulfate (SDS) and Triton-X100 [161]. This is an important step as retaining any cell membrane epitopes would trigger a host response *in vivo*. [162] Once the mechanical integrity has been established and decontaminated, the acellular scaffold undergoes the process of repopulation with organ-specific cells, thus reconstituting the organ from a functional perspective.

For the liver, *in vitro* studies of drug-induced hepatotoxicity, liver function and regenerative potential have advanced the development of primary hepatocyte isolation and the generation of two- and three-dimensional liver models, spheroids, organoids, slice cultures and microfluidic models [163, 164]. Decellularized human liver scaffolds have been repopulated with human cell lines and resulted in liver scaffold cubes that could be successfully xenotransplanted subcutaneously into immunocompetent mice, without any resulting immunogenic responses [165]. Other investigations in animal models have shown that decellularization of the whole organ liver is possible with preservation of the macrostructure [166]. While decellularization has been demonstrated using a variety of methodologies, there is no consensus on the most advantageous approach nor have any unified criteria been accepted [167].

Similarly, studies have reported successful recellularization of kidney scaffolds with preservation of microarchitecture and ECM components such as glycosaminoglycans (GAGs) [168–170]. After preloading of the vascular matrix with vascular endothelial growth factor (VEGF) and angiopoietin 1, pluripotent stem cell-derived endothelial cells can be delivered with efficient adherence, proliferation, and survival [168].

The lung is a challenging organ to bioengineer given the complexity of the cell population that constitutes it. Scaffold generation has been approached both from the angle of using human-derived acellular scaffolds and artificial scaffolds made from synthetic polymers [171–173]. Because there are over 40 different cell types that make up the lung, recellularization is a challenging task and a variety of sources including tissue-isolated progenitor cells, differentiated pluripotent stem cells, and mesenchymal stem cells are being investigated for their potential [174–177].

While these methodologies have a long way to go before they can be considered for clinical application, the progress being made is promising for the advent of engineered organs to meet the demand for transplantation.

CONCLUSION

As the definitive management for end-stage organ disease, transplantation remains an in-demand procedure. While surgical and perioperative techniques have progressed, there is still a notable shortcoming, notably the mismatch between the number of available organs and the need for grafts by potential recipients on the waiting list. In order to bridge the gap between supply and demand, new methods are needed for the recuperation of suitable organs. Machine perfusion provides an emerging platform for the recovery and regeneration of organs that would otherwise be discarded. Advancements made in gene therapy, applications of mesenchymal stem cells, cross-circulation methodologies, and the advent of advanced bioengineering principles have been covered in this review as promising avenues for organ recovery. While many of these approaches are in various stages of development and clinical application, they represent important advances that can be leveraged in the search for meaningful ways to increase the donor pool.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Orthostatic *Ex-Vivo* Lung Perfusion (EVLP): A Proof of Concept

Massimo Boffini^{1*}, Andrea Costamagna², Matteo Marro¹, Erika Simonato¹, Paola Cassoni³, Luca Bertero³, Vito Fanelli², Cristina Barbero¹, Luca Brazzi² and Mauro Rinaldi¹

¹Cardiac Surgery Division, Surgical Sciences Department, Citta della Salute e della Scienza, University of Torino, Turin, Italy, ²Anesthesiology and Intensive Care Division, Surgical Sciences Department, Citta della Salute e della Scienza, University of Torino, Turin, Italy, ³Pathology Unit, Medical Sciences Department, Citta della Salute e della Scienza, University of Torino, Turin, Italy

The key goal in lung donation remains the improvement of graft preservation with the ultimate objective of increasing the number and quality of lung transplants (LTx). Therefore, in recent years the field of graft preservation focused on improving outcomes related to solid organ regeneration and restoration. In this contest Ex-Vivo Lung Perfusion (EVLP) plays a crucial role with the purpose to increase the donor pool availability transforming marginal and/or declined donor lungs suitable for transplantation. Aim of this proof of concept is to test the safety, suitability and feasibility of a new tilting dome for EVLP designed considering the dorsal lung areas as the “Achilles’ heel” of the EVLP due to a more fluid accumulation than in the supine standard position.

Keywords: lung transplant, animal model, EVLP, organ preservation, perfusion

INTRODUCTION

Although nowadays lung transplantation (LTx) is a well-established treatment for patients with end-stage lung diseases, shortage of suitable lung grafts is still a major limitation for an extensive application of this therapy and the low number of acceptable grafts negatively impacts waitlist mortality [1]. However, increasing the quantity of grafts by accepting donor organs of inferior quality might increase the incidence of primary graft dysfunction and worsen recipients’ outcomes [2]. *Ex Vivo* Lung Perfusion (EVLP) has emerged as an effective technique for preserving, evaluating and eventually reconditioning donor lungs [2–7]. Thus lung transplant activity may be increased by 15%–30% in Lung Transplant Programs adopting EVLP protocols [8–10].

In the last years we faced an increase in clinical experience and an implementation of this technology, configurating EVLP as a tool providing an opportunity to treat initially rejected donor lungs before lung transplantation: antimicrobial therapy [11, 12], interleukin gene therapy [13], cytokines absorption [14] are some examples of potential therapeutical applications. Despite this wider application, there are still remaining issues concerning EVLP use and one of these might be the supine position of the graft during the procedure. This can result in hyper-perfusion of the lower-dependent regions and hypo-perfusion of the nondependent regions according to the West three-zone model [15]. The subsequent asymmetric ventilation-perfusion distribution might increase capillary shear stress resulting in increased vascular permeability with pulmonary edema accumulation [16]. Thus Niikawa et al. [17], in a porcine model study comparing prone and supine positioning of lungs during EVLP, showed less ischemia-reperfusion injury and improved lung function in the prone group. The same authors confirmed this results in human lungs rejected from clinical use, showing in addition a selective improvement in terms of lung function and cytokine load in the lower lobes of the prone lungs [18]. In the same way Ordies et al. [19]

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*Correspondence

Massimo Boffini,
✉ massimo.boffini@unito.it

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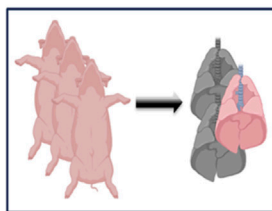
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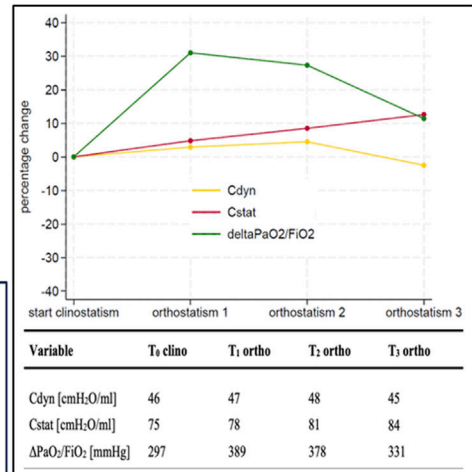
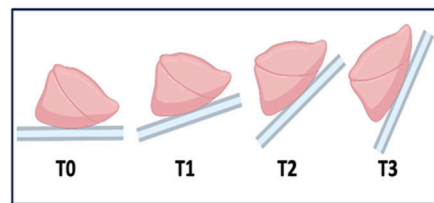
Orthostatic Ex-Vivo Lung Perfusion (EVLP): a proof of concept

- Experimental study to test the **suitability, feasibility** and **safety** of a **new tilting dome** hypothesizing its better impact on the dorsal area of lungs during EVLP.
- The **orthostatic EVLP** provides a **stability of ventilation** parameters, **blood gas** and **histology analysis**.

Grafts harvesting



EVLP



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GRAPHICAL ABSTRACT

highlighted a more concentrated fluid accumulation in lower-dependent regions in the prone group of porcine lungs mounted on a normothermic EVLP compared to the supine group, despite oxygenation (P/F), dynamic compliance and peak airway pressures were comparable between both groups. To the best of our knowledge, only two cases of successful lung transplantation after normothermic acellular EVLP with prone positioning, indicated by the clinical evidence of lung oedema, have been published so far [20].

Although promising, prone positioning of the EVLP lungs raises some technical concerns about feasibility and safety in the routine clinical practice. First, pulmonary arterial and left atrial cuffs and cannulas are non-accessible during prone position. Second, the anterior face of the grafts might be compressed or injured during lung manipulation and positioning. Third, the anterior regions might in turn experience hyper-perfusion during the procedure. The current evidence focuses on the comparison between supine and prone position, but theoretically, the best option would be the possibility of switching between supine and prone position (and vice-versa) during the perfusion. Although potentially beneficial in terms of ventilation/perfusion mismatch, this approach could raise some concerns related to the risk of repeated graft manipulation, break in sterility, accidental mispositioning of vascular cannulas and endotracheal tube and eventually lung lesions.

Considering the dorsal lung areas as the “Achilles’ heel” of the EVLP, we experimented a self-designed tilting organ dome allowing a graft positioning change from 0° to approximately 90° during the procedure, miming the physiological position of

human lungs in a standing subject. This would allow to overcome the aforementioned concerns about sterility break and technical issues. Aim of this experimental study was to test the feasibility and safety of the new dome hypothesizing its better impact on the dorsal extravascular fluid accumulation observed during EVLP.

METHODS AND MATERIALS

Study Design

Three adult pigs (around 200 kg each) of a slaughterhouse were included in the study. All animals were treated according to the European Commission guidelines about animal care during butchery [21]. After euthanasia, the heart-lung blocks were procured by the veterinary surgeon in a standard fashion and then anterogradely flushed with cold (4°C) Perfadex® solution (70 mL/kg). Only one of the three blocks presented with no injuries due to the retrieval and/or macroscopic signs of pulmonary damage thus it was considered feasible for the experiment. One was excluded for macroscopical edema from the trachea and one for pleuro-parenchymal lesions with significative air leak. A second retrograde cold flush of 1,000 mL of the same solution was then performed in the block and then subjected to 4 h of cold static storage, mimicking the setting of donation after circulatory death (DCD). Differently from other study protocols [17, 19], due to the slaughterhouse setting and rules, the animals were not intubated prior death and grafts were harvested without

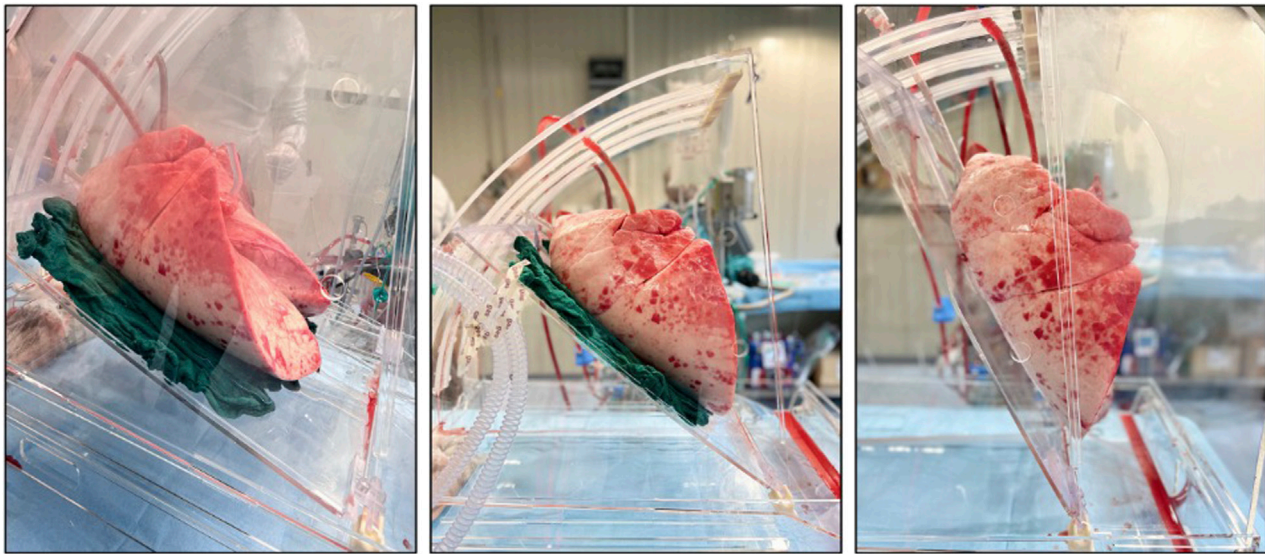


FIGURE 1 | Lung block positioned in the dome with different degree of inclination.

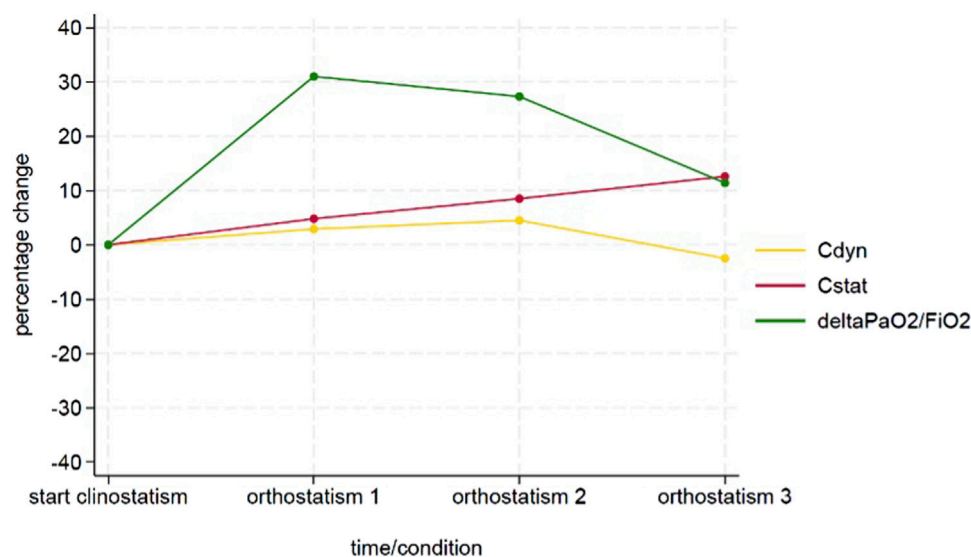


FIGURE 2 | Time course of the delta PaO₂/FiO₂, Cdyn and Cstat variables.

modification of the butchery production line due to slaughterhouse regulation and according to the animal welfare.

Ex Vivo Lung Perfusion

Once in the dedicated facility, the left atrial (LA) cuff was trimmed and sewn to a dedicated cannula with a 4-0 polypropylene running suture and the pulmonary artery (PA) cannula simply inserted proximally to the artery bifurcation and secured with two heavy silk ties. A conventional endotracheal tube was then inserted and secured. The components of the circuit were: a centrifugal pump, a

leukocyte filter, a hollow-fiber oxygenator, a heat exchanger and a hard-shell reservoir. The circuit was primed with 2.0 L of acellular Steen solution. 500 mg methylprednisolone, 3000 IU of unfractionated heparin were also added to the solution of perfusion. The new dome (PerLungs[®], Aferetica s.r.l., Bologna, Italy) (**Figure 1**) is a novel chamber with a mobile base manually tilting from 0° to approximately 90° on the coronal plane. A hook in the proximal part of the tilting plate serves to anchor the lungs at the carina and it keeps the graft suspended when in orthostatic position. The hook can be placed at different lengths according to the dimensions of the graft. On the side of the dome, there are

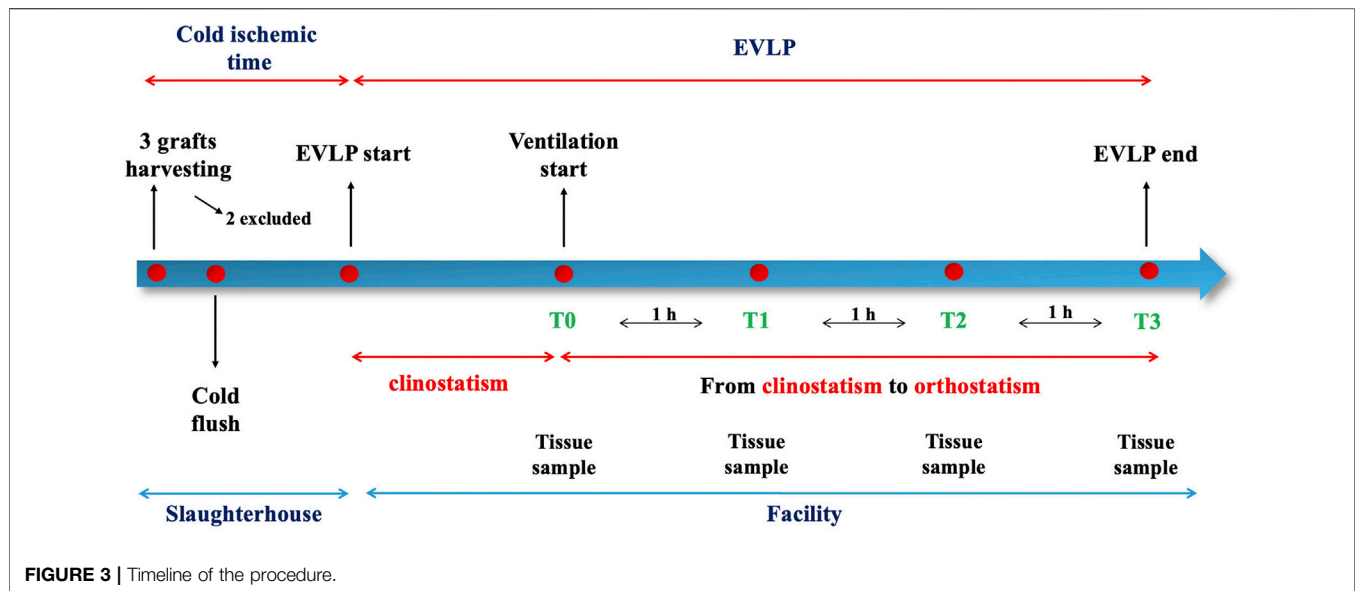


TABLE 1 | Time course of physiologic and respiratory mechanic variables during EVLP.

Variable	T ₀ clino	T ₁ ortho	T ₂ ortho	T ₃ ortho
Cdyn [cmH ₂ O/ml]	46	47	48	45
Cstat [cmH ₂ O/ml]	75	78	81	84
ΔPaO ₂ /FiO ₂ [mmHg]	297	389	378	331

List of abbreviations: Cdyn dynamic compliance; Cstat static compliance; ΔPaO₂/FiO₂ difference between left atrial PaO₂ and pulmonary artery PaO₂ with an 1.0 oxygen concentration.

working ports to allow the crossing of additional catheters used during perfusion. The anterior face of the chamber presents three slits the circuit can pass through. The lungs were thus taken to the EVLP dome and connected to the circuit: the PA cannula was then connected to the circuit and anterograde flow was started. The LA cannula was then deaired and connected to the circuit. According to our protocol, the target perfusion flow consists of 40% of the donor predicted cardiac output. Following the principles of gradual rewarming and stepwise increase of vascular flow, the perfusion is then initiated with subsequent increase of the calculated target flow to reach the target cardiac output in 1 hour. At 10 min, the flow is increased to 20% and the temperature is set to 30°C. The flow is progressively increased to 30%, 50%, 80% and finally 100% of target, respectively every 10 min. The temperature of the heat exchanger is set to 37°C after 20 min from the beginning of perfusion and ventilation is initiated (FALCO 202 EVO, Siare Engineering International Group Srl, Italy) with a standard ventilation (Vt 7 mL/kg, PEEP 5 cm H₂O and 7 cycles/min, FiO₂ 21%) when the temperature reaches 32°C. Just before the lungs are ventilated, the oxygenator is connected to a gas mixture (86% N₂, 8% CO₂ and 6% O₂) tank. The sweep gas is set to achieve a pCO₂ between 35–40 mmHg. Lastly, the left atrial

pressure is carefully maintained to a stable value of 3–5 mmHg by adjusting the venous return to the reservoir. The lungs reached the steady state and they were subsequently perfused for up to 4 h. The tilting plate is progressively moved to reach a nearly orthostatic position during the first hour of perfusion and the graft is kept in the orthostatic positing till the end of perfusion.

Lung Function Assessment on EVLP

After the beginning of ventilation the dome base was positioned from 0°C to approximately 90°C and lungs were derived hourly with physiologic parameters (Vt 10 mL/kg, PEEP 5 cm H₂O and 10 cycles/min, FiO₂ 100% - **Figure 2**). The following measurements were performed:

- dynamic compliance (cdyn) that was calculated according to the following formula:

$$C_{dyn} = V_t / (P_{iP} - PEEP) \text{ [mL/cmH}_2\text{O]}$$

where PiP was peak inspiratory pressure.

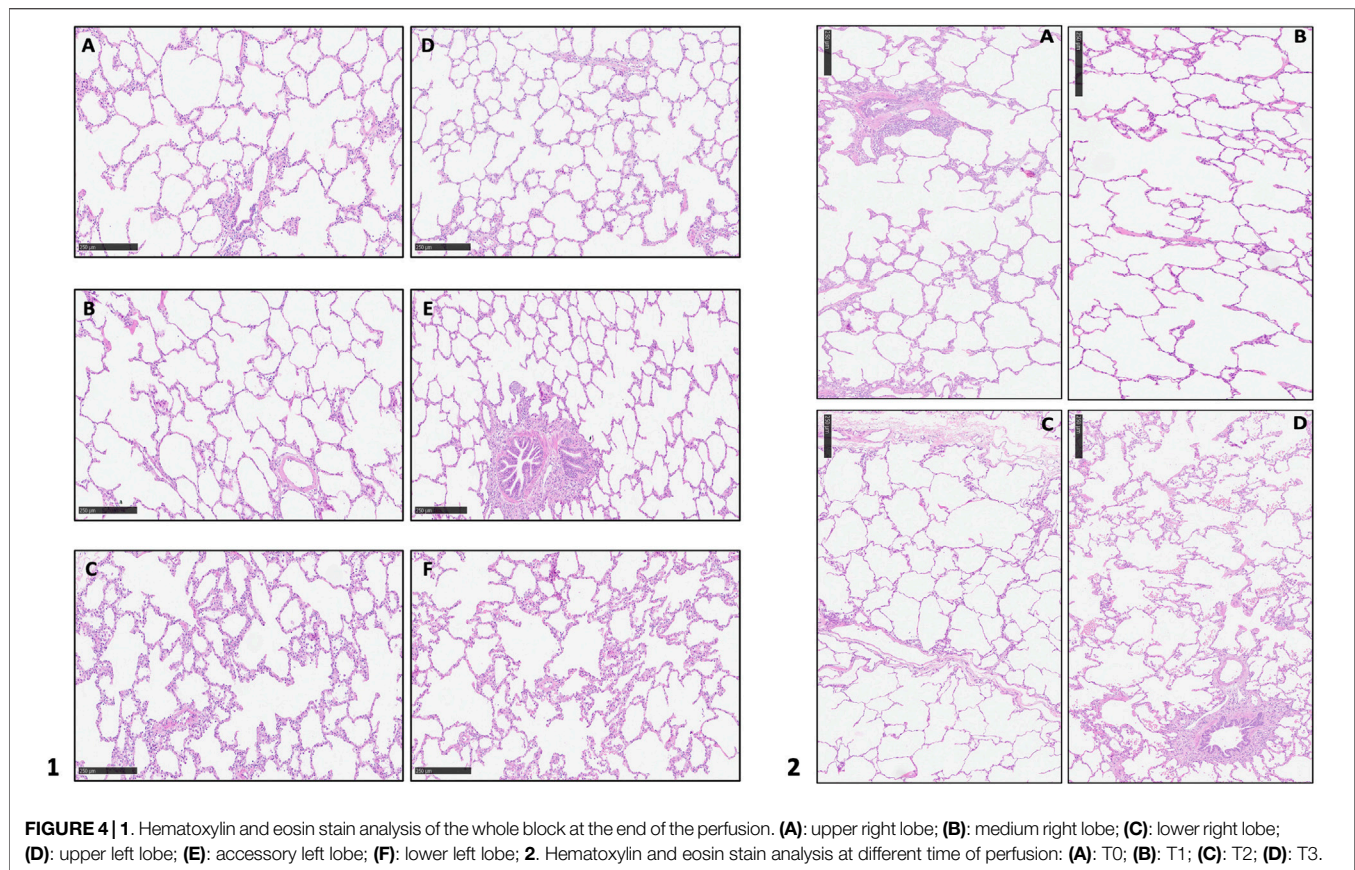
- static compliance (cdyn) that was calculated according to the following formula:

$$C_{stat} = V_t / (P_{plat} - PEEP) \text{ [mL/cmH}_2\text{O]}$$

where Pplat was plateau inspiratory pressure, calculated during an inspiratory hold maneuver.

- assessment of gas exchange: blood gas tests were performed on perfusate samples to calculate the difference between left atrial PaO₂ and pulmonary artery PaO₂ with an Oxygen concentration on the ventilator of 100% for 5 min

$$\Delta PaO_2 / FiO_2 = PaO_2 / FiO_{2LA} - PaO_2 / FiO_{2PA} \text{ [mmHg]}$$



All the variables were reported as absolute values and percentage change between the value measured at each timepoint during orthostatism and its baseline measured in supine position. **Figure 3** represents the study timeline.

Lung Tissue Sample Collection

Lung tissue samples were collected from the lower left lobe at the beginning of the procedure (supine position) and every hour of EVLP (in the orthostatic position), then fixated in formaldehyde 6%, embedded in paraffin, sliced (4 mm) and stained with hematoxylin and eosin. A lung pathologist studied each biopsy for presence of congestion, alveolar edema, alveolar granulocyte infiltration, interstitial granulocyte infiltration, alveolar microhemorrhage and micro-thrombosis. Additional histological analysis were performed on the entire block at the end of the procedure, one for each of the six lobes.

RESULTS

During the entire procedure, lungs maintained a stable gross aspect with no evident lobar congestion and/or atelectasis. Physiological ventilation parameters and blood gas analysis variation during EVLP are shown in **Figure 2**. Lungs maintained satisfying gas exchange parameters throughout the experimental time: the ΔPaO_2 (PaO_2 pre - PaO_2 post) was >300 mmHg for 4 h, with a mean value of 366 ± 30 mmHg. Peak and plateau airway maintained a stable value during the procedure; similarly dynamic and static compliance did not

worse significantly over time (**Table 1**). A representative histology sample of each region at the end of the perfusion is presented in **Figure 4.1**. The hematoxylin and eosin stain analysis showed neither edema nor congestion in all the samples from the first to the fourth hour of the procedure (**Figure 4.2**), with a minimum focal alveolar septal thickening; the granulocytic infiltration was sporadic in the lung interstitium and absent in the alveolus. Moreover, neither hemorrhage nor micro-thrombosis have been detected.

DISCUSSION

The key goal in lung donation remains the improvement of graft preservation with the ultimate objective of increasing the number and quality of transplants. Therefore, in recent years the field of graft preservation focused on improving outcomes related to solid organ regeneration and restoration. In this contest EVLP plays a crucial role with the purpose to increase the donor pool availability transforming marginal and/or declined donor lungs suitable for transplantation. Aim of this proof of concept is to test the safety, suitability and feasibility of a new tilting dome for EVLP designed considering the dorsal lung areas as the “Achilles’ heel” of the EVLP due to a more fluid accumulation and a ventilation-perfusion mismatch in the supine standard position.

Mechanical ventilation in the prone position, first reported in 1970 [22], has been evaluated as a strategy to enhance oxygenation and lung recruitment in acute respiratory failure. The mechanisms

by which no-supine positioning may benefit patients with acute respiratory distress syndrome (ARDS) undergoing mechanical ventilation include increasing end-expiratory lung volume, improving ventilation–perfusion matching and preventing ventilator-induced lung injury by more uniform distribution of tidal volume through lung recruitment and alterations in chest wall mechanics [23]. Based on this postulate, the porcine model study of Niikawa et al. [17] demonstrated that prone positioning during EVLP was significantly associated with less lung weight gain, better P/F ratio and improved compliance, lower inflammatory cytokines, and lower ALI grade difference than supine positioning in the control group. Some months later Ordies et al. [19] demonstrated that prone positioning of the donor porcine graft during EVLP is feasible and resulted in more homogenous distribution of interstitial fluid.

In our experience, the positioning of graft at almost 90° provided a stability of ventilation parameters and blood gas analysis data. The macroscopic evaluation (Video 1) also demonstrated a better expansion of the dorsal graft areas, miming the physiological movement of the lungs of a standing subject.

This proof-of-concept experiment has several limitations related with the experimental setting and the limited number of tests. The animal model mimics DCD donation with some differences mainly related with the suspension of mechanical ventilation and a delayed flush of preservation solution. The paper describes a single experiment without a control group of standard EVLP. This does not allow to speculate about a potential positive and meliorative effect of the orthostatic position during the ex-vivo perfusion. The paper wants to be only a “proof of concept” regarding the feasibility of tilting the graft during EVLP. A direct comparison with a control group may add specific insights regarding its efficacy and/or potential improvement and it may allow a deeper evaluation exploring if the tilting dome only moves the fluid according to the gravity law or a more “physiological” position might act through more complex pathways. Moreover, the absence of a portable x-ray machine did not allow a radiological evaluation of the graft in terms of lobar congestion. However, our initial test might suggest that this approach is feasible without compromising the usual stability obtained maintaining the graft in the standard position.

CONCLUSION

Further experimental tests are mandatory to better analyze the safety, feasibility and potential benefits of a “stand-up position” of the graft on the physiological respiratory mechanics and blood gas analysis data.

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was approved by the Comitato Etico Territoriale AOU Città della Salute e della Scienza di Torino. The study was conducted in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; MB, AC, MM, and ES conducted the experiment. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13178/full#supplementary-material>

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Ex Situ Left Ventricular Pressure-Volume Loop Analyses for Donor Hearts: Proof of Concept in an Ovine Experimental Model

I. A. Ertugrul^{1*}, R. A. D. A. Puspitarani², B. Wijntjes³, M. T. Vervoorn⁴, E. M. Ballan^{4,5,6}, N. P. van der Kaaij⁴, H. van Goor⁷, B. D. Westenbrink², A. van der Plaats³, F. Nijhuis³, V. van Suylen¹ and M. E. Erasmus¹

¹Department of Cardiothoracic Surgery, University Medical Centre Groningen, University of Groningen, Groningen, Netherlands,

²Department of Cardiology, University Medical Centre Groningen, University of Groningen, Groningen, Netherlands, ³XVIVO, Gothenburg, Sweden, ⁴Department of Cardiothoracic Surgery, University Medical Centre Utrecht, Utrecht, Netherlands,

⁵Department of Cardiology, Laboratory of Experimental Cardiology, University Medical Center Utrecht, Utrecht, Netherlands,

⁶Netherlands Heart Institute, Utrecht, Netherlands, ⁷Department of Medical Biology and Pathology, University Medical Centre Groningen, University of Groningen, Groningen, Netherlands

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*Correspondence

I. A. Ertugrul,
✉ i.a.ertugrul@umcg.nl

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Ex situ heart perfusion (ESHP) has emerged as an important strategy to preserve donation after brain death (DBD) and donation after circulatory death (DCD) donor hearts. Clinically, both DBD and DCD hearts are successfully preserved using ESHP. Viability assessment is currently based on biochemical values, while a reliable method for graft function assessment in a physiologic working mode is unavailable. As functional assessment during ESHP has demonstrated the highest predictive value of outcome post-transplantation, this is an important area for improvement. In this study, a novel method for *ex situ* assessment of left ventricular function with pressure-volume loop analyses is evaluated. Ovine hearts were functionally evaluated during normothermic ESHP with the novel pressure-volume loop system. This system provides an afterload and adjustable preload to the left ventricle. By increasing the preload and measuring end-systolic elastance, the system could successfully assess the left ventricular function. End-systolic elastance at 60 min and 120 min was 2.8 ± 1.8 mmHg/mL and 2.7 ± 0.7 mmHg/mL, respectively. In this study we show a novel method for functional graft assessment with *ex situ* pressure-loop analyses during ESHP. When further validated, this method for pressure-volume assessments, could be used for better graft selection in both DBD and DCD donor hearts.

Keywords: donation after circulatory death (DCD), *ex situ* heart perfusion, viability assessment, functional evaluation, heart transplantation

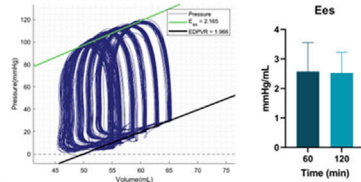
Abbreviations: DBD, Donation after brain death; DCD, Donation after circulatory death; Ees, End systolic elastance; ESHP, *ex situ* heart perfusion; HTx, Heart transplantation; LV, Left ventricle; PV, Pressure-volume; SVR, Systemic vascular resistance.

Ex situ left ventricular pressure-volume loop analyses for donor hearts: proof of concept in an ovine experimental model

Aim

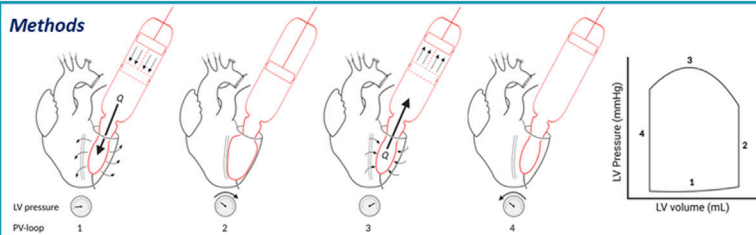
To introduce and test an ex situ PV loop system which provides an after- and adjustable preload to the LV during unloaded ex situ heart perfusion.

Results



- Functional assessment of the LV was feasible by increasing the pre-load.
- End systolic elastance (Ees) at 60 min and 120 min was 2.8 ± 1.8 mmHg/mL and 2.7 ± 0.7 mmHg/mL ($p=0.9$), respectively.

Methods



- PV loop assessments during normothermic ex situ heart perfusion with ovine hearts
- Fluid tight system including an intra ventricular balloon and a cylinder-piston combination with a pre-set volume, which is connected to a motor and control unit
- Real-time pressure and LV flow shift (Q) measurements during cardiac cycle
- Virtual afterload + adjustable preload by pressurizing the piston

Conclusion

Pressure-volume loops with pre-load increments can be successfully generated during unloaded ex situ heart perfusion. Validation of this method is required before clinical implementation.



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GRAPHICAL ABSTRACT |

INTRODUCTION

Ex situ heart perfusion (ESHP) has been successfully implemented in the field of donor heart preservation worldwide [1, 2]. ESHP prevents ischemia during preservation by providing oxygenated, nutrient-rich perfusate to the heart and provides a platform for graft assessment prior to heart transplantation (HTx) [1]. In donation after brain death (DBD), both hypothermic and normothermic ESHP have been shown to be non-inferior to the static cold storage and have been successfully applied for extended criteria DBD donors [3–5]. In addition, ESHP has emerged as a successful preservation technique in donation after circulatory death (DCD). In DCD heart donation, hearts are retrieved from donors whose death is determined by circulatory arrest, as opposed to the golden standard DBD. Transplantation of DCD hearts has expanded the donor pool and number of heart transplantations substantially, thereby offering a viable solution for the persistent shortage of donor hearts [6]. However, the utilization of DCD hearts presents challenges, given the inherent ischemic injury and thus the need for optimized preservation techniques, including ESHP [6, 7].

The Transmedics Organ Care System™ (Andover, MA, United States) is currently the only clinically available normothermic ESHP device and provides coronary perfusion without loading the ventricles [8]. Viability can only be assessed by measuring lactate levels, and the platform does not offer a module for the assessment of myocardial function. Lactate was previously shown to be a poor predictor of outcome after

transplantation [9]. In addition, an increasing number of studies indicate that evaluating donor heart viability with functional ventricle loaded parameters is superior compared to metabolic parameters. This suggests that the assessment of cardiac contractile function could emerge as a reliable tool for graft evaluation and decision-making in HTx [10–13]. However, there is currently no clinically available device capable for *ex situ* assessment of contractile function of the heart including clinically important factors such as pre- and afterload.

In this study we describe our initial experience with a novel device that enables functional graft assessment during unloaded normothermic ESHP with pressure-volume (PV) loop analyses, in an ovine abattoir model. This device provides virtual afterload and preload during ESHP in contrast to the non-compressible classical Langendorff balloon. Using the ovine abattoir model, we aimed to develop a widely available and reproducible model, recapitulating the DCD setting.

MATERIALS AND METHODS

Animals

Female sheep (10 months old, 30–35 kg) from the abattoir were included in this study. The protocol is consistent with regulation number 1069/2009 of the European Parliament and Council regarding abattoir animal products for diagnosis and research, which were authorized by the relevant legal animal welfare authorities (Food and Consumer Product Safety Authority). The sheep were terminated employing a captive bolt pistol to

the head. Subsequently, the carotid arteries were cut open and the animal was suspended by its hind legs to exsanguinate. During exsanguination, blood was collected and heparinized (5,000 IU/L). A median sternotomy was performed, and the heart and lungs were harvested *en bloc*.

Heart Procurement

On the back table, the heart was immediately topically cooled and visually inspected for abnormalities or damage due to the termination process. The heart was dissected from the lungs by transecting the pulmonary artery and veins. Approximately 10 cm of the ascending aorta was retained and cannulated by securing the cardioplegia flush line in the aperture of the ascending aorta. Therefore, both coronary sinuses were perfused simultaneously. The cardioplegic solution (Custodiol histidine-tryptophan-ketoglutarate solution, Essential Pharmaceuticals, LLC, Durham, United States) was supplemented with adenosine (200 $\mu\text{mol/L}$), lidocaine (500 $\mu\text{mol/L}$) and nitroglycerin (100 mg/L) and administered at a pressure of 80–100 mmHg (1L, 4°C) [14, 15]. Warm ischemic time was defined as the time between the termination of the animal and delivery of cardioplegia. A maximum of 10 min of warm ischemia was accepted.

Subnormothermic Ex Situ Heart Perfusion

All hearts were preserved with oxygenated subnormothermic ESHP (15°C) for 2 h. The subnormothermic ESHP system (modified Kidney Assist Transport, XVIVO, Gothenburg, Sweden) consists of a reservoir, an oxygenator (Medos Hilite 1000 XENIOS, Heilbronn, Germany), and a centrifugal pump (Medos DP2 XENIOS, Heilbronn, Germany). Antegrade perfusion of the coronary arteries was delivered in a pressure-controlled and pulsatile fashion by cannulating the aorta root. The aortic valve was tested for insufficiency. Oxygen (100%) was delivered at a flow of 100 mL/min. Hearts were continuously perfused with a hyperkalemic (20 mmol/L), buffered dextran-albumin perfusion solution supplemented with nitroglycerin (25 mg/L). Perfusion temperature was targeted at 15°C. The hearts were prepared for subnormothermic ESHP by cannulating the aorta and by venting the LV to avoid LV distention. Subsequently, the hearts were submerged within the reservoir and the perfusion cannula was attached to the arterial end of the perfusion system. The perfusion pressure was gradually increased to 45 mmHg, aiming at a perfusion flow of >150 mL/min. The right atrium was left open, enabling the free return of the perfusate from the coronary sinus into the reservoir. Blood gases and electrolytes were analyzed to ensure the perfusate composition (pH: 7.1–7.4; HCO_3^- : 20–25 mmol/L; pO_2 : 40–60 kPa; pCO_2 : 4.7–6 kPa; K^+ : >20 mmol/L; Ca^{2+} : 0.18–0.25 mmol/L).

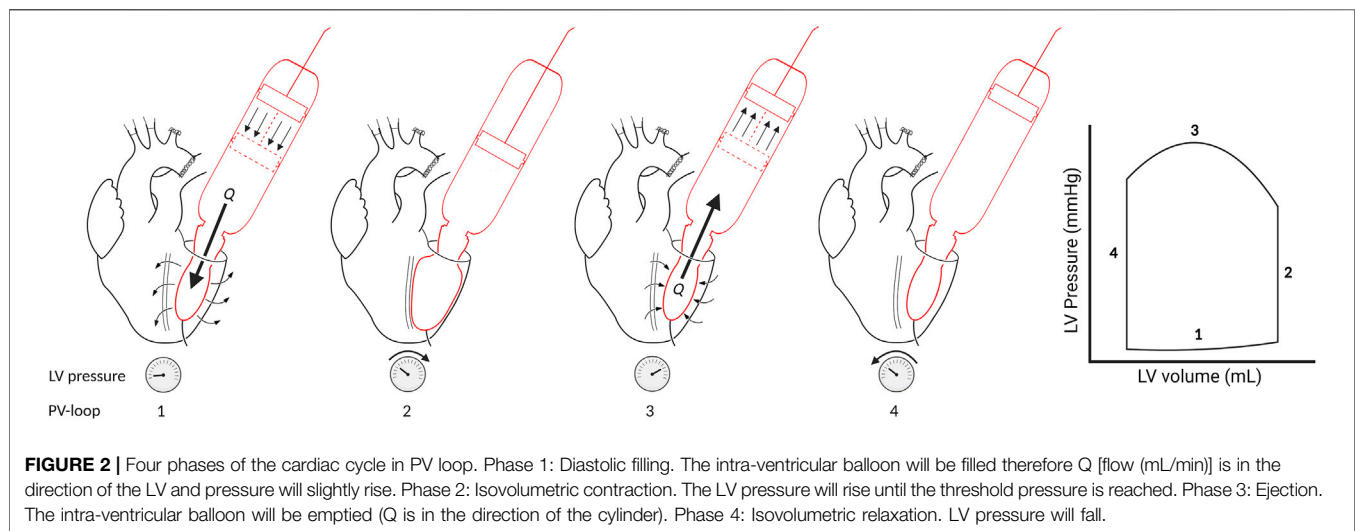
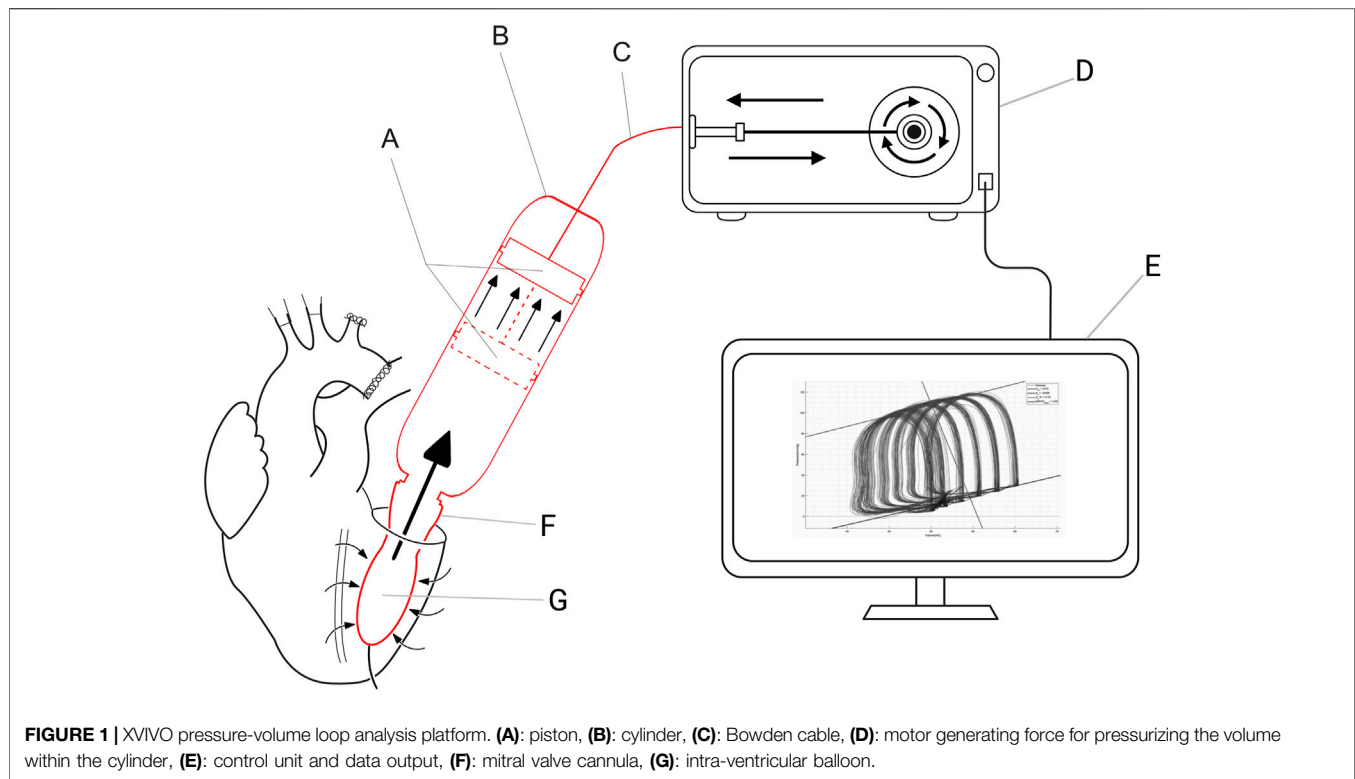
Normothermic Ex Situ Heart Perfusion

At the end of preservation, the hearts were submerged in cold cardioplegic solution in order to minimize injury during the preparations for normothermic evaluation. First, hearts were flushed with Perfadex (XVIVO, Gothenburg, Sweden) to flush out the hyperkalemic subnormothermic ESHP perfusate. Subsequently, hearts were weighted. For normothermic

evaluation, a Langendorff perfusion system comparable to Langendorff perfusion was used. The normothermic ESHP system (modified Kidney Assist, XVIVO, Gothenburg, Sweden) consists of a heater-cooler and a pump housing unit, which must be connected to a disposable set, consisting of a reservoir, an oxygenator (Medos Hilite 2800 XENIOS, Heilbronn, Germany) and a centrifugal pump (Medos DP2 XENIOS, Heilbronn, Germany). The normothermic perfusion is pressure-controlled and delivers a continuous flow to the coronary arteries using the same cannula as for subnormothermic ESHP. Initial reperfusion was initiated in a controlled way, with a gradual increase in both pressure and temperature [16]. Perfusion temperature was initiated at 27°C with 3°C increments every 5 min over 30 min. After 30 min, the pressure was increased from 40 mmHg to 60 mmHg. Rewarming continued for another 30 min. The perfusate was based on albumin solution supplemented with Perfadex (XVIVO, Gothenburg, Sweden), to which washed RBCs (± 500 mL) were added. The gas flow of carbogen (O_2 95%; CO_2 5%) was adjusted to maintain physiological pH and arterial carbon dioxide pressure (pCO_2). Blood gases and electrolytes are analyzed to ensure a perfusate composition within the physiological range (pH: 7.3–7.4; HCO_3^- : 20–25 mmol/L; pO_2 : 40–60 kPa; pCO_2 : 4.7–6 kPa; K^+ : 3.0–5.9 mmol/L; Ca^{2+} : 1.0–1.3 mmol/L; Hb: 4.0–4.5 g/dL). Reperfusion was initiated with a low calcium concentration (<0.8 mmol/L). During normothermic *ex situ* perfusion, hearts were ventricular paced at a rate of 70 beats/min. Normothermic ESHP was performed for a total duration of 2 h.

Ex situ Pressure Volume Loop Analyses

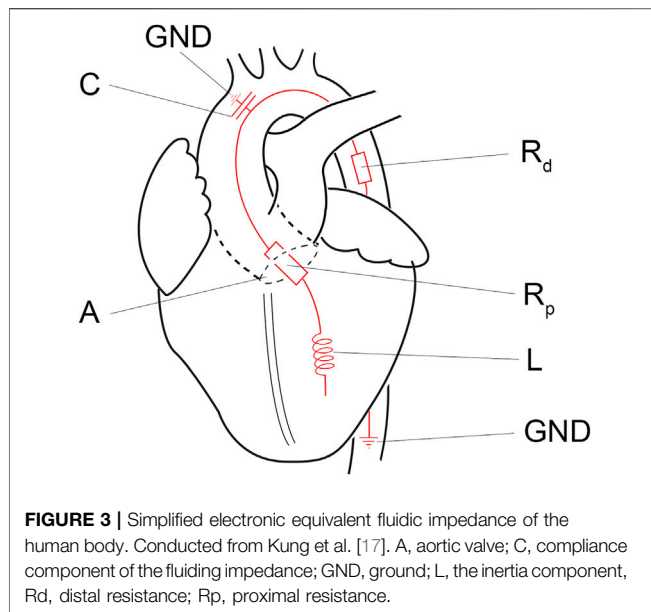
The novel XVIVO *ex situ* PV loop system consists of a cylinder-piston combination that is placed on the annulus of the mitral valve, a balloon, a motor, and a control unit (Figure 1). The balloon is placed in the LV through the mitral valve, secured with a transapical line, and filled with saline. As stated before, the LV is vented. This assures that there will be little to no fluid surrounding the intra-ventricular balloon. To make sure that the balloon does not protrude from the LV to the left atrium, the mitral valve annulus is closed by placing two polyamide nylon tapes (Ethicon 3 mm $\times$ 70) behind the chordae tendineae of both the posterior and anterior leaflet of the mitral valve. Subsequently, the nylon bands are secured using a tourniquet kit, which ensures that the mitral valve annulus completely encloses the mitral valve cannula. A WheatStone bridge pressure catheter is placed in the balloon for real-time pressure measurements. The balloon is connected to the mitral valve cannula, which forms the connection to the piston-cylinder combination (Figures 1A, B). As a result, the volume within the balloon is in continuity with the volume within the cylinder-piston combination, providing a fluid-tight system with a constant and preset fluid volume. As the LV contracts, the volume is pushed out of the balloon into the cylinder, moving the piston (Figure 1A). This movement of the piston is transmitted through a Bowden cable (Figure 1C) which is connected to the motor pulley (Figure 1D), registering the volume changes in the LV during the cardiac cycle. Both the motor and pressure sensor are connected to the control unit which includes a computer with XVIVO software (Figure 1E).



During the cardiac cycle the software switches between the four phases of the cardiac cycle (**Figure 2**). The software detects the changes in the cardiac cycle by an algorithm dedicated to every phase of the cardiac cycle. Change in the cardiac phase is detected by the software as follows. The software switches from the diastolic filling phase to the isovolumetric contraction phase when a small outward volume displacement from the ventricular cavity is detected. It switches from the isovolumetric contraction phase to the systolic ejection phase when a certain predetermined threshold pressure is reached. The systolic ejection phase changes

to the isovolumetric relaxation phase when a small inward volume displacement to the ventricular cavity is detected. And lastly, the software switches from the isovolumetric relaxation phase to the diastolic filling phase when the ventricular pressure is lower than the predetermined preload pressure.

Furthermore, the volume within the cylinder-piston combination is pressurized by force generated by the motor. Therefore, the internal volume can be pressurized by changing the motor power. This enables the simulation of a virtual physiologic load on the ventricle (pre- and afterload). Both the

**TABLE 1 |** Components of virtual impedance.

Components of virtual impedance	Symbol	Standard setting
Inertia component	L (Barye s ² /cm ³)	0.7
Inverse compliance	1/C	420
Flow resistance aortic valve	R _p (Barye s/cm ³)	24.5
Flow resistance distal track/SVR	R _d (Barye s/cm ³)	404.6

power and speed delivered by the motor can be controlled with the XVIVO software. The software reads the pressure measured in the LV, compares this with the desired preload pressure, and subsequently increases or decreases the motor power to adjust the LV pressure. The desired preload pressure can be adjusted in the XVIVO software after every cardiac cycle. As a result, the LV pressure will increase to the desired preload pressure within one cardiac cycle, enabling the ability to effortlessly execute a preload challenge to assess LV function.

In the systolic ejection phase, the software controls the afterload. The afterload is calculated from the measured ventricular outward flow and a virtual impedance based on the analogue described by Kung and colleagues [17]. The simplified virtual impedance is constructed out of four components (Figure 3; Table 1). The inertia component (L) counteracts a flow change. Compliance (C), primarily the aortic compliance, is the stretching capacity of the arteries. R_p is the virtual flow resistance of the aortic valve, and R_d is the virtual flow resistance of the distal track/the systemic vascular resistance (SVR).

The afterload is calculated using the following equation:

$$P = L \frac{dQ}{dt} + Q \cdot R_p + \frac{1}{C} \int (Q - Q_{Rd}) dt + P_{C_0}$$

In which P is the target pressure in mmHg. The software will control the motor force in such a way that the measured pressure in the ventricle will equal this targeted pressure or afterload. Q is the

flow (mL/min) out of the ventricle and Q_{Rd} is the flow through the virtual flow resistance of the SVR. The inertia component (L) is dependent on the flow change (dQ) over time (dt, in seconds) out of the ventricle. R_p is the flow resistance of the aortic valve. Pressure generated by the compliance component (C) is determined by the volume in the aorta. Volume in the aorta can be calculated by summation of the flow from the ventricle (Q), minus the flow to the body (Q_{Rd}). P_{C0} is the pressure (in mmHg) in the aorta at the start of the systolic ejection phase. The targeted pressure of the ventricular cavity is recalculated at intervals of dt, and dt is defined as to be smaller than 4 milliseconds.

The equation above is valid in the systolic ejection phase, as in the other phases the aortic valve would be closed. If the aortic valve is closed, the aorta is still loaded and stretched as a result of the systolic ejection phase. During unloading of the aorta, the aortic pressure falls. The formula for decreasing the pressure of the aorta, is based on a resistance-capacitor circuit, the electronic equivalent of resistance and compliance:

$$P_c = P_{ces} \cdot e^{-\frac{t}{R_d \times C}}$$

In which P_c is the virtual pressure in the aorta, P_{ces} is the end systolic pressure compliance in the aorta and t is the progression in time since closure of the aortic valve in seconds, C is the compliance and R_d is the SVR. When a heartbeat is in the isovolumetric systolic phase, P_c is the threshold pressure for switching to the ejection phase.

Data Collection

Blood Gas Analysis

Blood gas analysis was performed with the ABL90 Flex plus blood gas analyzer (Radiometer Benelux BV). Analyses were performed during subnormothermic ESHP (5, 60 and 120 min), after priming of the normothermic ESHP system and during normothermic ESHP (5, 30, 60, 90 and 120 min).

PV Loop Analysis

At 60 and 120 min of normothermic ESHP, PV loop analyses were performed. Before testing, the pacing was halted, and hearts were checked for their rhythm. During PV loop analyses, pre-load was started at 10 mmHg and increased to 25 mmHg in steps of 5 mmHg. After each increment of 5 mmHg, PV loops were recorded for 60 s. Calculations and figures were made in MATLAB (The Mathworks).

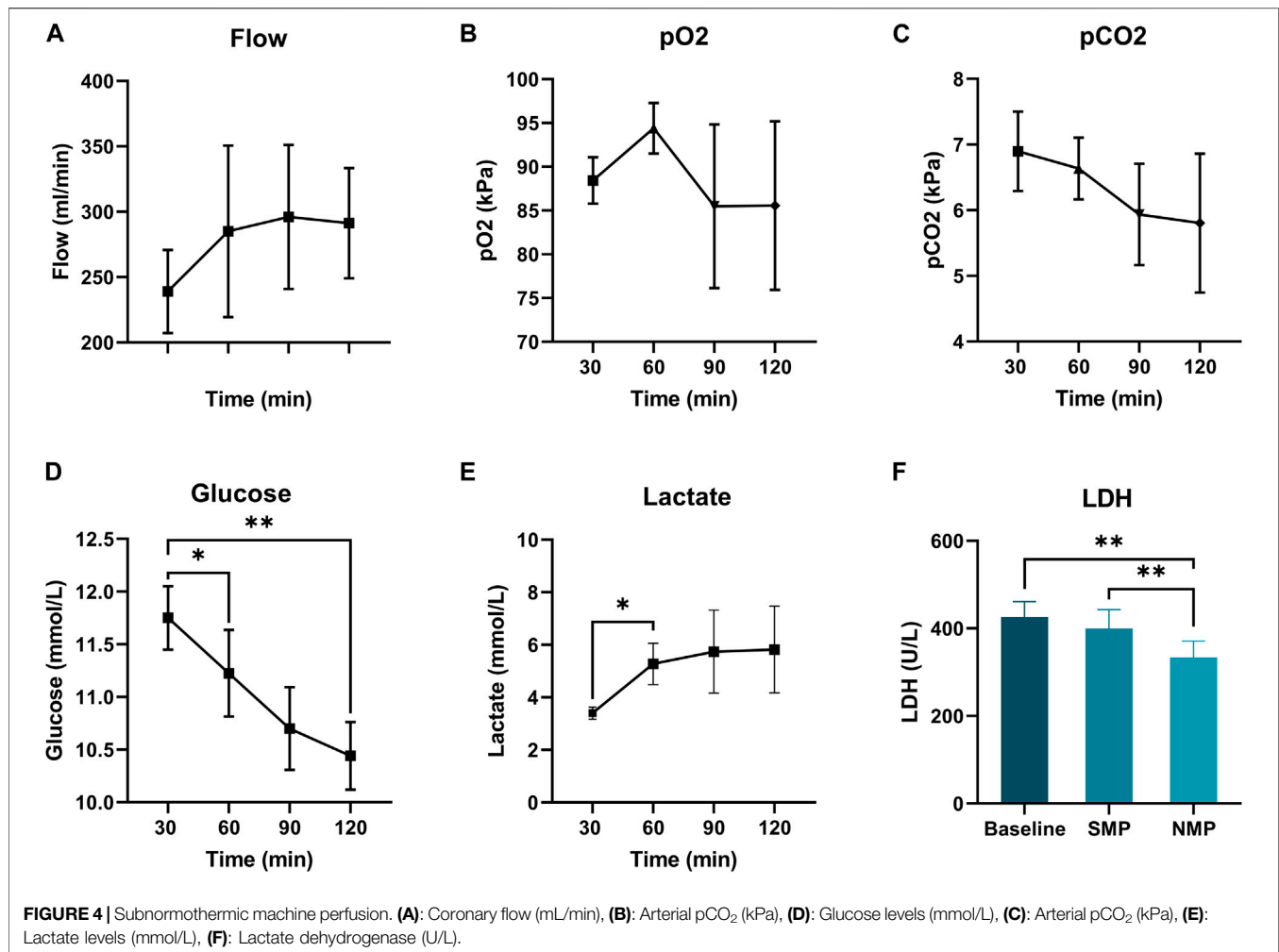
Statistical Analysis

Data is presented as mean ± standard deviation (SD). A repeated measures ANOVA was performed to analyze differences over time. p-value <0.05 was considered statistically significant. Data analysis was performed using GraphPad Prism 9.4 (GraphPad Software, CA, United States).

RESULTS

Baseline Data, Ischemic and Preservation Time

A total of 5 female sheep of 10–12 months old (25–35 kg) were included in the study. The functional warm ischemic time was



8.4 ± 0.9 min. The cold ischemic time during preparations for subnormothermic ESHP was 5 ± 0.5 min. Hearts were perfused with subnormothermic ESHP for 135 ± 13.3 min and evaluated during normothermic ESHP for 122 ± 1.6 min. The cold ischemic time during preparations for normothermic ESHP was 9 ± 3.4 min. The baseline weight of the heart was 328.4 ± 3 g. Weight gain during preservation was 71 ± 12.7 g ($p = 0.0005$) and during normothermic ESHP 41 ± 16 g ($p = 0.010$).

Subnormothermic Machine Perfusion

All hearts were perfused with a pressure of 45 mmHg. Coronary flow increased non-significantly over time until 60 min of preservation, and stabilized hereafter (Figure 4A). pO₂ and pCO₂ concentration during subnormothermic ESHP are shown in Figures 4B, C, respectively. Glucose concentration decreased over the course of preservation (Figure 4D), baseline: 11.8 ± 0.3, end of subnormothermic ESHP: 10.4 ± 0.3 ($p = 0.005$). A significant increase in lactate was seen in the first hour of preservation (Figure 4E). However, after 60 min of preservation, lactate remained rather stable. Lactate dehydrogenase after 5 min of

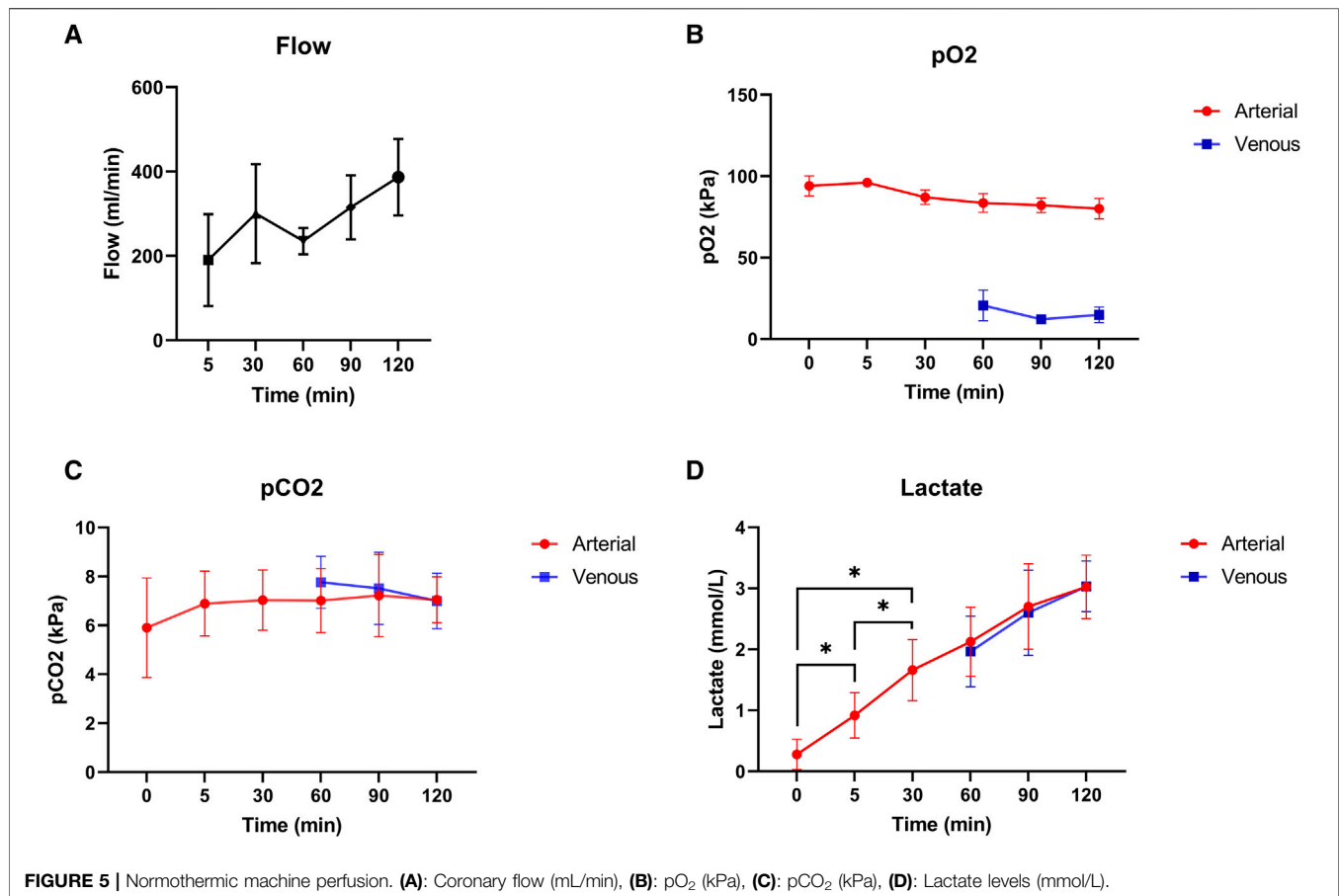
preservation was 426 ± 35 U/L (Figure 4F). At the end of preservation, lactate dehydrogenase decreased to 400 ± 43 U/L ($p = 0.3$).

Normothermic Machine Perfusion

Mean pressure during normothermic ESHP was 62 ± 6 mmHg. Changes in flow during normothermic ESHP, are shown in Figure 5A. Changes in both arterial and venous pO₂ and pCO₂ are shown in Figures 5B, C. In the first 30 min of normothermic ESHP, lactate increased significantly (baseline: 0.28 mmol/L, 5 min: 0.92 mmol/L, 30 min: 1.66 mmol/L, $p = 0.02$). Hereafter, a non-significant increase in lactate was seen between each timepoint (Figure 5D). At the end of normothermic ESHP, lactate dehydrogenase level was 333 ± 37 U/L ($p = 0.005$, Figure 4D).

PV Loop Analyses

PV loop analyses were performed at 60 and 120 min of normothermic ESHP. A simulated output from one of the PV loop assessments with increasing pre-load is shown in Figure 6. Dp/dt-min and -max and maximally generated pressure values



are shown in **Figure 7**. End-systolic elastance (Ees) at 60 min and 120 min of normothermic ESHP was 2.8 ± 1.8 mmHg/mL and 2.7 ± 0.7 mmHg/mL ($p = 0.9$), respectively (**Figure 7C**). Tau, the exponential decay of the ventricular pressure during isovolumic relaxation, is shown in **Figure 7E**.

DISCUSSION

In this study, a novel method for *ex situ* PV loop analyses during unloaded normothermic ESHP is shown in an ovine abattoir model, which is recapitulating the DCD setting. This PV loop system provides a virtual afterload and an adjustable preload to the LV. Pre-load increments of 5 mmHg, led to measurable PV loops for LV function assessment. Our results, including end-systolic elastance, are in line with experimental studies on LV function in ovine models [19–21].

Given the uncertainty about transiency and reversibility of myocardial dysfunction in DCD hearts, assessment of cardiac function might be of added value. In line with this, it is important to state that several donor hearts initially thought to be transplantable were discarded after perfusion on the Transmedics OCS system [1]. Furthermore, in DBD HTx,

DBD donors might suffer from brain stem death-induced myocardial dysfunction, which could prove to be reversible over time [22]. Prolonging the interval between brain death and graft assessment, may therefore increase the number of successful heart procurements, which stresses the transiency of myocardial dysfunctions over time, especially from extended criteria DBD donors [22, 23]. Therefore, donor heart assessment in both DCD and DBD could be of additional value.

Several methods have been applied to assess graft function during ESHP. Echocardiography is the preferred imaging technique for myocardial contractility. Two-dimensional echocardiography has been used during ESHP to determine myocardial contraction by assessing fractional area reduction [24]. Lowalekar et al. showed that cardiac functional parameters and ventricular and septal wall thicknesses can be assessed with a two-dimensional transesophageal probe during ESHP [25]. Furthermore, more extensive methods including the quantitative assessment of shear wave velocity using ultrasound have been used as a biomarker for cardiac viability in a porcine model. This methodology includes ultrasound elastography and assessment of myocardial stiffness evolution during ESHP. Shear wave velocity has been shown to correlate with cardiac function index, suggesting shear wave velocity as a non-invasive marker of graft viability [26].

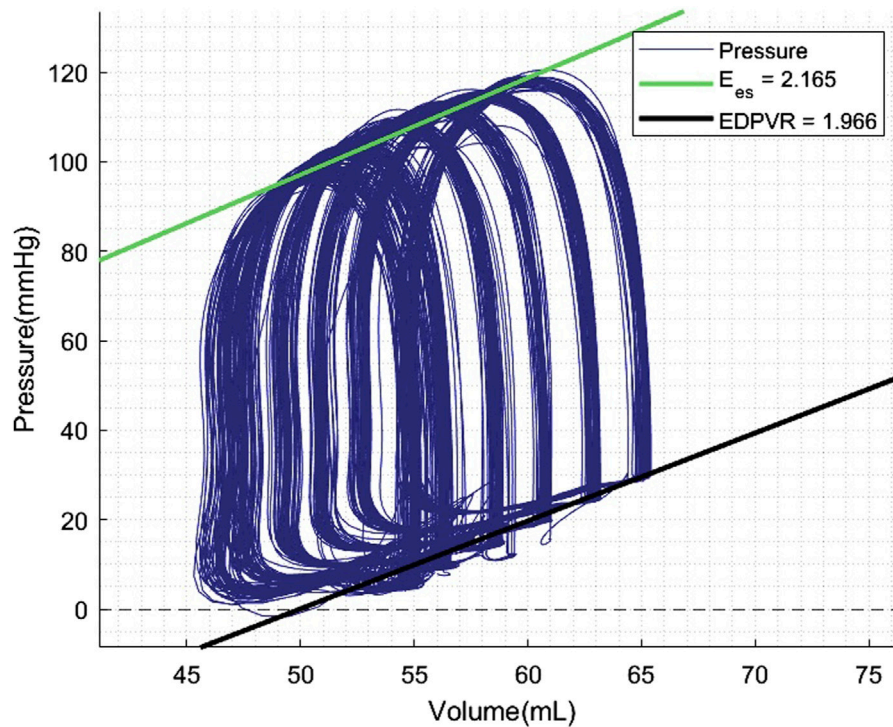


FIGURE 6 | Pressure-volume loop analyses output. Preload was increased with increments of 5 mmHg. EDPVR, End-diastolic pressure-volume relationship; Ees, End-systolic elastance.

PV loop analyses are widely used in basic research and are considered the gold standard for *in situ* hemodynamic assessment of the heart [27]. PV loop assessments have also been applied during normothermic regional perfusion in DCD HTx to assess the donor heart before retrieval [12]. Messer et al. demonstrated the effectiveness of this functional assessment method for DCD HTx with successful transplantation and 100% survival rate [28]. PV loop analyses are also performed during EHSP using an intraventricular conductance catheter and isovolumetric pressure measurements [12, 18, 29].

The novel *ex situ* PV loop system includes a pre-determined virtual afterload and an adjustable preload, and therefore simulates a physiological loading state, which is a valuable novel addition to ESHP PV loop assessments. Contrary to current PV loop methods, this novel system allows LV fluid shuttling. In addition, previous studies have shown that ESHP in working mode may result in improved preservation compared to non-working or unloaded perfusion [30]. This novel *ex situ* PV loop analyses platform provides an accessible working mode ESHP system during Langendorff perfusion and may therefore optimize ESHP. In addition, complicated working-mode or loaded perfusion systems can be avoided. Furthermore, its compact and transportable size, easy application and modest design might facilitate clinical application.

For optimal graft viability assessment, we believe that a multi-assessment approach including real-time PV loop assessment with our novel system, myocardial perfusion and metabolic- and damage markers would be an adequate strategy for evaluation of contractility, coronary flow, and overall cardiac function. Several

metabolic markers, including myocardial oxygen consumption, lactate, glucose, and metabolomics have been applied in ESHP graft assessment [31–34]. These markers are accessible and could provide additional insight in graft viability.

Several experimental rodent models of cardiac DCD are available, providing a relatively easily manageable model with respect to technical and financial aspects [28, 35, 36]. However, large animal models provide a more clinically relevant platform for DCD heart research. Although these models are clinically relevant, costs are relatively high and challenging to perform. Utilizing animal organs obtained from an abattoir is an alternative source of animal donor hearts and fits well into the 3R's principle (refinement, reduction, and replacement) of humane experimental techniques. Since these animals are specifically bred and terminated for consumption, repurposing their organs for research after termination allows for scientific research without causing additional animal harm [37]. Other advantages of abattoir models include low costs and unlimited access. In large-animal models, the porcine heart is one of the preferred choices considering its anatomical similarity to the human heart [38, 39]. However, ovine hearts are also comparable on a physiological and anatomical level to human heart and have been studied comprehensively [40–42]. The advantage of using ovine hearts for experimental studies is that the slaughter process for sheep is shorter than that for pigs. Therefore, acceptable warm ischemic times can be reached. The described ovine model is a reproducible model for ESHP DCD studies.

Further enhancements of the PV loop system could include the possibility to adjust the afterload and assessment of right-

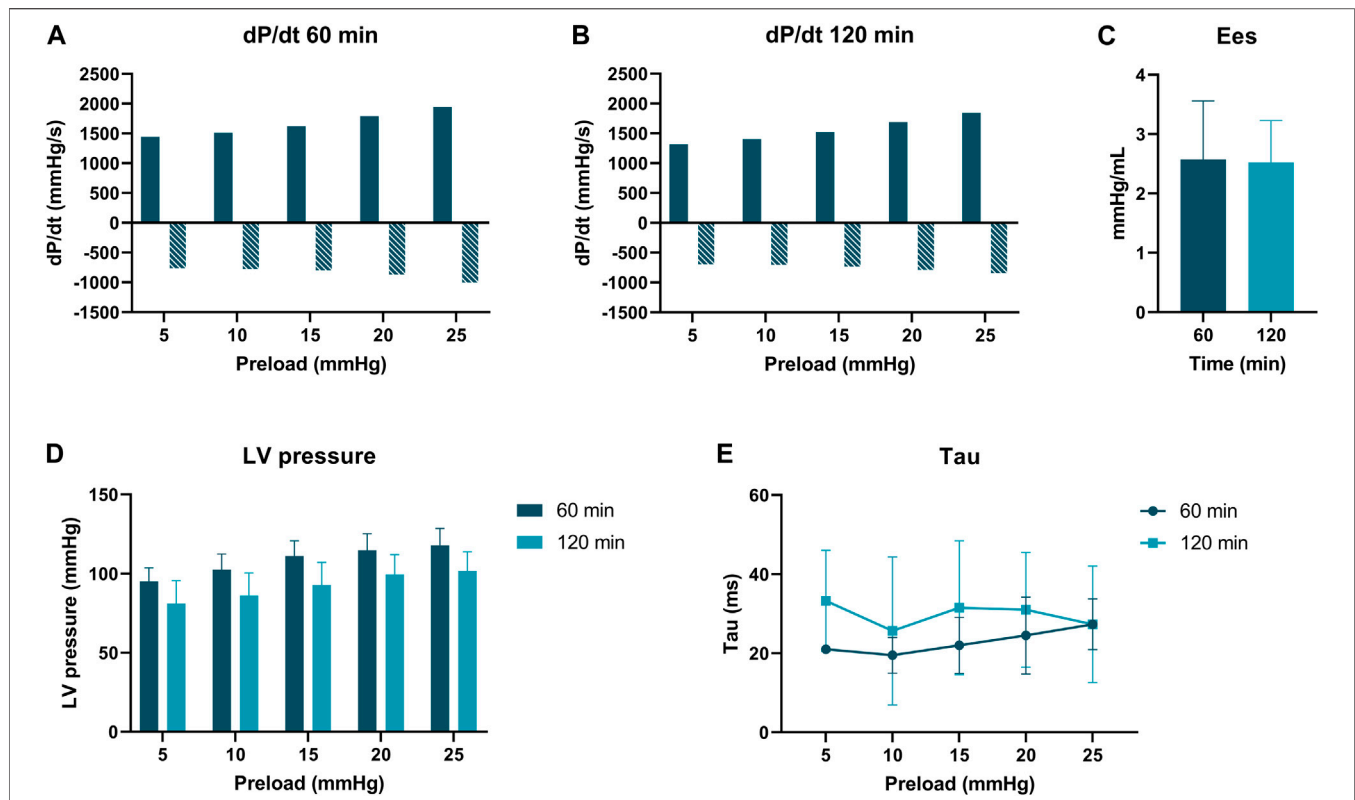


FIGURE 7 | Pressure-volume loop analyses. **(A)** dP/dt min and max (mmHg/s) at 60 min of *ex situ* heart perfusion. **(B)** dP/dt min and max (mmHg/s) at 120 min of *ex situ* heart perfusion **(C)** End-systolic elastance (mmHg/mL), **(D)** LV pressure (mmHg) **(E)** Tau (ms).

ventricular function. As this is a proof-of-concept study, we did not include a cut-off value for adequate cardiac function. Future studies validating this novel *ex situ* PV loop system are required to determine a cut-off value relevant for clinical DCD HTx. In addition, these studies should test the safety of the intra-ventricular balloon. The intra-ventricular balloon and mitral valve canula affects the geometry of the mitral valve and might therefore also affect the geometry of the LV, which can be considered as a limiting factor of this study. Further limiting factors include the low number of hearts and lack of a control group (e.g., laboratory-raised sheep). However, this paper aimed at showing a novel animal DCD model and system for *ex situ* PV loop analyses as a proof of concept, not to validate these methods. Although hearts retrieved from an abattoir have many advantages, there are also limitations to abattoir organs for research. Variability in farms of origin, stress due to transport and sacrifice, and a high-fat diet, are some of the factors which should be considered when using abattoir models for experimental studies [39].

In conclusion, this study shows a reproducible method for DCD heart preservation and proves the concept of functional assessment of these ovine hearts. We tested a novel clinically applicable method for functional assessment of the graft with *ex situ* PV loop analyses including a virtual afterload and preload during ESHP. We believe that this method could become important in more optimal graft selection and enhancing overall outcome in both DBD and DCD HTx. Validation of

this system is the next step in the development of this method and is required before clinical implementation.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The requirement of ethical approval was waived by European Parliament and Council for the studies involving animals because the protocol is consistent with regulation number 1069/2009 of the European Parliament and Council regarding abattoir animal products for diagnosis and research, which were authorized by the relevant legal animal welfare authorities (Food and Consumer Product Safety Authority). The studies were conducted in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

Concept and design: ME, VvS, FN, AvP, and IE. Acquisition of data: IE, FN, RP, and BW. Analysis and interpretation of data: IE, ME, and FN. Drafting of the manuscript: IE, VvS, and ME. Critical

revision: FN, NvK, MV, EB, AvP, BW, HvG, and RP. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

Authors BW, AvP, and FN were employed by XVIVO. ME holds a patent with XVIVO.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A Volume-Adjustable Artificial Womb for Extremely Preterm Infants

Jan Heyer^{1*}, Franziska Schubert¹, Alexander L. Seitz¹, Yannick Steinle¹, Jutta Arens², Thorsten Orlikowsky³, Ulrich Steinseifer¹, Thomas Schmitz-Rode¹, Sebastian V. Jansen^{1†} and Mark Schoberer^{3†}

¹Department of Cardiovascular Engineering, Institute of Applied Medical Engineering, Helmholtz Institute, RWTH Aachen University and University Hospital, Aachen, Germany, ²Engineering Organ Support Technologies Group, Department of Biomechanical Engineering, University of Twente, Enschede, Netherlands, ³Pediatric Clinic, Neonatology Section, RWTH Aachen University and University Hospital, Aachen, Germany

More than 13 million children are born preterm annually. Prematurity-related mortality accounts for 0.9 million deaths worldwide. The majority of those affected are Extremely Preterm Infants (gestational age less than 28 weeks). Immaturity causes organ failure and specific morbidities like germinal matrix hemorrhage, bronchopulmonary dysplasia, and necrotizing enterocolitis. Artificial womb and placenta technologies address these issues. As a bridge-to-life technology, they provide a liquid environment to allow organ maturation under more physiological conditions. The proposed artificial womb can adapt to fetal growth. Volume adjustment is achieved by removing fluid from the interspace between an inner and outer chamber. Results of the *in vitro* tests showed a temperature constancy of $36.8^{\circ}\text{C} \pm 0.3^{\circ}\text{C}$ without pressure loss over 7 days. The volume of the inner sac was variable between 3.6 and 7.0 L. We designed a filtration and disinfection system for this particular purpose. This system has proven strong disinfection capabilities, effective filtering of metabolic waste, and the ability to avoid phospholipid washout. The presented artificial womb has sufficient volume variability to adapt to the physiologic growth of an extremely preterm neonate over a 4-week period. We regard this as an important step in the development of this bridge-to-life technology.

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*Correspondence

Jan Heyer,

✉ heyer@ame.rwth-aachen.de

[†]These authors have contributed equally to this work

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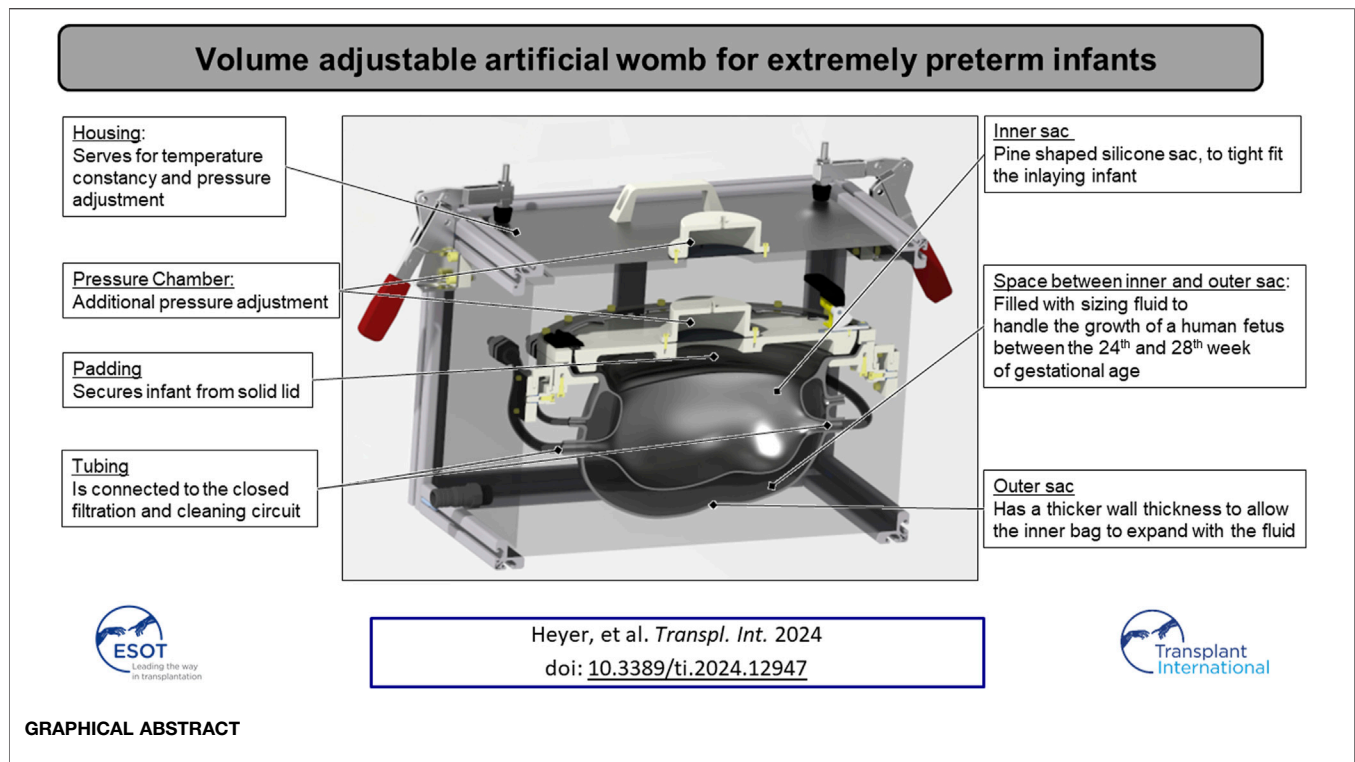
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Keywords: artificial womb, artificial amniotic sac, adjustable silicone sac, perinatal life support, extremely preterm infant

INTRODUCTION

In 2020, more than 13 million children worldwide were estimated to be born preterm [gestational age (GA) less than 37 weeks], with more than 5% of mortality due to complications of preterm birth [1]. Neonatal intensive care serves to improve prematurity outcomes. Modern neonatal intensive care treatment uses a microenvironment to control respiratory gases, temperature, and humidity. Antenatal steroid prophylaxis, postnatal surfactant replacement therapy, and mechanical ventilation have enabled pulmonary gas exchange at the canalicular stage of lung development. This has allowed survival in the first place [2].

However, extremely small-for-gestational-age neonates (ELGANs, GA less than 28 weeks) have the highest therapy-related morbidity and mortality rates. Their vulnerability can be attributed to organ immaturity [3, 4], especially of the lungs and brain. Lung surfactant is detectable as early as 24 weeks of gestation [5], and is essential for the ability to breathe air as well as a prerequisite for functional lung development. Surfactant deficiency causes neonatal respiratory distress syndrome



[5, 6]. The 23rd to 24th week of GA is currently the limit of viability, with survival rates below 10% at 23 weeks of gestation and 35% at 24 weeks of gestation [3]. Acute respiratory distress is the leading cause of mortality in this group [2]. Survival comes at the expense of lifelong structural and functional pulmonary morbidity [2].

Searching for a way out of this paradox, several research groups are developing artificial womb (AW) and artificial placenta (AP) technologies [7–10]. These technologies aim to overcome the current complications in therapy and establish a bridge-to-life approach. Recent achievements in animal models have demonstrated the potential of this technology [7, 11]. Patridge et al. and Usuda et al. were able to support lambs in their AW for 19–25 days [7], with a maximum of 28 days [11]. Fetal growth at mid-term was observed to be exponential [12]. The fetus almost doubles its weight between the 24th and the 28th weeks of GA [12, 13]. Furthermore, during the 24th week of GA, the multisensory and pain perception of the fetus [14] and the haptic system are at a decisive stage of development [15–17]. At this age, the fetus learns to discriminate between the environment and its own body through touch [15–17].

All animal studies published to date have required fixation of the animal in its surrounding bag in order to prevent movement and decannulation within the proposed artificial wombs [7]. Current AW Systems manage growth using an end-stage-size biobag where the fetus is held in position using manually adjusted magnets [7]. Such fixation of the fetus can have a serious impact on neurosensory and motor development. Furthermore, the artificial amniotic fluid is constantly drained of, flushing away the autologous surfactant in the fluid produced by the fetus [7–10]. This rather simple design of the currently used

“biobags” has shown good functionality in the animal experimental setup [18] but has practical limitations for clinical use and does not cover the core needs of a developing fetus. The need for constant flushing with sterile fluids would be a practical limitation and an enormous logistic burden, even more so in environments that have a developing infrastructure. Keeping a closed, fluid-filled polymeric system sterile means a significant challenge in terms of filtration and disinfection technology. In addition to hygienic issues, the system should support neurodevelopment. Current biobags purposefully restrict fetal movement as described above. Moreover, biobags do not well simulate the elastic forces in the interaction between the developing fetal musculature and the uterus.

The AW approach presented here, the Liquid Filled Chamber (LFC), provides an adaptable volume that allows for fetal movement and growth without fixation. Furthermore, a closed-loop filtration system is added to prevent flushing of the autologous surfactant from the artificial amniotic fluid, as suggested by Bie et al. [18].

MATERIALS AND METHODS

Requirements for the LFC

As a first step, the requirements for the LFC were identified by dividing the LFC into four functional units: the artificial amniotic sac (AAS), housing, amniotic fluid, and filtration system. The requirement specifications are shown in **Table 1**.

Requirements R1, R2 and R3 result from the anatomy and growth dynamics of the neonate, and the maternal uterus.

TABLE 1 | Requirement specifications for LFC.

Unit	Requirement	Requirement specification	No.
AAS	Mimicking the physiological womb	A fluid environment for the AAS with materials that are elastic and can be shaped and sized to fit a fetus of the appropriate GA	R1
	Tight-fitting but not restrictive of physiological fetal movement	The AAS must be continuously or repeatedly adjustable relative to the size and growth of the neonate [12, 13, 19]	R2
	Growing system		R3
	Transfer and accessibility	Ability to open the AAS from outside the system for perinatal transfer as part of the EXIT procedure ^a , and for emergency interventions	R4
	Closed system	The AAS needs to generate a sealed ecosystem similar to the physiological womb	R5
	Umbilical cord interface	The AAS needs to incorporate a sealable interface for the throughput of the umbilical cord or an appropriate cannula	R6
Housing	Temperature control	The temperature needs to be physiological (37°C) and stable according to the body temperature	R7
	Pressure level	Pressure needs to be physiological and stable but adjustable according to the intrauterine pressure [20]	R8
Amniotic fluid	Mimicking the physiological fluid	Artificial amniotic fluid should have a similar composition to natural amniotic fluid	R9
	Leakage detection	Fluid leakage from or into the AAS should be detectable	R10
Filtering system	Excretion elimination	Toxic excretions need to be constantly filtered from the artificial amniotic fluid stream	R11
	Microorganism elimination	Infectious agents need to be eliminated to prevent sepsis [18]	R12
	Prevention of phospholipid washout	Phospholipid washout by the filtration system needs to be prevented [5, 6]	R13

^aEXIT-procedure: ex utero intrapartum treatment procedure.

TABLE 2 | Parameters of the maternal womb and the neonate relative to GA.

GA in weeks	Crown to rump length (CRL) in mm [21, 22]	Chest circumference (CC) in mm [21]	Weight of the neonate in g [12]
22	195 (15)	161 (19)	477 (61)
23	204 (15)	170 (20)	568 (72)
24	213 (16)	180 (21)	670 (85)
25	233 (17)	189 (22)	785 (100)
26	232 (18)	199 (23)	913 (115)
27	242 (19)	209 (24)	1,055 (134)
28	250 (21)	217 ^a (25 ^a)	1,210 (154)

^aExtrapolated from literature data.

These are presented in **Table 2** according to the literature values, with the standard deviation given in brackets. In the case of different values in the literature, the largest variation was chosen to obtain a conservative result. For intrauterine pressure, different values are reported in the literature [23–25]. For LFC, the minimum pressure at 24 weeks of GA was estimated to be 9.5 mmHg, according to Wiener et al. [25]. A peak pressure of 27.7 mmHg caused by uterine contraction was measured by Beard et al. [23], and this value was used as the upper pressure limit for the LFC.

Design Targets for LFC

Design targets for the LFC System can be derived from the requirements defined in **Table 1** and from the physiological parameters given in **Table 2**. The resulting design targets are listed in **Table 3**.

All parts of the LFC and its components were designed using CAD software (Autodesk Inventor, San Francisco, United States). Silicone parts were manufactured by hand (Silicone 620 A/B, Wacker Chemie AG, München, Germany). Special molding techniques were developed for each silicone component. Other parts were manufactured in the in-house mechanical workshop, and commercially available parts were integrated into the system.

Verification Testing

The identified requirements (**Table 1**) were tested in individual setups. R1, R2 and R3 were tested in a volume adaptability test. R5, R7, R8, and R10 were tested in a 7-day sealing and stability test. R12 and R13 were tested in a filter test. R4 and R6 are determined by the selected lid construction and the respective sealing technique. No verification is required for the structural part. For R9 and R11, commercially available CE-marked medical products were used.

In the volume adaptability test, the principle of the sizing fluid system was tested. The AAS was filled using water as a surrogate for the artificial amniotic fluid according to the volumes given by the state at 24 weeks of GA. Manikins representing the 3D reconstruction of the fetal body from MRI data were manufactured by our consortium partners from TU/e Eindhoven, van Haren et al. [26]. The fetal manikins were laid in the AAS to visualize the volume adjustability. At the beginning of the test, a manikin at 24 weeks of GA was used. After increasing the volume, the manikin was replaced by one mimicking 28 weeks of GA. This would represent the body size of a fetus at the expected end of the treatment period. The volume of adaptive sizing fluid between the AAS walls was removed stepwise in

TABLE 3 | Design targets for LFC.

Unit	No.	Design target
AAS	R1	The AAS is a fluid-filled system made of transparent silicone to ensure elasticity and to allow observation of the neonate. The shape and size were chosen with the values given in Table 2 plus two standard deviations to allow 95.5% of neonates to fit into the ASS
	R2	The AAS consists of a double-walled encasement (silicone). A sizing fluid is placed between the two walls. Passive or active removal of the sizing fluid enables volume adjustment. The sizing fluid system is isolated from the rest of the LFC
	R3	
	R4	The AAS features a lid that seals its inner system from the environment, but can be opened to have access to the neonate. The lid is designed to be atraumatic
	R5	The AAS is a closed system attached to the filtration system
	R6	The umbilical cord or its cannulas are routed outside the AAS for connection with the Artificial Placenta (AP)
Housing	R7	The outer housing is a fluid-filled chamber that surrounds the AAS. The heat capacity of water is used to ensure a stable temperature of 37°C ± 1°C. The internal pressure can be adjusted between 10 mmHg and 30 mmHg
	R8	
Amniotic fluid	R9	A commercially available medical-grade artificial amniotic fluid is chosen (Anmion Flush, Serumwerk Bernburg AG, Brenburg, Germany)
	R10	Leakage of different fluids is detectable in the system by photometric measurement
Filtration system	R11	The filtration system is designed as a closed circuit with different functional components: gross filtration, fine filtration (dialyzer), and disinfection (UV-light). The prevention of phospholipid washout in all systems is secured by an adjusted concentration gradient. The maximum flow rate of the filtration system is estimated to be 800 mL/min, resulting in the filtration of the entire fluid in 12.5 min
	R12	
	R13	

500 mL increments, and the same amount of fluid was added to the AAS to mimic neonatal growth. After each fluid removal step, a photograph of the system was taken for visual evaluation. The steps were repeated until the complete removal of the sizing fluid. The internal “intrauterine” pressure of the AAS LFC was measured using two calibrated pressure sensors at the in- and outlet of the AAS (Xtrans, CODAN Companies, Rodan, Denmark).

For the sealing and operability test, the completely assembled LFC was tested for leakage, temperature stability, and AAS intrauterine pressure constancy for 7 days. We believe that 7 days is a representative time frame for the maximum therapy duration of 4 weeks in terms of leakage, temperature, and pressure constancy. Water was again used as a surrogate for the artificial amniotic fluid, and the volume flow was set at 800 mL/min, the maximum filter flow as depicted in **Table 3**. The umbilical cord interface was sealed with a closed silicone tube mimicking the umbilical cord.

Leakage detection was performed visually by a red coloring ((Reaktiv-Rot 120 ($\lambda = 509$ nm), Merck, Darmstadt, Germany) of the surrounding water inside the LFC housing, such that leakage into the AAS could be detected. At the beginning of the test, a 100 mL sample of the dyed surrounding water was kept as a positive reference and 5 mL of the water inside the AAS was taken as a negative reference. Every 24 h of the test, a 5 mL sample was taken from each circuit. Samples and references were analyzed with respect to absorption at $\lambda = 509$ nm (Ultrospec 2100 pro, Amersham Bioscience, Amersham, United Kingdom). Temperature was measured (Omgea, Deckenpfronn, Germany) inside the AAS, inside the housing, and inside the heating circuit as a reference. The sensors were calibrated in a heating bath at 37°C using an analog laboratory thermometer. The LFC was filled with fluid 12 h prior to the beginning of the test to ensure a homogeneous starting temperature of 22°C in all components. Temperature values were taken automatically every 5 minutes. Two-point calibrated pressure sensors (Xtrans, CODAN Companies, Rodan, Denmark) were placed at the top of the

lid and at the in- and outlet of the inner AAS to measure the AAS “intra uterine” pressure of the system. Automatic recording of the pressure sensors every 60 min was performed using the institute’s data acquisition system.

The filter was tested for elimination of microorganissms and potential washout of phospholipids. In total, 500 mL of artificial amniotic fluid in contact with ambient air was incubated for 14 days at 37°C to allow microorganisms to grow within the fluid. From this fluid, three 50 mL samples (Falcon, Thermo Fisher Scientific, Waltham, Massachusetts, United States) were prepared. Two of these were exposed to the filtration system’s UV-C lamp for 5 min, and one was left untreated as a reference. All three samples were tested for microorganisms with a standard smear test. The washout of phospholipids through the dialyzer of the filtration system was tested in two separate experiments. Phospholipid EMPIGEN® BB Detergent (Merck, Darmstadt, Germany) (271.44 DA) was used to mimic the autologous phospholipid Dipalmitoylphosphatidylcholine (735 DA) of the neonate on the AAS side. To prevent washout, a phospholipid 3-(N,N-Dimethylmyristylammonio)-propanesulfonate (Merck, Darmstadt, Germany) of similar size (391.65 Da) was used in the filtration system to eliminate a concentration gradient across the dialyzer membrane. For the test, both fluids (AAS side and filtration system side) were fully saturated using the respective phospholipids. The filtration system was run at 800 mL/min for a duration of 240 min. A reference test circuit was set up using fully saturated fluid on the AAS side and no phospholipids on the filtration side of the dialyzer. Repeated samples were collected between 0 and 360 min and subsequently analyzed using the bubble test proposed by Pattle et al. [27].

RESULTS

Liquid Filled Chamber (LFC) Design

The fully assembled LFC is shown in **Figure 1**. The housing (**Figure 1 1**) represents the outer subsystem of the LFC. The outer

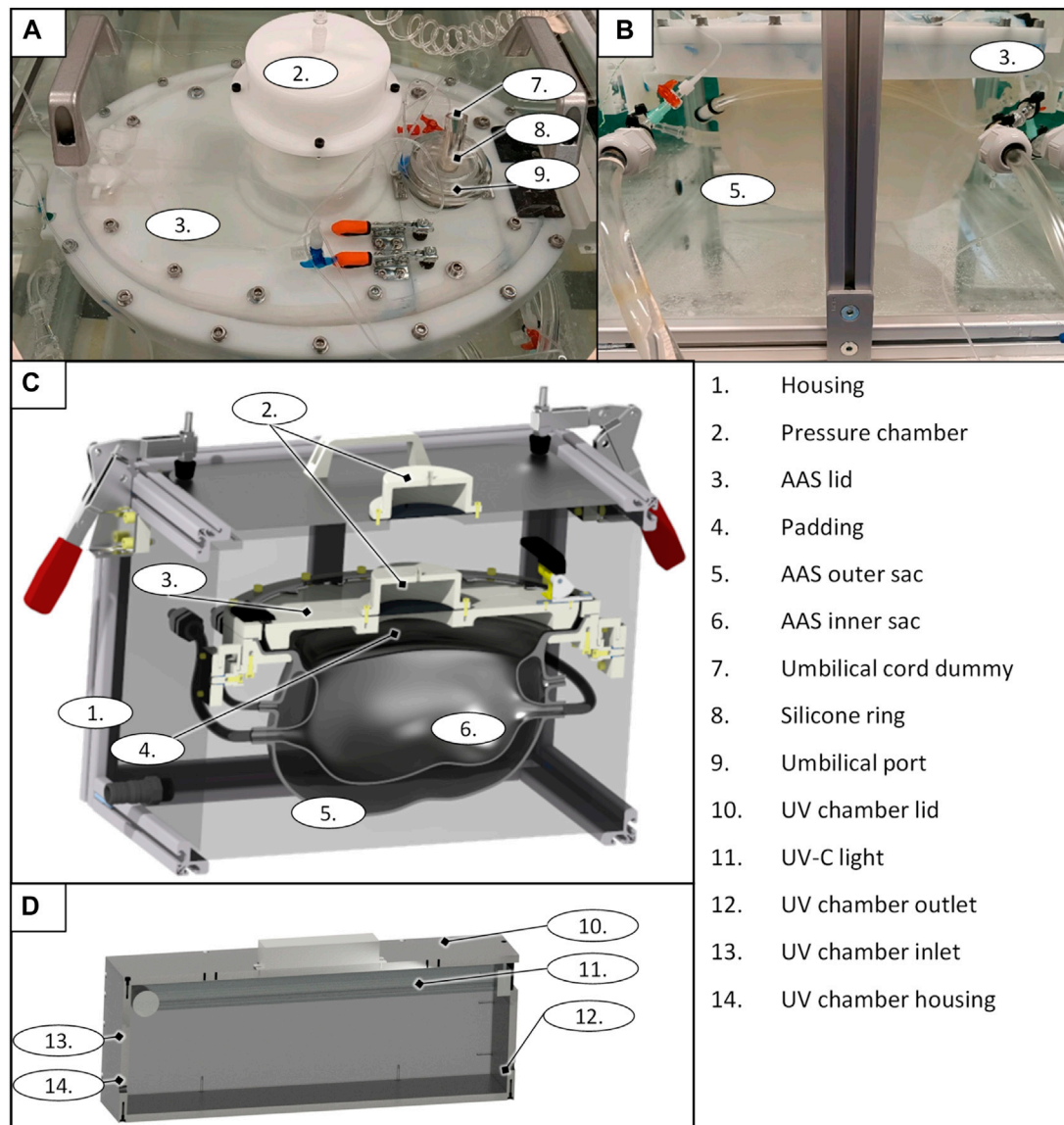


FIGURE 1 | LFC prototype top view (A); side view (B), general design of the liquid-filled chamber as a CAD rendering sectional view (C), and the UV chamber of the filtration system as a CAD rendering sectional view (D).

subsystem is intended to ensure a constant temperature at 37°C (R7) using a heating unit (Figure 1 5). The internal pressure (R8) on the AAS (Figure 1 6) is set by the geodetic pressure of the water level above the AAS and additionally by a pressure chamber, which increases the overall pressure in the housing unit if needed.

The AAS consists of an inner sac, an outer sac, and its lid (Figure 1 3). The sizing fluid (R2 & R3) is enclosed between the inner sac and outer sac of the AAS and can either be pushed out passively (due to growth) or externally adjusted. Both sacs and the sizing fluid mimic the elasticity of the maternal womb (R1). The outer sac has a wall thickness of 3 mm–4 mm, and the inner sac has a wall thickness of 1.5 mm–2 mm (R2 & R3). The volume of

the outer sac is 7.2 L and the volume of the initial inner sac is 3.6 L, leaving 3.6 L of volume for growth in the interspace between the two sacs. The shape of the initial inner sacs was defined along the CRL and CC (Table 2) as the axes of two perpendicular ellipses. The pine-shaped bottom of the two sacs (Figure 1) predefines the position of the neonate. The shape and growth-adaptive system provides enough space for the growing neonate, especially in the hip and head regions (R1). The AAS lid closes and seals the inner sac opening (R5), providing padding (Figure 1 4) on the inside to protect the neonate. The housing and the AAS lid have a built-in pressure chamber (Figure 1 2), consisting of an air-filled chamber and a flexible membrane connected to the housing system and the

padding. Air can be compressed in this chamber to generate extra pressure on the corresponding system. The AAS lid further includes a port for the umbilical cord (**Figure 1 9**). The umbilical port acts as an interface between the inner sac and the outer system to allow the neonate's umbilical cord or the cannulation tubing to pass to the artificial placenta (R6). The port seals the umbilical cord (**Figure 1 7** illustrated with an umbilical cord dummy) or the cannulation tubing using a pressurized flexible silicone ring (**Figure 1 8**).

The filtration system is attached to the inner sac and prevents contamination of the neonate's environment within the LFC. It comprises a roller pump (HL 20, Marquet, Raststatt, Germany), a changeable gross filter (0.5 mm–0.7 mm), a UV–C disinfection unit, and a hemodialyzer (High Flux FX 50, Fresenius medical care, Bad Homburg, Germany). The roller pump pumps the artificial amniotic fluid from the AAS system through the filtration system. The gross filter removes skin and hair particles. The UV–C unit uses a UV–C lamp (Osram UV-Lamp HNS-L 95W, Osram, Garching, Germany) (**Figure 1 11**) assembled in a PMMA box (135 mm × 580 mm × 176 mm) (**Figure 1 10** and **14**) with UV sublimated coating (Clear UVC, Solarscreen, Foetz, Luxembourg). It additionally serves as a reservoir for the AAS circuit. The UV–C lamp is automatically switched off for 15 min every 30 min to prevent overheating (R12). If changes in the water level exceed ± 1 L (e.g., due to leakage), the pump and UV lamp are automatically switched off, and an acoustic alarm is triggered. The hemodialyzer removes harmful excretions from the artificial amniotic fluid (R11) using a conventional dialysate with added phospholipids (EMPIGEN® BB Detergent, Merck, Darmstadt, Germany) to prevent washout of the neonate's autologous phospholipids (R13).

In Vitro Verification of LFC

The results of the volume adaptability test of the system to compensate for the growth of the neonate are shown in **Figure 2**. A total of 3.4 L of sizing fluid was manually removed from the initial state (**Figure 2A**) to the maximum expanded state of the AAS (**Figure 2B**). It was not possible to extract the full 3.6 L of sizing fluid, so 0.2 L remained in the interspace between the sacs. Falling of the folds leads to an inversion of the outer sac. As the volume increased, the AAS pressure increased linearly from 8 to 21 mmHg.

The seal and operability test results are shown in **Table 4**. The Absorption, Temperature, and Pressure parameters are listed according to their measurement location for each day starting after the heating period, which took 5 h.

In the filter test, the standard smear showed a negative result for both samples without any detectable growth of microorganisms, in contrast to the reference sample, which showed a positive result and the formation of a bacterial lawn. Results for the phospholipid washout are given in **Figure 3**. With an equal concentration of phospholipids of the same size on both sides of the hemodialyzer membrane, no change in the phospholipid concentration could be observed (**Figure 3A**). In the negative reference without adjusted phospholipid concentration in the dialysate, a washout of phospholipids was detected with an apparent decrease in total phospholipid concentration (**Figure 3B**).

DISCUSSION

The size and shape of the AAS were designed to appropriately fit a human neonate (at the 24th week of GA) according to growth data reported in the literature (R1). The elasticity of the double-walled AAS provides a gentle resistance to the movements of the neonate (R2). Published data on uterine compliance or uterine muscle E-module were not sufficient for our purpose. Theoretically, the compliance of the AAS can be adjusted by the thickness of its wall or by choosing a different medical grade silicone. The AAS allows the interaction between fetal movements and an elastic but still restrictive surrounding just like *in utero*. This interaction makes it possible to develop the necessary steps in the haptic sense and in neuro-muscular and musculoskeletal development [28]. The lid of the AAS is designed to enable the two different transfer procedures (R4) that are currently the focus of our team. Furthermore, the lid provides access (R4) to the neonate, e.g., for emergency procedures.

The ability of the AAS to compensate for neonatal growth (R1, R2, R3) was demonstrated by a 3.4 L (94%) increase in the inner sac volume. According to the data given in **Table 2**, the resulting volume adjustability should fit 90% of all neonates from GA 24 to GA 28. The AAS intrauterine pressure can be manually adjusted from 0 mmHg to 30 mmHg (R8). External adjustment is possible via water level variations in the housing. Internal adjustment can be achieved through the pressure chamber. Increasing pressure due to the growth of the neonate can be compensated by the same mechanisms, enabling the AAS to maintain a constant pressure for the neonate.

The long-term operability of the LFC System was verified in a 7-day test. No leakage could be detected. The variation in the absorption values of the colored liquid was within the measurement accuracy of the UltroSpec 2100. Temperature and pressure values were found to be constant over time and within the predefined range (R5, R6, R7, R8).

For the filtration system, a commercially available CE-marked hemodialyzer (R11) was used so that the elimination of metabolic waste in the amniotic fluid could be assumed. The elimination of microorganisms using the UV disinfection unit proved successful in the static test. Exposure of the fluid samples to UV light for 5 min was sufficient to disinfect the sample. Given the current volume of the UV-irradiated reservoir (6.2 L) and the intended flow rate of the filtration system of 800 mL/min, this results in an average passage time through the UV-irradiated reservoir of 7.75 min, giving sufficient time for disinfection (R12).

A stable level of phospholipids by harmonizing the concentration gradient on the dialysate side of the hemodialyzer showed no washout of the phospholipids within the AAS. However, it is still possible that an exchange of phospholipids across the dialyzer membrane takes place. Although it would be favorable to keep the autologous phospholipids completely within the AAS circuit, a mixture of autologous and artificial phospholipids is not expected to be harmful to the neonate. However, this needs to be further investigated.

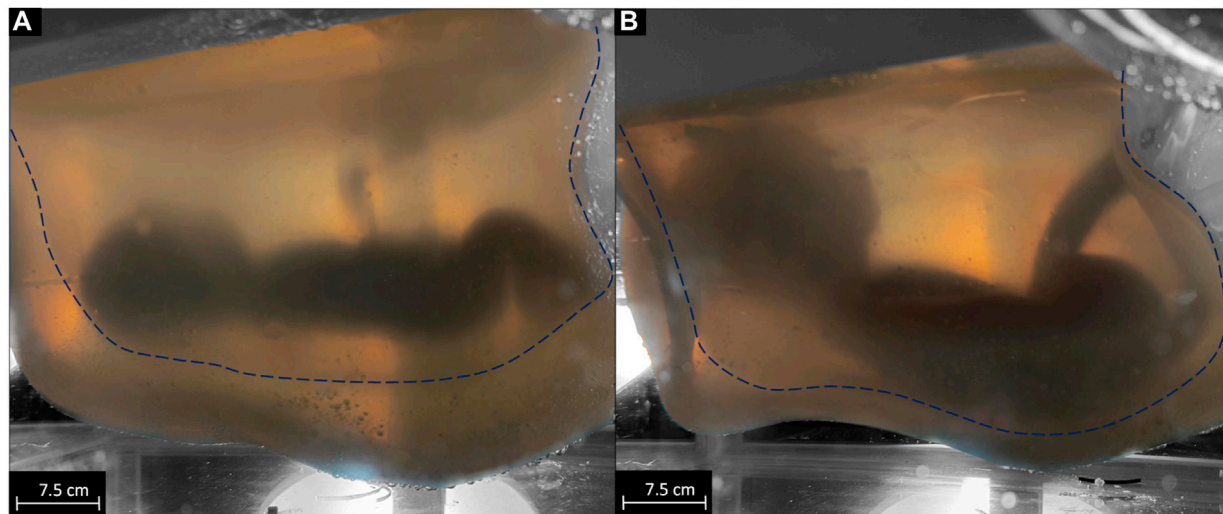


FIGURE 2 | visualization of the growth of the system from 3.6 L filling volume of the inner sac [dashed line (A)] to 6.8 L filling volume of the inner sac [dashed line (B)].

TABLE 4 | Resulting absorption rates, temperature and pressure data from the long-term sealing and operational stability test, and standard deviations are given in brackets.

Days		1	2	3	4	5	6	7
Absorption	ϵ_{outer}	0.303	0.289	0.304	0.299	0.308	0.308	0.293
	ϵ_{inner}	0.003	0.024	0.015	0.010	0.011	0.010	0.012
Temperature	T_{inner}	37.37 (0.27)	36.88 (0.33)	36.66 (0.05)	36.69 (0.05)	36.69 (0.07)	36.71 (0.07)	36.71 (0.06)
	T_{outer}	39.24 (0.54)	38.51 (0.12)	37.55 (0.14)	37.30 (0.09)	37.57 (0.09)	37.87 (0.48)	37.46 (0.40)
	$T_{\text{reference}}$	39.85 (0.50)	39.36 (0.08)	39.43 (0.05)	39.41 (0.04)	39.43 (0.05)	39.43 (0.05)	39.41 (0.04)
Pressure	p_{padding}	27.80 (0.22)	27.61 (0.15)	27.03 (0.40)	27.00 (0.10)	26.51 (0.25)	26.60 (0.15)	26.65 (0.23)
	$p_{\text{inner_in}}$	30.35 (0.48)	30.17 (0.32)	29.56 (0.08)	29.09 (0.10)	28.86 (0.05)	29.04 (0.19)	29.29 (0.05)
	$p_{\text{inner_out}}$	28.72 (0.38)	28.50 (0.51)	29.28 (0.16)	28.81 (0.06)	28.54 (0.09)	28.60 (0.21)	28.26 (0.22)

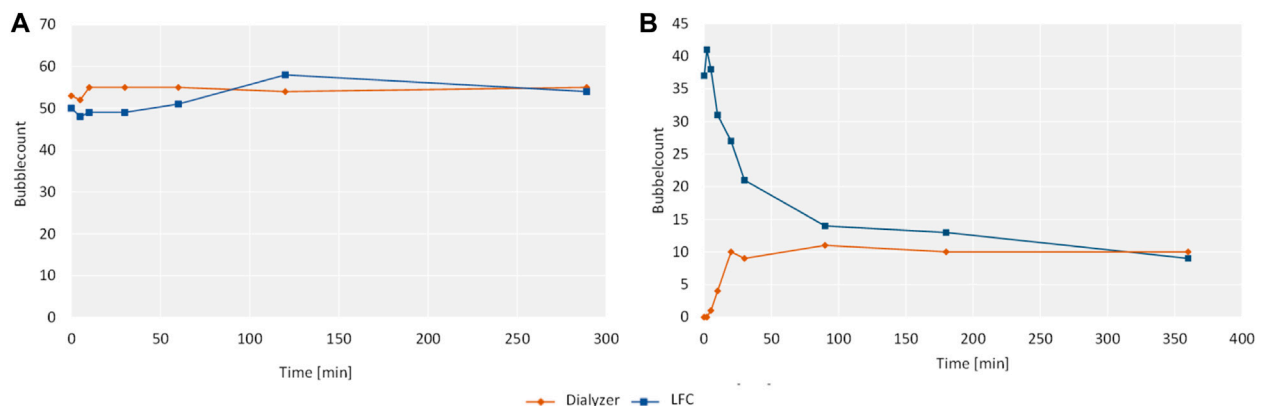


FIGURE 3 | Results of the filtration system washout with bubble count on the y-axis and time on the x-axis.

All LFC surfaces that form the inner fluid circuit are made of sterilizable materials. Therefore, a sterile starting point can be achieved with the system. A static test for sterility has been presented in this work. Both conditions lead to the assumption

of complete operational sterility. Nevertheless, this must be proven in a further study during the operation of the prototype.

Current artificial womb systems tested in animal models by other researchers are designed to place the neonate on its side or back and

compensate for growth by adjusting magnets on the biobag. Our novel design lets neonates float in our system without contact with a potentially harmful solid floor. The buoyancy generated by the different fluid-filled sacs and the surroundings, in combination with the potential for neonatal movement within the system, is an evolution toward a more physiologic artificial womb. This is ensured by the inner shape and the tight-fitting elasticity of the inner chamber together with the neonate being in a state of buoyancy. We expect that this will positively influence the naturalness of movements and thus motor development. However, the movements are also restricted by the tight fit and shape of the inner sac analogous to the physiological womb. A definitive statement on the possibilities of fetal movement will have to be made with an LFC prototype adapted in shape and elasticity to the chosen animal model in future tests.

It is an inherent characteristic of artificial womb technology that it creates an artificial barrier to sensory contact between neonates and parents. This is the most substantial contrast to the dyadic relationship between mother and child. The importance of early bonding through skin-to-skin contact between mother and child for attachment in later life has been well examined [29]. Kangaroo-Mother-Care has been proven to reduce preterm morbidity and mortality [30].

Introducing delivery room skin-to-skin care into ELGANs has proven to improve subsequent mother-infant interactions and reduce the expression of stress-response genes [31, 32]. Current WHO recommendations for the care of preterm or low birth weight infants strongly advocate early and continuous Kangaroo mother care (KMC) [33]. We acknowledge the potential of artificial womb technology to negatively impact parent-child attachment. It is essential to develop procedures and techniques to reduce this risk. Technical solutions aimed at supporting visual and auditory contact could be well imagined: a clearer picture of the child could be transmitted through a camera. Acoustic signals like speech and physiological noises from the mother, like a heartbeat, could be transmitted by appropriate techniques. Furthermore, removing the outer housing would allow for closer sensory contact between parents and neonates. Therefore, additional concepts must be developed to maintain the pressure adaptability, temperature stability and buoyancy generated by the outer chamber.

CONCLUSION

This study demonstrates the *in vitro* functionality of the volume-adjustable artificial womb. The system was verified to be adjustable in volume and pressure. It was temperature stable during a 7-day operation. The size of the system fits the size of the

target patient group. It was demonstrated that phospholipid washout can be prevented within a closed-circuit disinfection and filtration system. In addition, the successful elimination of metabolic waste products and the effective antiseptic treatment of amniotic fluid were proven in a static *in vitro* test and mathematically transferred to the dynamic system. We have presented an artificial uterus that allows neonatal growth and movement. This is an important step in the transition to artificial womb technology as a bridge-to-life technology for the smallest of patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JH: Conceptualization, Methodology, Validation, Visualization, Writing—original draft; FS: Conceptualization, Writing—review and editing; AS: Conceptualization, Writing—review and editing; YS: Conceptualization, Writing—review and editing; JA: Supervision, Writing—review and editing; TO: Supervision, Writing—review and editing; US: Resources, Supervision, Writing—review and editing; SJ: Project administration, Supervision, Writing—review and editing; MS: Project administration, Supervision, Writing—review and editing.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Assessment of Mitochondrial Respiration During Hypothermic Storage of Liver Biopsies Following Normothermic Machine Perfusion

Julia Hofmann^{1†}, Alexander Kofler^{1†}, Melanie Schartner¹, Madita L. Buch¹, Martin Hermann¹, Bettina Zelger², Dietmar Öfner¹, Rupert Oberhuber¹, Theresa Hautz¹, Stefan Schneeberger¹ and Andras T. Meszaros^{1*}

¹OrganLife Laboratory, Department of Visceral, Transplant and Thoracic Surgery, Medical University of Innsbruck, Innsbruck, Austria, ²Institute of Pathology, Neuropathology and Molecular Pathology, Medical University of Innsbruck, Innsbruck, Austria

Organ quality can be assessed prior to transplantation, during normothermic machine perfusion (NMP) of the liver. Evaluation of mitochondrial function by high-resolution respirometry (HRR) may serve as a viability assessment concept in this setting. Freshly collected tissue is considered as optimal sample for HRR, but due to technical and personnel requirements, more flexible and schedulable measurements are needed. However, the impact of cold storage following NMP before processing biopsy samples for mitochondrial analysis remains unknown. We aimed at establishing an appropriate storage protocol of liver biopsies for HRR. Wedge biopsies of 5 human livers during NMP were obtained and assessed by HRR. Analysis was performed after 0, 4, 8, and 12 h of hypothermic storage (HTS) in HTK organ preservation solution at 4°C. With HTS up to 4 h, mitochondrial performance did not decrease in HTS samples compared with 0 h (OXPHOS, 44.62 [34.75–60.15] pmol·s⁻¹·mg wet mass⁻¹ vs. 43.73 [40.69–57.71], median [IQR], $p > 0.999$). However, at HTS beyond 4 h, mitochondrial respiration decreased. We conclude that HTS can be safely applied for extending the biopsy measurement window for up to 4 h to determine organ quality, but also that human liver respiration degrades beyond 4 h HTS following NMP.

Keywords: liver transplantation, mitochondria, normothermic machine perfusion, high-resolution respirometry, hypothermic storage

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*Correspondence

Andras T. Meszaros,

✉ andras.meszaros@i-med.ac.at

[†]These authors have contributed equally to this work and share first authorship

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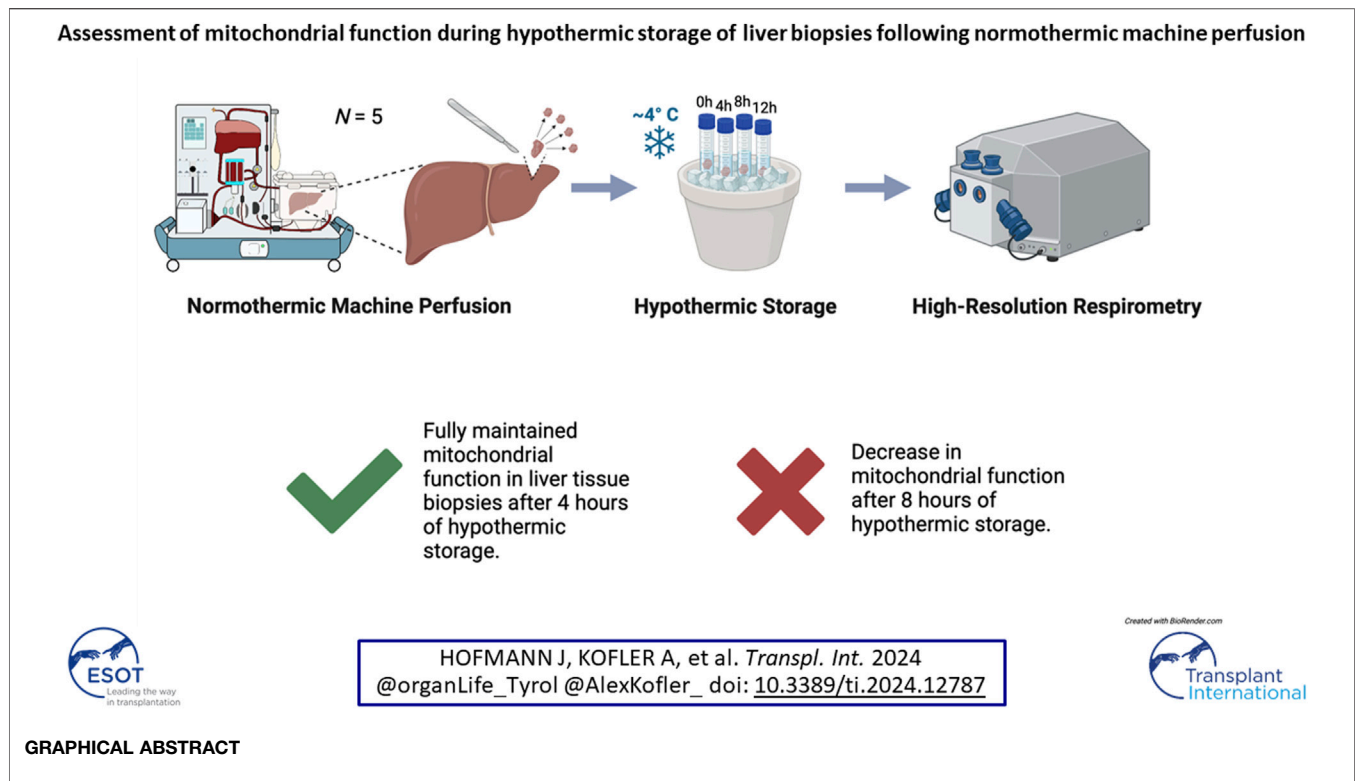
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INTRODUCTION

The serious shortage of donor organs is a daily challenge in liver transplantation (LT). To overcome this limitation and reduce waiting list mortality, extended criteria donor (ECD) organs are increasingly utilized for LT [1]. However, a substantial proportion of retrieved organs are not transplanted since they are considered inferior in their macroscopic appearance and expected function. Thus, it is essential to evaluate the quality of these grafts and predict the clinical outcome with the help of reliable biomarkers [2]. Machine perfusion is an emerging organ preservation strategy. Donor livers are perfused under normothermic conditions, which also allows for assessment of the organ function prior to transplantation. For this purpose, several biomarkers have been recently suggested. Transplant centers usually rely on a combination of different parameters for decision-making, including lactate clearance, level of perfusate transaminases, bile production and its



composition. However, since robustness of those parameters with regard to their predictive capacity is limited, new biomarkers are under investigation [3–5]. Recently, our group demonstrated that mitochondrial bioenergetics assessed by high-resolution respirometry (HRR) in liver biopsies during the first 6 h of NMP predicts graft function after LT [6]. Different methods to analyze mitochondrial status are available, including monitoring free flavin mononucleotide [7–10], a mitochondrial damage parameter. In contrast, HRR allows for evaluation of the actual bioenergetic capacity by assessing mitochondrial respiration. Oxygen consumption of mitochondria is modulated by various substrates and inhibitors and is measured in real-time, thus enabling a comprehensive characterization of energy production, mitochondrial (dys) function and metabolic pathways.

In the above study, we performed HRR in tissue homogenates of freshly collected biopsies. However, this might be challenging in the clinical routine, especially in serial biopsies requiring immediate measurements, each taking approximately 2 h. To reduce this logistical and technical burden, extended hypothermic storage (HTS) would require little effort and ease the logistics. HRR analyses of tissue samples collected in the early phase of NMP could be performed in parallel, and the results would be available in a relevant period of the decision-making process for transplantation [11, 12]. The effect of cold ischemia on mitochondrial respiration has been extensively studied in endothelial cells [13], in heart [14], and in small samples of porcine livers [15]. However, no data on

preservation protocols for liver biopsies taken during NMP exist. In this study we aimed to investigate the effect of HTS on mitochondrial respiration and coupling control in clinical liver NMP.

MATERIAL AND METHODS

Study Design and Ethics

Tissue samples of liver allografts ($N = 5$) subjected to NMP (Metra[®], OrganOx Limited, Oxford, United Kingdom) prior to transplantation were included in this single center experimental study between January and August 2023. The study was approved by the institutional ethics committee (Ethics Commission of the Medical University of Innsbruck, EK Nr. 1175/2018). NMP was performed in accordance with our center's protocols as described by Cardini *et al.* [16, 17]. After organ acceptance and indication for NMP subsection, livers were included in the study on a rolling-basis when (i) logistics could be arranged and (ii) repetitive high-resolution respirometry measurements during the next 12 h could be ensured. At the time point of inclusion, the performance of the livers during NMP was still under investigation. Wedge biopsies ($20\text{ mg} \pm 2\text{ mg}$ wet mass) were taken during clinical NMP and divided into 4 approximately equal parts ($\sim 5\text{ mg}$ each). The biopsy samples were then directly placed in ice-cold HTK solution (Custodiol[®], Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) and transferred to the laboratory at

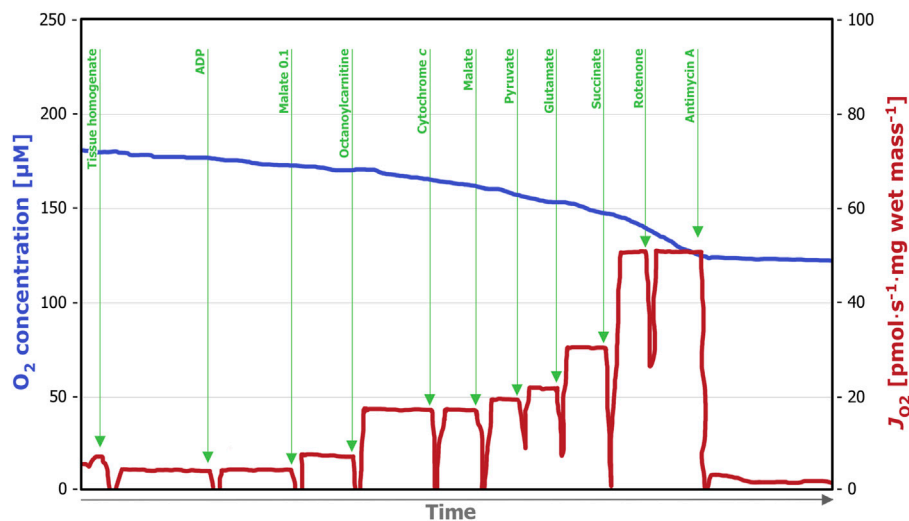


FIGURE 1 | Representative trace of a high-resolution respirometry measurement applying the SUIT (i) protocol. After addition of the tissue homogenate (0.5 mL), residual oxygen consumption (ROX) was assessed in the absence of fuel substrates and adenosine diphosphate (ADP). Next, ADP (5 μ M) was added, followed by malate (0.1 mM) and octanoylcarnitine (0.5 mM), which allows for assessment of the F_P OXPHOS capacity. Integrity of the outer mitochondrial membrane was tested by titration of cytochrome *c* (10 μ M). The subsequent titration of N_P -linked substrates (2 mM malate, 5 mM pyruvate and 10 mM glutamate) enables evaluation of combined F_P - and N_P -linked OXPHOS capacity. Next, F_P -, N_P - and S_P -linked OXPHOS capacity was measured by the addition of succinate (10 mM), followed by the titration of Complex I inhibitor rotenone (0.5 μ M), which allows the measurement of S_P -linked OXPHOS capacity only. Lastly, the Complex III inhibitor antimycin A (2.5 μ M) was added, which allows for evaluation of ROX. OXPHOS, oxidative phosphorylation; F_P , fatty acid oxidation-linked pathway; N_P , nicotinamide adenine dinucleotide-linked pathway; S_P , succinate-linked pathway; ROX, residual oxygen consumption.

4°C on ice for further processing. The first biopsy was immediately analyzed for baseline mitochondrial respiratory function values. The further samples were stored at 4°C on ice and analyzed after 4, 8, or 12 h of HTS.

High-Resolution Respirometry (HRR)

Mitochondrial respiration was assessed by HRR (O2k, Oroboros Instruments, Innsbruck, Austria) with devices equipped with two identical 0.5 mL measurement chambers at 37°C and under continuous stirring at 750 rpm [18]. Measurements were preceded by air-calibration of the devices using the mitochondrial respiration medium MiR05 (MiR05-Kit, Oroboros Instruments, Innsbruck, Austria) containing 0.5 mM EGTA, 3 mM $MgCl_2 \cdot 6 H_2O$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4 , 20 mM HEPES, 110 mM D-sucrose, 1 g·L⁻¹ essentially fatty acid free bovine serum albumin). Liver wedge biopsies were weighted in a 4°C environment, and subsequently homogenized in 4°C MiR05 using a PBI-Shredder O2k-Set (Oroboros Instruments, Innsbruck, Austria), to disrupt cellular structures and extract mitochondria. The crude tissue homogenate, containing freely accessible mitochondria, was adjusted to a final concentration of 1 mg wet mass·mL⁻¹, and 500 μ L was transferred into the O2k chambers. Reagents were added using Hamilton glass microsyringes (Hamilton Bonaduz AG, Bonaduz, Switzerland) following pre-defined Substrate-Uncoupler-Inhibitor Titration (SUIT) protocols. All measurements were performed in technical duplicates [6]. Oxygen concentration and flux were recorded at an interval of 2 s using DatLab 7 software (Datlab 7.4, Oroboros Instruments, Innsbruck, Austria).

Substrate-Uncoupler-Inhibitor Titration (SUIT) Protocols

We applied two different SUIT protocols. SUIT(i), corresponding SUIT-025 in the online protocol library Bioblast¹, (Figure 1) allows for evaluation of the substrate pathway control in the OXPHOS state. After addition of ADP in saturating concentrations (5 mM), the substrates for the fatty acid oxidation-linked pathway (F_P), malate (0.1 mM) and octanoylcarnitine (0.5 mM) were titrated, followed by cytochrome *c* (10 μ M) for the evaluation of the integrity of the mitochondrial outer membrane. Next, substrates for the NADH-linked pathway (N_P); pyruvate (5 mM), malate (2 mM) and glutamate (10 mM) were added. The overall capacity of convergent electron transfer pathways was determined after succinate addition (10 mM) (FNS_P). For determination of the succinate-linked pathway alone, (S_P) rotenone (0.5 μ M) was titrated. Finally, to inhibit Complex III and determine residual oxygen consumption (ROX), antimycin A (2.5 μ M) was added.

SUIT(ii), corresponding SUIT-006 in the online protocol library Bioblast² (Figure 2) provides detailed information on the S_P -linked mitochondrial respiration. Rotenone (0.5 μ M) was added for inhibition of the mitochondrial Complex I, which was followed by succinate (10 mM) titration. Next, ADP (5 mM) for evaluation of the OXPHOS capacity was added followed by cytochrome *c* (10 μ M) for the evaluation of the integrity of the mitochondrial outer membrane. Carbonyl cyanide

¹<https://wiki.oroboros.at/index.php/SUIT-025>

²<https://wiki.oroboros.at/index.php/SUIT-006>

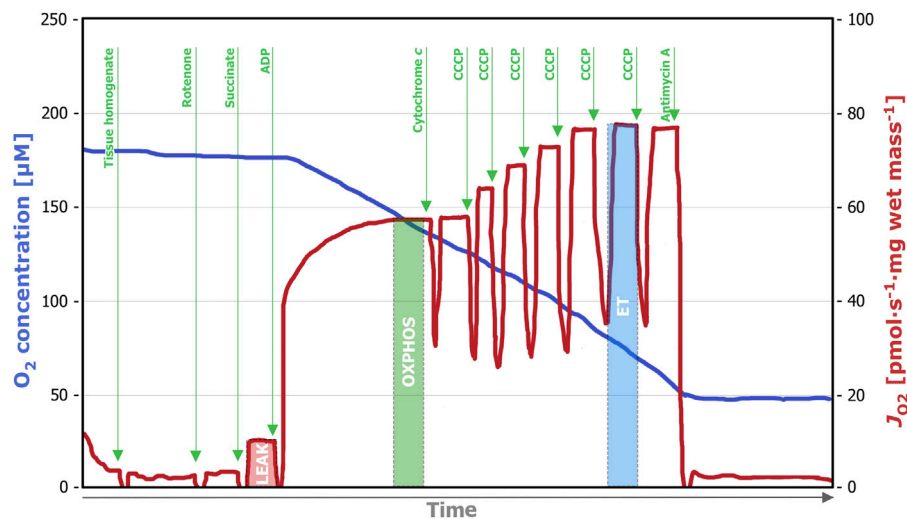


FIGURE 2 | Representative trace of a high-resolution respirometry measurement applying the SUIT (ii) protocol. After addition of the tissue homogenate (0.5 mL), residual oxygen consumption (ROX) was assessed in the absence of fuel substrates and adenosine diphosphate (ADP). Titration of rotenone (0.5 μ M) inhibits Complex I and after the subsequent addition of succinate (10 mM), LEAK respiration could be measured. The addition of ADP (5 mM) enables S_P -linked OXPHOS capacity assessment and with the subsequent titration of cytochrome *c* (10 μ M) the integrity of the outer mitochondrial membrane can be evaluated. To assess the electron transfer (ET) capacity, step-wise titration (0.5 μ M per step) of CCCP until maximum respiration was performed. Lastly, the Complex III inhibitor antimycin A (2.5 μ M) was added, inducing ROX. OXPHOS, oxidative phosphorylation; S_P , succinate-linked pathway; CCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone.

p-trifluoromethoxyphenyl hydrazone (CCCP) was titrated stepwise (0.5 μ M per step) to assess non-coupled respiration. Finally, antimycin A (2.5 μ M) was added to achieve ROX.

Mitochondrial Coupling Control States

Respiration rates were expressed as O_2 flux per wet mass tissue [$\text{pmol} \cdot O_2 \cdot s^{-1} \cdot \text{mg}^{-1}$]. In addition, respiratory capacities were normalized to an internal reference state for each measurement to determine flux control ratios (FCR) for evaluation of SUIT(i). For SUIT(ii), the coupling states LEAK (*L*), OXPHOS (*P*), OXPHOS(*c*) (*P_c*), and Electron Transfer capacity (*ET*) were evaluated. Based on the above, the following control efficiencies were calculated: *P-L* control efficiency ($1-L/P$), to evaluate the efficiency of the ATP production within S_P , and cytochrome *c* control efficiency ($1-P/P_c$) to evaluate the damage to the mitochondrial outer membrane [17, 19].

Real-Time Confocal Microscopy (RTCM)

Tissue integrity and cell viability of the obtained biopsies were assessed by real-time confocal microscopy (RTCM) as previously described [20, 21]. Briefly, biopsies were incubated with the fluorescent stains Hoechst 33,342 (blue, staining all nuclei, Molecular Probes; final concentration 5 $\mu\text{g} \cdot \text{mL}^{-1}$), propidium iodide (PI; red, staining the nuclei of dead cells, Molecular Probes; final concentration 500 nM), and wheat germ agglutinin (WGA; green, staining of cell morphology, Molecular Probes, Eugene, OR, United States; 5 $\mu\text{g} \cdot \text{mL}^{-1}$ final concentration) at room temperature for 15 min. Image acquisition was performed with an UltraVIEW VoX spinning disk confocal system (Perkin Elmer, Wellesley, MA) connected to a Zeiss AxioObserver Z1 microscope (Zeiss, Oberkochen,

Germany). Quantification relied on a semi-quantitative scoring system (0-1-2): 0—high number of viable cells, 1—an equal count of viable and dead cells, and 2—low number of viable cells [6].

Histopathology

Liver biopsies were fixed in 10% formalin for 24 h, then embedded in paraffin and cut in 3 μm sections. Hematoxylin and eosin (H&E) staining was performed following standard protocols to assess necrosis, inflammation, steatosis, fibrosis, and vascular changes. For scoring a modified Suzuki score was applied [6].

Statistical Analysis

Shapiro-Wilk test was employed to examine the normality of the data. Continuous variables are represented as median [interquartile range (IQR)]. Categorical variables are expressed as percentage (%). To assess differences compared to baseline values (0 h sample), Friedman's test with a post-hoc comparison to examine specific pairwise differences by the Dunn's method was performed. Statistical analysis was performed using GraphPad Prism 10 and a *p*-value of <0.05 was considered statistically significant.

RESULTS

Donor and Preservation Characteristics

5 human livers undergoing NMP were included in this study. Four grafts (80%) originated from donors after brain death (DBD). The median age of the donors was 72 [54–78] years, with a median body mass index of 29 [23–33] $\text{kg} \cdot \text{m}^{-2}$. The median donor risk index (DRI) was 2.04 [1.94–2.37], and 80% of organs were procured from ECD. One liver was found suitable

TABLE 1 | Donor and preservation characteristics.

Donor characteristics	Liver 1	Liver 2	Liver 3	Liver 4	Liver 5
Age (years)	65	43	72	79	77
Sex	M	M	M	M	M
Body mass index (kg·m ⁻²)	29	23	37	29	23
Cause of death	CVA	CVA	CVA	CVA	CVA
Donor type	DBD	DCD	DBD	DBD	DBD
ECD status	no	yes	yes	yes	yes
DRI	1.92	2.43	2.04	2.31	1.96
Steatosis ^a	mild	no	mild	no	no
Fibrosis ^a	mild	no	no	no	no
Hepatitis ^a	minimal	no	no	no	no
Preservation	Liver 1	Liver 2	Liver 3	Liver 4	Liver 5
CIT (min)	538	599	492	306	365
Functional WIT ^b (DCD; min)		18			
Lactate ≤2.5 mmol·L ⁻¹ after 2 h NMP	yes	yes	yes	yes	no
AST peak (NMP) (U·L ⁻¹)	2,417	11,221	1,048	2,159	12,800
ALT peak (NMP) (U·L ⁻¹)	2,588	3,327	909	1,515	11,655
LDH peak (NMP) (U·L ⁻¹)	5,596	16,339	3,036	3,270	25,507
Bile production during NMP	no	yes	yes	yes	yes
Reason for decline	pH instable, no bile production	Inhomogeneous perfusion after complex arterial reconstruction	Transplanted	Recipient tested positive for COVID-19, liver was reallocated	Late lactate clearance, cholestasis, high LDH

^aAssessed in frozen tissue sections.

^bIn the context of DCD donors, functional warm ischemia time refers to the interval starting when the mean arterial pressure falls below 50 mmHg or arterial saturation drops below 80%, and ending when cold perfusion starts.

CVA, cerebrovascular accident; ECD, extended criteria donor; DBD, donation after brain death; DCD, donation after circulatory death; DRI, donor risk index; CIT, cold ischemic time; WIT, warm ischemic time; NMP, normothermic machine perfusion; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase.

for transplantation after NMP, while four livers deemed unsuitable and had to be declined. The individual donor and preservation characteristics, viability parameters and reasons for decline can be found in **Table 1**.

High-Resolution Respirometry for Assessment of Hypothermically Stored Liver Biopsies

With the progression of hypothermic storage, gradual, time-dependent changes in mitochondrial respiration were observed (**Figure 3**).

Characterization of Pathway Contribution

We investigated the NADH- (N_p), FAO- (F_p), and succinate-linked (S_p) oxidative phosphorylation (**Figure 4; Table 2**). Respiration was highest for S_p and lower for N_p and F_p . Concerning S_p , fresh and 4 h HTS measurements revealed similar respiration rates (53.59 [49.51–65.87] pmol·s⁻¹·mg wet mass⁻¹ vs. 51.01 [38.31–66.91] pmol·s⁻¹·mg wet mass⁻¹, $p > 0.999$), while after 8 h HTS a marked decrease in respiration was observed (43.32 [27.22–55.48] pmol·s⁻¹·mg wet mass⁻¹, $p = 0.811$). At 12 h, the respiration was even lower: (37.99 [19.61–47.79] pmol·s⁻¹·mg wet mass⁻¹, $p = 0.06$). In line with this, N_p and F_p remained stable for 4 h of HTS and declined after 8 h: N_p decreased to 3.89 [3.10–4.94] pmol·s⁻¹·mg wet mass⁻¹ after 12 h ($p = 0.006$). Furthermore, the flux control ratios for each pathway were calculated, to take advantage of the internal normalization relative to the maximal convergent electron pathway respiration (**Figure 4; Table 2**). In the present

cohort, in fresh biopsies FCRs of 0.27 and 0.14 were found for F_p and N_p , respectively, which also remained constant over storage time. For S_p a FCR of 1.0 was observed for fresh biopsies and all analyzed HTS biopsies, which indicated that succinate alone is sufficient to saturate OXPHOS capacity.

Succinate-Linked Mitochondrial Respiration

The succinate-linked respiration was analyzed in greater detail as it relates to the prominent substrate pathway in the present and previous studies (**Table 3; Figure 5**). Measurement of LEAK respiration, representing the non-phosphorylating component of mitochondrial respiration, yielded similar results between fresh tissue biopsies and those measured after 4 h HTS (6.75 [6.69–8.50] pmol·s⁻¹·mg wet mass⁻¹ vs. 7.24 [7.03–8.09] pmol·s⁻¹·mg wet mass⁻¹, $p > 0.999$). In line with this, OXPHOS capacity, measured with saturating substrate, ADP, and inorganic phosphate concentrations remained stable for 4 h HTS (fresh 43.73 [40.69–57.71] pmol·s⁻¹·mg wet mass⁻¹ vs. 4 h HTS 44.62 [34.75–60.15] pmol·s⁻¹·mg wet mass⁻¹, $p > 0.999$). The same pattern was also observed for the electron transfer capacity in the non-coupled state (fresh 82.64 [78.22–93.51] pmol·s⁻¹·mg wet mass⁻¹ vs. 4 h HTS 71.09 [68.24–96.07] pmol·s⁻¹·mg wet mass⁻¹, $p > 0.999$). All assessed parameters showed decrease after 8 h HTS and beyond. However, significance was not reached.

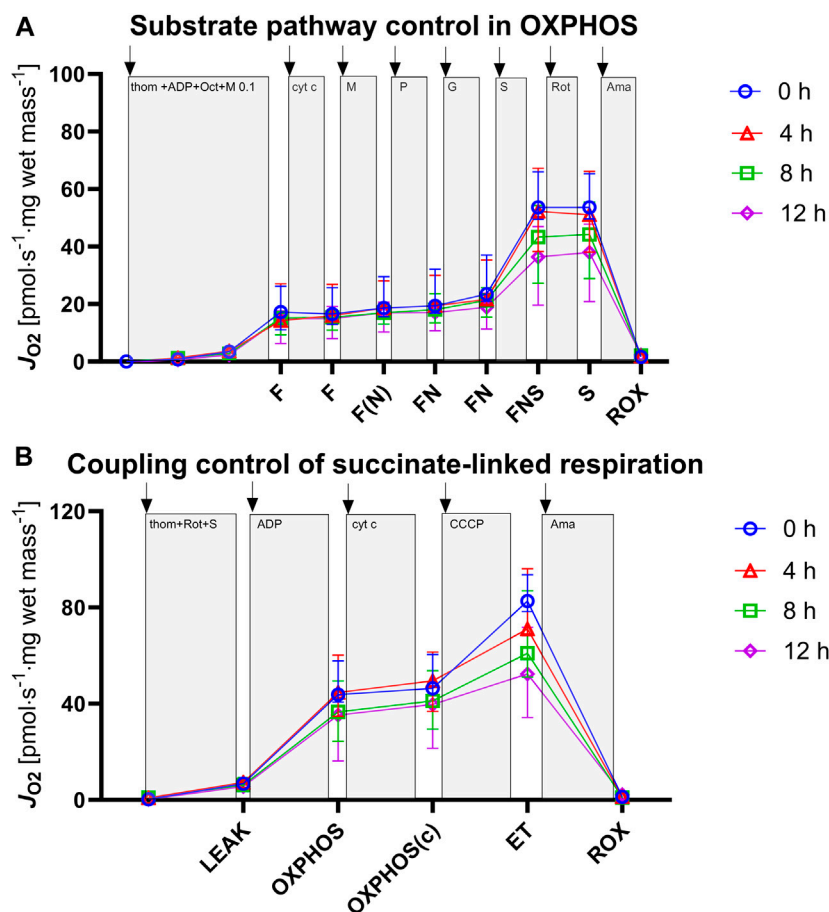


FIGURE 3 | Assessment of mitochondrial respiration in tissue biopsies after 0, 4, 8, and 12 h of hypothermic storage. **(A)** ROX was measured after addition of tissue homogenate and was followed by titration of ADP to evaluate OXPHOS capacities for the F, FN and FNS pathway states. **(B)** ROX was measured after addition of tissue homogenate followed by Complex I inhibition and succinate titration to evaluate S-linked LEAK respiration. ADP and cytochrome c addition allowed for measurement of OXPHOS capacity and OXPHOS (c) capacity, respectively. Uncoupler titrations were performed to analyze ET capacity. ADP, adenosine diphosphate; F, fatty acid oxidation; FN, fatty acid oxidation and NADH; FNS, fatty acid oxidation-NADH-succinate; OXPHOS, oxidative phosphorylation; ET, electron transfer; ROX, residual oxygen consumption; thom, tissue homogenate; Oct, octanoylcarnitine; M, malate; P, pyruvate; G, glutamate; S, succinate; Rot, rotenone; Ama, antimycin A; cyt c, cytochrome c; CCCP, Carbonyl cyanide p-trifluoromethoxyphenyl hydrazone. Data are represented as median and interquartile range.

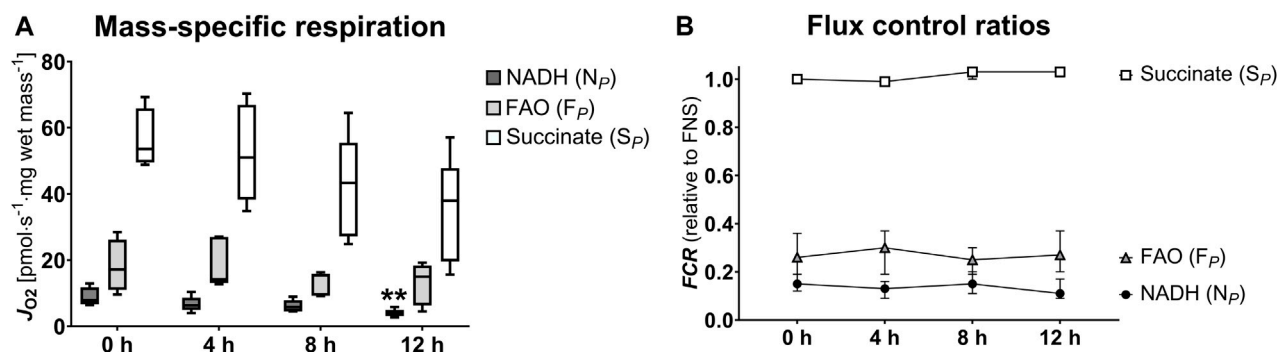


FIGURE 4 | Pathway-specific mitochondrial respiration **(A)** and flux control ratios **(B)**. NADH, nicotinamide adenine dinucleotide hydrogen; FAO, fatty acid oxidation; S, succinate; FCR, flux control ratio. **(A)**: Data are represented as median, interquartile range and min-max. **(B)**: Data are represented as median and interquartile range. Friedman's test with Dunn's multiple comparisons was applied to assess differences compared to 0 h samples. $^{**}p < 0.01$.

TABLE 2 | Pathway-specific oxidative phosphorylation.

Mitochondrial respiration (pmol·s ⁻¹ ·mg wet mass ⁻¹)	0 h	4 h	p-value	8 h	p-value	12 h	p-value
NADH (N _P)	7.84 [6.65–11.78]	6.30 [4.95–8.66]	0.981	5.86 [4.52–7.88]	>0.999	3.89 [3.10–4.94]	0.006
FAO (F _P)	17.20 [11.00–26.18]	14.17 [13.08–27.03]	>0.999	15.40 [9.26–15.89]	0.534	14.99 [6.31–18.38]	0.334
Succinate (S _P)	53.59 [49.51–65.87]	51.01 [38.31–66.91]	>0.999	43.32 [27.22–55.48]	0.811	37.99 [19.61–47.79]	0.06
FCR (relative to FNS)	0 h	4 h	p-value	8 h	p-value	12 h	p-value
NADH (N _P)	0.15 [0.13–0.18]	0.13 [0.10–0.15]	0.662	0.15 [0.12–0.19]	>0.999	0.11 [0.10–0.17]	>0.999
FAO (F _P)	0.26 [0.17–0.35]	0.30 [0.21–0.34]	0.534	0.25 [0.22–0.30]	>0.999	0.27 [0.23–0.35]	0.259
Succinate (S _P)	1.00 [0.99–1.00]	0.99 [0.99–0.99]	>0.999	1.03 [1.01–1.08]	0.199	1.03 [1.02–1.07]	0.150

NADH, nicotinamide adenine dinucleotide hydrogen; FAO, fatty acid oxidation; FCR, flux control ratio; FNS, fatty acid oxidation-NADH-succinate. Data are represented as median, interquartile range and min-max. Friedman's test with Dunn's multiple comparisons was applied to assess differences compared to 0 h samples.

TABLE 3 | Mitochondrial respiratory capacities of the S-pathway.

Coupling states (pmol·s ⁻¹ ·mg wet mass ⁻¹)	0 h	4 h	p-value	8 h	p-value	12 h	p-value
LEAK	6.75 [6.69–8.50]	7.24 [7.03–8.09]	>0.999	6.19 [5.33–8.30]	>0.999	5.51 [5.02–8.54]	>0.999
OXPHOS	43.73 [40.69–57.71]	44.62 [34.75–60.15]	>0.999	36.56 [24.38–49.41]	0.811	35.29 [16.18–46.77]	0.199
OXPHOS(c)	46.31 [44.55–60.41]	49.42 [36.75–61.42]	>0.999	41.17 [29.47–53.69]	0.811	39.71 [21.42–50.43]	0.199
ET	82.64 [78.22–93.51]	71.09 [68.24–96.07]	>0.999	60.95 [51.07–86.91]	0.811	52.33 [34.26–75.40]	0.06

OXPHOS, oxidative phosphorylation; OXPHOS(c), OXPHOS capacity after cytochrome c addition; ET, electron transfer. Data are represented as median, interquartile range and min-max. Friedman's test with Dunn's multiple comparisons was applied to assess differences compared to 0 h samples.

Coupling Control Efficiencies

The calculated mitochondrial coupling control efficiencies of the succinate-pathway did not show significant alterations during HTS up to 12 h compared to fresh biopsies (Table 4). Calculation of the *P-L* control efficiency allows for evaluation of the efficiency of ATP production. It was sufficiently high in fresh biopsies (0.85 [0.83–0.86]) but a trend towards lower values has been observed after storage for 12 h (0.79 [0.65–0.84], *p* = 0.112). Furthermore, cytochrome *c* control efficiency, which reflects the integrity of the mitochondrial outer membrane, revealed a slight increase at 8 h and 12 h HTS, indicating higher membrane permeability (*p* = 0.199 and *p* = 0.112) (Figure 5).

Real-Time Confocal Microscopy and Conventional Histology

In contrast to mitochondrial respiration, tissue integrity and cell viability could be preserved for up to 12 h of HTS, as demonstrated by RTCM analysis (Figure 6). The semiquantitative RTCM score revealed no changes during the storage period (data not shown). The microscopic evaluation of H&E sections also showed no differences regarding histopathology when comparing fresh biopsies with 12 h HTS biopsies (Supplementary Figure S1). This also underlines the sensitivity of HRR in comparison to other assessment methods during cold storage.

DISCUSSION

Herein we investigated the feasibility of prolonged hypothermic storage of tissue biopsies taken during NMP for later HRR

analysis. Testing of mitochondrial respiration in livers undergoing NMP can provide information on organ function and displays a reliable biomarker for decision-making [6, 17]. The HRR parameters cytochrome *c* control efficiency, *P-L* control efficiency, and LEAK respiration of the succinate-linked pathway correlated with the clinical outcome after transplantation in a previous clinical study. While HRR is a sensitive method, it requires technical proficiency of the operators and measurement immediately after sample collection. Enabling postponement of mitochondrial respiratory assessment would allow to analyze more biopsies and reduce the burden on personnel. This aspect is particularly relevant in scenarios where a higher number of biopsies need to be assessed for a comprehensive understanding of organ viability.

Our findings shed light on the feasibility of preservation of mitochondrial function in hypothermically stored liver biopsies. It indicates that prolonged periods of cold storage following liver NMP might be harmful, while shorter storage times of up to 4 h may be acceptable. Our results demonstrated that mitochondrial respiration remained stable within this timeframe, and thus reliable results on mitochondrial function can be obtained after this storage period. This could guide decision-making processes pertaining to organ acceptance or utilization. Interestingly, beyond the 4-h mark mitochondrial respiration and efficiency tend to decrease, while the mitochondrial outer membrane damage increases. This contrasts earlier findings of our department [15], where the preservation of OXPHOS capacity and cytochrome *c* control efficiency during 48 h of hypothermic storage in porcine livers were analyzed. The first key difference between these experiments is that livers analyzed in the present study have variable and notably longer cold ischemic

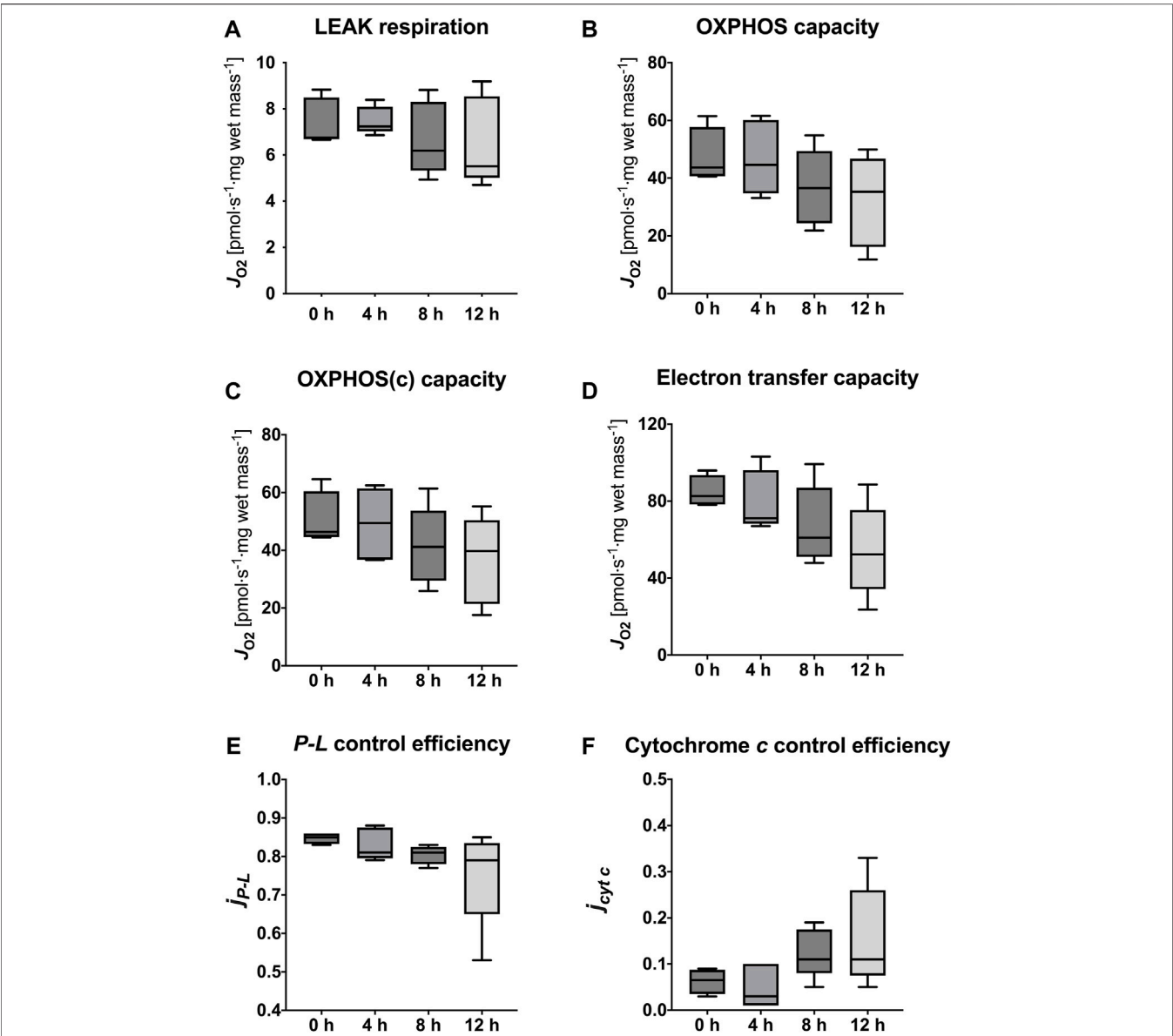


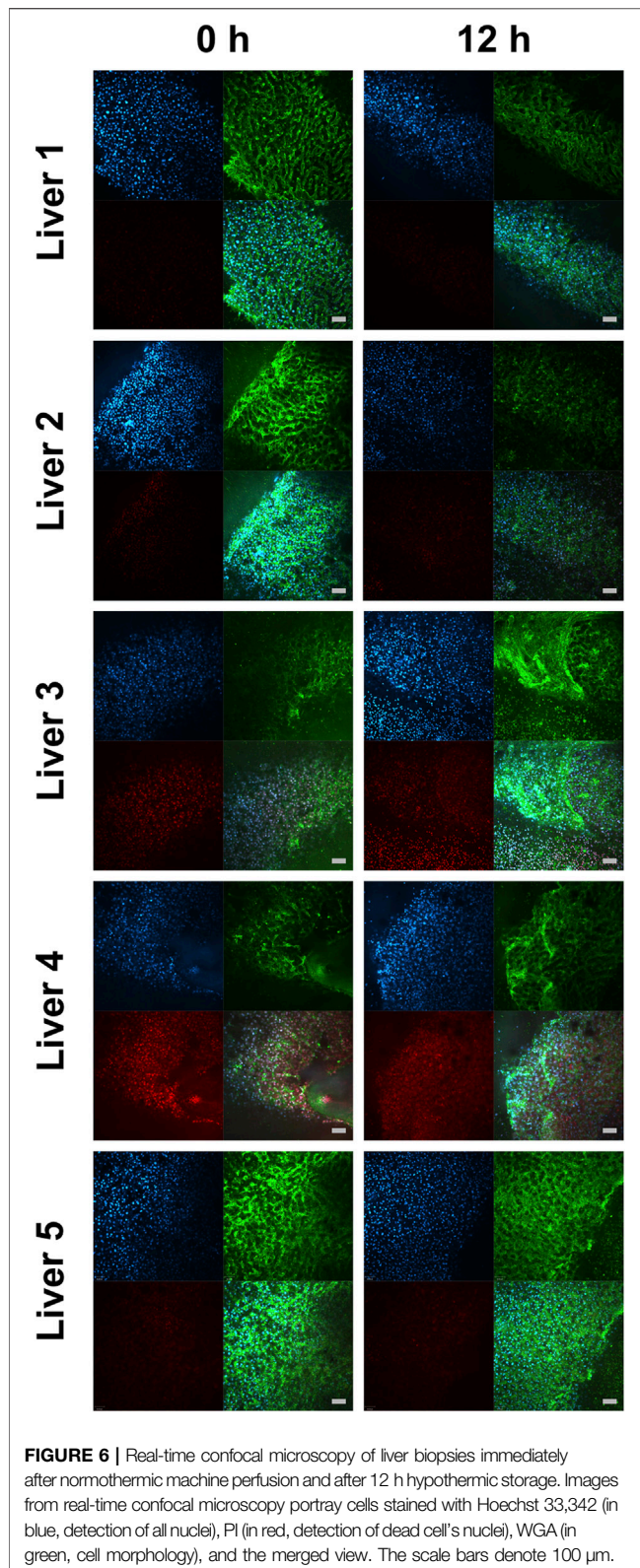
FIGURE 5 | Mitochondrial respiratory states and coupling control efficiencies. LEAK respiration (A), OXPHOS capacity (B), OXPHOS(c) capacity (C), Electron transfer capacity (D), P-L control efficiency (E) to evaluate efficiency of ATP production, and cytochrome c control efficiency (F) to evaluate integrity of the outer mitochondrial membrane. Data are represented as median, interquartile range and min-max. Friedman’s test with Dunn’s multiple comparisons was applied to assess differences compared to 0 h samples. No significant differences were found ($p < 0.05$).

TABLE 4 | Coupling control efficiencies of the succinate-linked pathway.

Coupling control efficiencies	0 h	4 h	p-value	8 h	p-value	12 h	p-value
P-L control efficiency	0.85 [0.83–0.86]	0.81 [0.80–0.88]	>0.999	0.81 [0.78–0.83]	0.150	0.79 [0.65–0.84]	0.112
Cytochrome c control efficiency	0.07 [0.04–0.09]	0.03 [0.01–0.10]	>0.999	0.11 [0.08–0.18]	0.199	0.11 [0.08–0.26]	0.112

Data are represented as median, interquartile range and min-max. Friedman’s test with Dunn’s multiple comparisons was applied to assess differences compared to 0 h samples.

times and have undergone reperfusion in the form of NMP, while in the previous study no machine perfusion was applied. This finding is in line with a recent study of Horváth *et al.* [22]. They investigated the effect of prolonged CIT and subsequent normothermic isolated perfusion on mitochondrial function in DCD rat livers. Mitochondrial respiration was decreased after



24 h of additional CIT compared to the control group, although in their setting no oxygen carrier was applied, which negatively affects mitochondrial metabolism under normothermic

conditions [23]. Furthermore, it has already been shown in small animal models that NMP leads to altered metabolomic signatures in liver tissue which affect mitochondrial biochemical pathways, such as reduced succinate levels. This holds also potential for using different perfusion models and various optimizing supplements to positively influence liver metabolism during NMP [24, 25]. The second major difference is the quality of the organs. Healthy porcine livers without preexisting damage seem to be less sensitive to mitochondrial damage when compared to human livers coming from aged, ECD and/or DCD donors. While in our recent study no decrease in mitochondrial respiration during the first 6 h of NMP was found in the overall cohort, individual changes in some livers could be demonstrated.

While the decreased respiration in biopsies at both the 8 h and 12 h HTS preservation did not reach statistical significance, the trends are clear, and this nonetheless underscores a potential threat of prolonged HTS following NMP. The decrease in mitochondrial function after reaching 8 h of HTS preservation could be attributed to several underlying factors such as the accumulation of metabolic byproducts, disruption of cellular homeostasis and ion gradients, oxidative stress, and consequent alterations in mitochondrial membrane integrity [1, 11]. This level of degradation at 8 h may warrant further exploration to better understand the mechanisms involved and potentially improve preservation methods. It is noteworthy that both the RTCM and the histopathological examinations did not show any appreciable differences between samples at 0 and 12 h of HTS.

Our study acknowledges certain limitations that pertain to both HRR and the organ quality. The reproducibility of HRR measurements can be influenced by sample variability, experimental conditions, technical factors, and from the measurement process itself, often influenced by the individual conducting the measurements. This can impact accuracy of the results, highlighting the importance of standardized protocols, training, quality control, and minimizing inter-operator variability [26]. In addition, the relatively small sample number of $N = 5$ livers and single-center design, indicate a possible frailty, which needs to be proven by further centers. While the stability of mitochondrial respiration up to 4 h upon sample collection in an NMP setting could be demonstrated, the actual clinical outcome could not be predicted in the 4 h samples as 4 of 5 livers have been rejected during NMP, based on conventional organ quality criteria.

In summary, this study accentuates the value of HRR in assessing the bioenergetic efficacy of livers during cold storage and the dynamics of mitochondrial stability and function during static cold storage following NMP. HTS of tissue biopsies of livers undergoing NMP can be performed for no longer than 4 h. While further research is needed, our findings underscore the potential of this simple method as a tool for informed decision-making during evaluation for organ transplantation, with the added benefit of easing the logistic challenges of HRR and facilitating concurrent assessment of a larger number of biopsies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the corresponding author upon request.

ETHICS STATEMENT

The studies involving humans were approved by the Ethics Commission of the Medical University of Innsbruck. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JH: study design, data acquisition, data analysis, interpretation of the data, drafting; AK: study design, data acquisition, data analysis, interpretation of the data, drafting; MS: study design, data analysis, and revising the manuscript; MLB: data analysis, and revising the manuscript; MH: data analysis, interpretation of the data, and revising the manuscript; BZ: data analysis, interpretation of the data, and revising the manuscript; DÖ: funding acquisition, revising the manuscript; RO: revising the manuscript, analysis and interpretation of clinical data, TH: revising the manuscript; SS: funding acquisition, revising the manuscript; ATM: study design, data analysis, interpretation of the data, drafting and revising the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontierspartnerships.org/articles/10.3389/ti.2024.12787/full#supplementary-material>

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GLOSSARY

ATP	adenosine triphosphate
CCCP	Carbonyl cyanide p-trifluoromethoxyphenyl hydrazone
CIT	cold ischemia time
CVA	cerebrovascular accident
DBD	donation after brain death
DCD	donation after circulatory death
DRI	donor risk index
ECD	extended criteria donor
ET	electron transfer
F	FAO-linked pathway
FAO	fatty acid oxidation
FCR	flux control ratio
FMN	flavin mononucleotide
HRR	high-resolution respirometry
HTS	hypothermic storage
IQR	interquartile range
L	LEAK respiration
LEAK	the state of non-phosphorylating resting respiration in the absence of ADP
LT	liver transplantation
N	NADH-linked pathway
NADH	nicotinamide adenine dinucleotide hydrogen
NMP	normothermic machine perfusion
OXPHOS	oxidative phosphorylation
P	OXPHOS: Respiratory capacity of mitochondria in the ADP-activated state of oxidative phosphorylation, at saturating concentrations of ADP and inorganic phosphate, oxygen, and defined substrates
PI	propidium iodide
ROX	residual oxygen consumption
RTCM	real-time confocal microscopy
S	succinate-linked pathway
SUIT	Substrate-Uncoupler-Inhibitor Titration
WGA	wheat germ agglutinin



Machine Perfusion Enables 24-h Preservation of Vascularized Composite Allografts in a Swine Model of Allotransplantation

Marion Goutard^{1,2,3†}, Pierre Tawa^{1,2,3†}, Yanis Berkane^{1,2,3,4,5}, Alec R. Andrews^{1,2,3}, Casie A. Pendexter^{2,3,6}, Reinier J. de Vries^{2,3,6,7}, Victor Pozzo^{1,2,3}, Golda Romano^{1,2,3}, Hyssem H. Lancia^{1,2,3}, Irina Filz von Reiterdank^{1,2,3,6,8}, Nicolas Bertheuil^{4,5}, Ivy A. Rosales^{9,10}, Ira Doressa Anne L. How^{9,10}, Mark A. Randolph^{1,2,3}, Alexandre G. Lellouch^{1,2,3}, Curtis L. Cetrulo Jr.^{1,2,3†} and Korkut Uygün^{2,3,6*†}

¹Division of Plastic and Reconstructive Surgery, Massachusetts General Hospital, Boston, MA, United States, ²Harvard Medical School, Boston, MA, United States, ³Shriners Children's Boston, Boston, MA, United States, ⁴Suivi Immunologique des Thérapeutiques Innovantes Laboratory, INSERM U1236, University of Rennes 1, Rennes, France, ⁵Department of Plastic, Reconstructive, and Aesthetic Surgery, Centre Hospitalier Universitaire de Rennes, Université de Rennes 1, Rennes, France, ⁶Center for Engineering in Medicine and Surgery, Massachusetts General Hospital, Boston, MA, United States, ⁷Department of Surgery, Amsterdam University Medical Centers—Location AMC, University of Amsterdam, Amsterdam, Netherlands, ⁸University Medical Center Utrecht, Utrecht University, Utrecht, Netherlands, ⁹Immunopathology Research Laboratory, Center for Transplantation Sciences, Massachusetts General Hospital, Boston, MA, United States, ¹⁰Department of Pathology, Harvard Medical School, Boston, MA, United States

OPEN ACCESS

*Correspondence

Korkut Uygün,

✉ kuygun@mgh.harvard.edu

[†]These authors share first authorship

[‡]These authors share senior authorship

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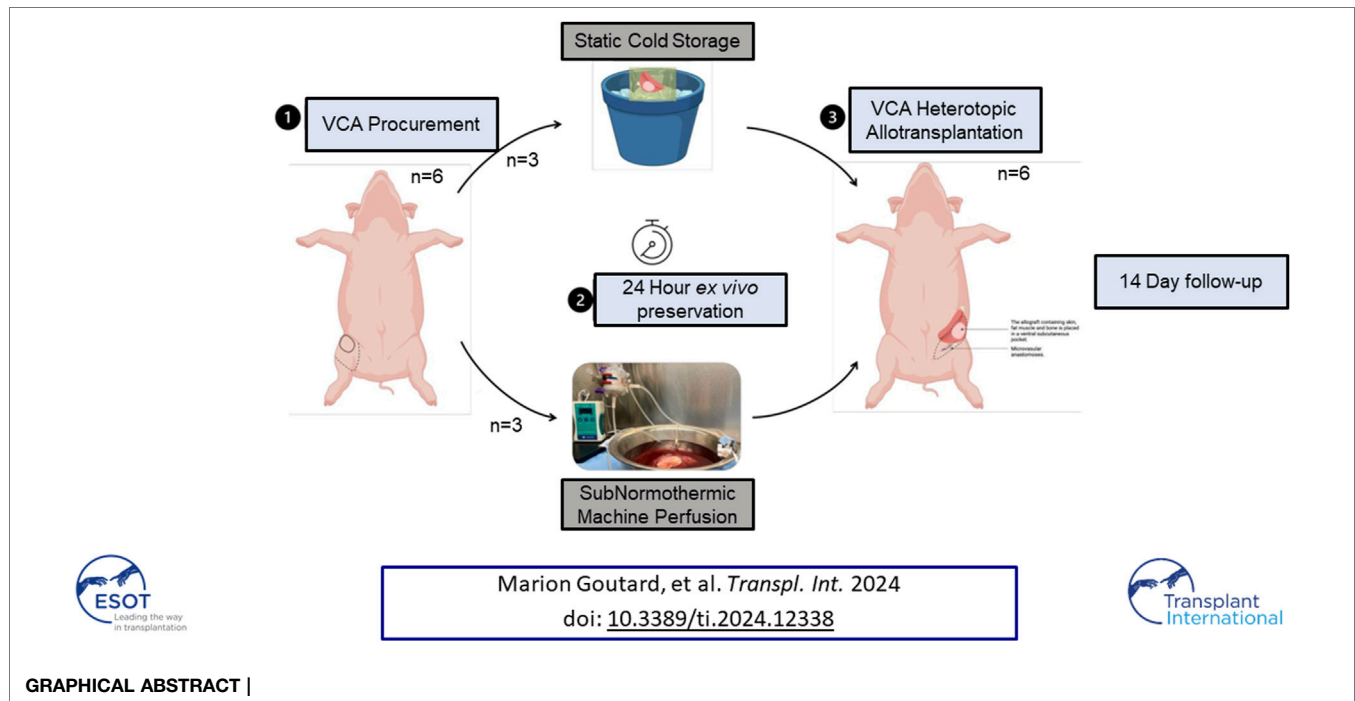
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The current gold standard for preserving vascularized composite allografts (VCA) is 4°C static cold storage (SCS), albeit muscle vulnerability to ischemia can be described as early as after 2 h of SCS. Alternatively, machine perfusion (MP) is growing in the world of organ preservation. Herein, we investigated the outcomes of oxygenated acellular subnormothermic machine perfusion (SNMP) for 24-h VCA preservation before allotransplantation in a swine model. Six partial hindlimbs were procured on adult pigs and preserved *ex vivo* for 24 h with either SNMP ($n = 3$) or SCS ($n = 3$) before heterotopic allotransplantation. Recipient animals received immunosuppression and were followed up for 14 days. Clinical monitoring was carried out twice daily, and graft biopsies and blood samples were regularly collected. Two blinded pathologists assessed skin and muscle samples. Overall survival was higher in the SNMP group. Early euthanasia of 2 animals in the SCS group was linked to significant graft degeneration. Analyses of the grafts showed massive muscle degeneration in the SCS group and a normal aspect in the SNMP group 2 weeks after allotransplantation. Therefore, this 24-h SNMP protocol using a modified Steen solution generated better clinical and histological outcomes in allotransplantation when compared to time-matched SCS.

Keywords: vascularized composite allotransplantation, machine perfusion, organ preservation, ischemia-reperfusion injuries, VCA

Abbreviations: ACURO, Animal Care and Use Review Office; EOS, End of the Study (euthanasia); H&E, Hematoxylin and Eosin; HISS, Histology Injury Scoring System; HTK, Histidine-tryptophan-ketoglutarate (solution); IACUC, Institutional Animal Care and Use Committee; MP, Machine Perfusion; POD, Postoperative Day; SCS, Static Cold Storage; SNMP, Subnormothermic Machine Perfusion; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; UW, University of Wisconsin (solution); VCA, Vascularized Composite Allograft.



INTRODUCTION

Traumatic injuries (ballistic, high-energy motor vehicle accidents, burns, etc.), congenital diseases, and neoplasia are responsible for severe disfigurements and amputation of functional aesthetic units in patients of all ages. For cases where autologous reconstructive options have been exhausted, vascularized composite allograft (VCA) transplantation offers significant quality of life improvements [1]. Although immense progress has been made in VCA procurement techniques, graft adjustments to the recipient, and post-operative care to achieve optimal results [2, 3], a more effective and longer graft preservation method is needed to make VCA transplantation a routine reconstructive procedure.

The method used for *ex vivo* preservation is a key factor in post-transplantation graft function and rejection risks [4–6]. For about half a century now, the combination of University of Wisconsin (UW) solution (or equivalent), an organ bag, and an icebox have been used to store all organs from procurement to transplantation. This method was chosen as a gold standard for its simplicity and cost-effectiveness [7]. However, limitations for this approach are obvious considering that at +4°C tissue metabolism continues at ~10% of nominal levels, resulting in a maximum preservation time of 4–6 h as the tissue effectively slowly suffocates. Further, cold ischemia causes ongoing injury to the graft, adversely affecting post-operative graft viability and functional outcomes [5, 8, 9].

Hence, the field has been pursuing better technologies to overcome *ex vivo* preservation injury. Machine perfusion (MP) is a promising alternative since it allows dynamic and monitored *ex vivo* organ preservation by supplying allografts with nutrients and oxygen at concentrations tailored to meet specific metabolic needs of

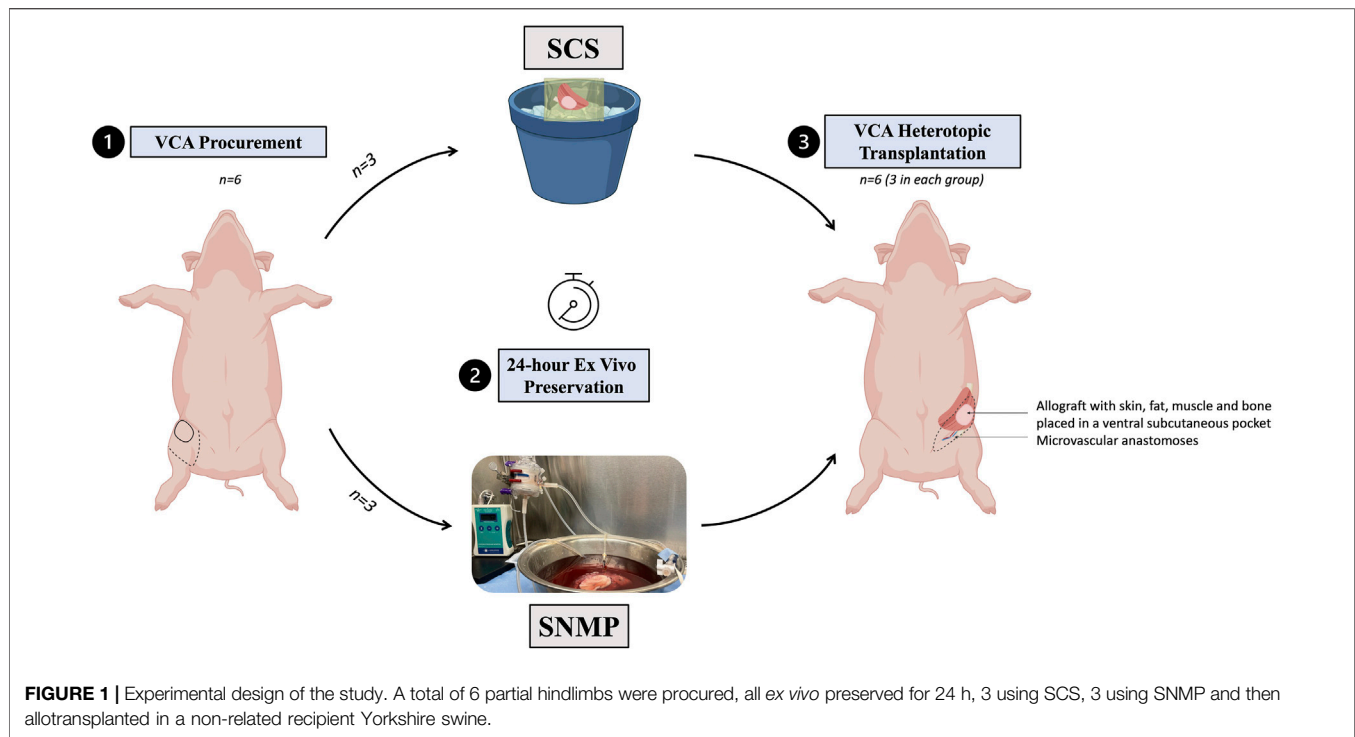
the tissue [10, 11]. Oxygenated MP was first optimized in solid organ transplantation and is now clinically used in kidney [12], liver [13], heart [14], and lung [15] transplantations. VCA *ex vivo* perfusion studies are rather recent in the literature and are still mostly confined to animal models. Swine limb models (forelimbs or hindlimbs) are frequently used for VCA preservation studies since they are easy to procure and contain common tissue types required for reconstructive procedures (skin, fat, muscle, nerve, tendon, and bone). Previous studies have suggested the superiority of different machine perfusion protocols in swine limb or myocutaneous flap preservation, when used either *ex vivo* [16–18] or *in situ* after replantation [19–21]. However, to our knowledge, no study reports the outcomes of *ex vivo* perfused VCAs after allotransplantation to assess the impact of immunological incompatibility between donor tissues and the recipient animals, which is a central component of clinical VCA procedures [22].

In this study we used a previously described heterotopic limb allotransplantation model in swine [23] to evaluate the possibility of enhanced *ex vivo* preservation of VCA using MP. Our work relied on 24-h *ex vivo* preservation of VCA using either SCS or subnormothermic machine perfusion (SNMP, +21°C), demonstrating that SNMP preserves VCAs for up to 24 h before allotransplantation, increasing the total viable duration for porcine partial hindlimbs 4–6 times beyond what can be achieved on ice.

MATERIALS AND METHODS

Animals

All animals received humane care in accordance with the National Institutes of Health Guide for the Care and Use of



Laboratory Animals. The MGH Institutional Animal Care and Use Committee (IACUC—protocol 2019N000176) and Department of Defense Animal Care and Use Review Office (ACURO) approved the animal protocols used in this study.

Study Design

This study was designed to compare two *ex vivo* preservation methods in VCA (Figure 1). A total of 12 female outbred Yorkshire pigs were used for all experiments: 6 served as donors for partial hindlimb procurement and 6 were allograft recipients, split equally between the control group of 24-h SCS (group SCS, $n = 3$) and the experimental group of 24-h SNMP (group SNMP, $n = 3$). Recipients and donors were randomly allocated to their group. Donors and recipients were not related in either group. The primary endpoint was the recipient animal survival at end of study (postoperative day 14).

Partial Hindlimb Procurement

All limb procurement surgeries were non survival procedures under general anesthesia, the animals were humanely euthanized at the end of the surgical procedure according to the American Veterinary Medical Association guidelines for animal euthanasia. Partial hindlimbs consisting of the distal femur, knee-joint, tibia-fibula, surrounding muscles, and a skin paddle vascularized through the femoral artery and vein were procured according to a previously described surgical protocol [23, 24]. An intravenous (IV) injection of 100 IU/kg heparin in the osteomyocutaneous allograft was performed 5 min before ligation of the femoral vessels with 2-0 silk ties just below the inguinal ligament. The femoral artery was then cannulated with a 14 or 16 G angiocatheter (BD Angiocath, Franklin Lakes, NJ,

United States) and the partial limb was flushed with 100 mL heparinized saline before being subjected to 24-h *ex vivo* preservation. Weight of the VCA and ischemia times were recorded for each limb.

Static Cold Storage (SCS)

Osteomyocutaneous flaps subjected to cold storage were flushed with 100 mL +4°C Histidine-tryptophan-ketoglutarate (HTK) solution (Custodiol, Essential Pharmaceuticals LLC, Durham NC, United States) and then immersed in a sterile bag containing 900 mL of cold HTK, transported on ice to a +4°C refrigerator. After 24-h, the allografts were removed from SCS, placed on a wet sterile gauze, and flushed with 100 mL of room temperature saline before being inset in the recipient animal.

Sub-Normothermic Machine Perfusion (SNMP)

Our continuous flow perfusion system was built in a sterile hood in a room at 21°C as displayed in Figure 2. A sterile container was filled with 2 L of modified Steen solution (Steen+) which presents a higher oncotic pressure than that of the original Steen® [25]. The system included a hollow-fiber membrane oxygenator (Affinity Pixie, Medtronic, Dublin, Ireland) Perfusion and a high precision peristaltic pump (Masterflex L/S, Cole Parmer, Vernon Hills, IL, United States). Perfusion was started an hour before VCA perfusion to oxygenate the circulating perfusate and bring the solution temperature to 21°C. A continuous flowrate of 0.5L/min carbogen (5% CO₂, 95% O₂) was used to ensure perfusate oxygenation. Before starting the experiment, the solution was tested for pO₂ and pH with an i-STAT machine

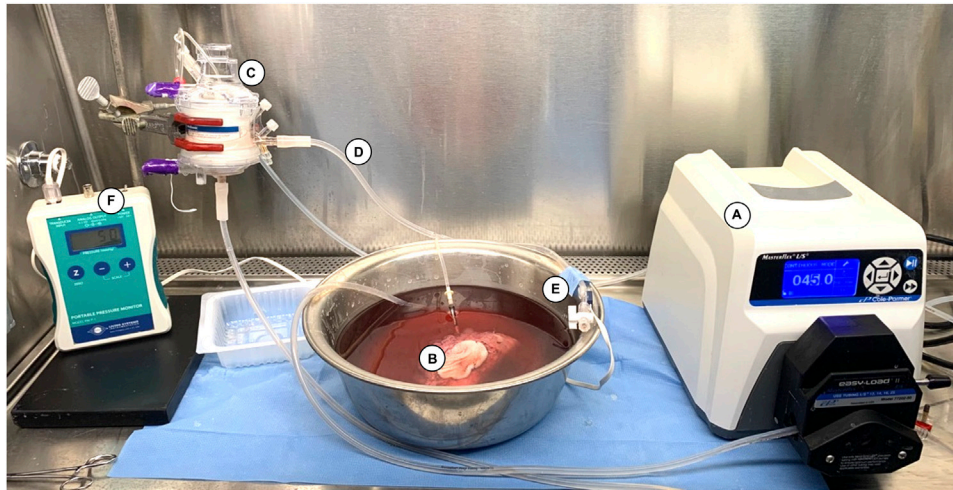


FIGURE 2 | Machine perfusion system used for 24-h SNMP. The system consists of (A) a roller pump (07522-20 DRIVE MFLEX L/S 600RPM 115/230, Cole-Parmer, Vernon Hills, IL, United States) delivering a continuous flow of Steen+ solution to (B) the VCA through an angiocatheter inserted in the femoral artery, (C) a hollow membrane oxygenator (Affinity Pixie™ Oxygenation System, Medtronic, Dublin, Ireland), and (D) sterile tubing (Masterflex® L/S Platinum-Cured Silicone Tubing, size 16, Cole Parmer, Vernon Hills, IL, United States). (E) A pressure transducer (PT-F, Living Systems Instrumentation, St Albans City, VT) was connected downstream to the inflow catheter and used to record the arterial pressure continuously on (F) the monitor (PM-P-1, Living Systems Instrumentation, St Albans City, VT, United States).

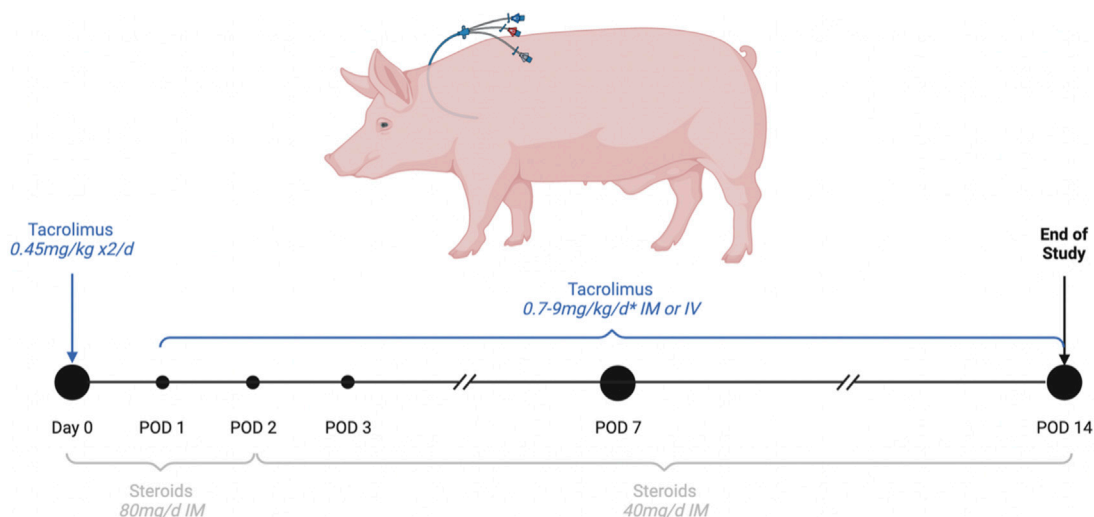


FIGURE 3 | Immunosuppression regimen. Drugs were given IV while the triple lumen central line was functional. *The targeted residual concentration of blood tacrolimus was between 0.4 and 0.6 ng/mL, blood concentration was measured every 2 days and the tacrolimus dose was adjusted to the tacrolimus trough level.

(Albott, Princeton, NJ, United States). A sodium bicarbonate 8.4% solution was used as a buffer to maintain a pH above 7.2 if necessary. The allografts were transported on ice to the machine perfusion setup. Once started, the first 50 mL of graft outflow were discarded to avoid accumulation of warm ischemia metabolites in the recirculating perfusate. One-liter (50%) perfusate exchange was performed after 12 h of SNMP. The flowrate was manually adjusted throughout perfusion to maintain an arterial pressure of 40 ± 5 mmHg as displayed by

continuous pressure monitoring in the closed system (PM-P-1, Living Systems, St. Albans, VT, United States). Pressure, flowrate, and metabolic parameters were measured at 0, 0.5, 1, 2, 3 h, then every 3 h during *ex vivo* perfusion. Circulating pH, pO_2 , pCO_2 , lactate levels, K^+ , Na^+ , HCO_3^- and glucose were tested from outflow and inflow perfusate samples with the iStat machine. Edema was estimated in percentage of weight gain (%) every 6 h (with no decannulation). All allograft manipulations were performed in sterile conditions.

Allotransplantation of Partial Hindlimbs Surgical Protocol

Recipient pigs were transplanted 24 h after VCA procurement under general anesthesia. The procedure started with isolation of the internal jugular vein through a 10 cm longitudinal incision in the neck and placement of a triple lumen vascular access line (Pressure Injectable Arrow[®] and Blue Plus[®] Three-Lumen CDC-45703-P1A) for later administration of IV drugs and blood draws. We followed a previously described method for VCA heterotopic transplantation in swine [23, 24]. In brief, an incision was made in the inguinal crease contralateral to the side of procurement and the femoral vessels were isolated and prepared for microvascular transfer. All other vessels outsourcing from the graft were ligated or coagulated to avoid leakage. When the allograft reached 24 h of *ex vivo* preservation, it was removed from the perfusion system or SCS and brought to the recipient site. The allograft was then placed in the ventral subcutaneous pocket and two vascular anastomoses (femoral artery, femoral vein) were performed (**Figure 1**). After observation of allograft revascularization, the surgical site was closed using absorbable sutures. Recipients were fully recovered within 2 h post-transplantation, singly housed, continuously monitored for the first 6 postoperative hours, and then twice daily. Recipient animals were humanely euthanized in case of graft failure, deteriorated general condition of the animal, or at the end of the study at day 14 post-transplantation. Upon euthanasia, allografts were thoroughly dissected before sampling.

Medications

Analgesia: A Fentanyl[®] patch delivering 50 µg/h was placed on the back on the animal preoperatively and for 72 h 4 mg/kg Carprofen[®] was administered per os (PO) for the first 72 h and more if needed.

Antibioprophylaxis: 50 mg/kg sulfamethoxazol-trimethoprim PO and enrofloxacin 5 mg/kg intramuscular (IM) were given daily for 14 days.

Immunosuppression (Figure 3**):** Tacrolimus was given once daily IV or IM to ensure a target residual blood concentration between 40 and 60 ng/mL. Steroids IV or IM were started at 80 mg daily and progressively tapered.

Postoperative Follow-Up

Recipient animals were monitored continuously during the first 6 h postoperatively. Physical examinations were then performed twice daily during the entire study to monitor signs of allograft rejection, allograft failure, or change in general condition. Blood draws were performed at 6 h, day 1, 3, 7, 10, and 14 post-transplantation for complete blood counts and assessment of potassium, sodium, and lactate blood levels. Tacrolimus residual blood concentration was measured on post-operative day (POD) 2 and every 48 h until levels were stabilized within the targeted therapeutic range (40–60 ng/mL).

Histology

Muscle biopsies were taken at 0, 6, 12, 18 and 24-h of *ex vivo* preservation and after transplantation at 1-h post-reperfusion and at the end of study. VCA skin punch biopsies (6 mm) were performed at 1-h post-transplantation, POD 1, 3, 7, 10 and 14. All

biopsy samples were fixed in formalin, paraffin embedded, sectioned and stained with hematoxylin and eosin (H&E), trichrome, and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling). All biopsies were evaluated by two independent pathologists blinded to the study using the Histology Injury Scoring System (HISS) for hypoxia-induced muscle injury [20], which include evaluation of the muscular architecture, structural myocyte injury and apoptosis. A Pathological Component Scoring System specific for skin-containing VCA and the 2007 Banff classification were used to grade skin components [26, 27].

Statistics

All statistical analyses were performed using Prism 9 for Mac OSX (GraphPad Software, La Jolla, CA). Population characteristics were compared using a Mann Whitney test. Comparison of categorical data was achieved using non-parametric tests. Mean histology scores were analyzed using a Sidak's multiple comparison test. *p* values less than 0.05 were considered significant.

RESULTS

VCA Characteristics

All procured VCAs had similar baseline characteristics (**Table 1**). Mean warm ischemia times (before and after *ex vivo* preservation) were comparable between both groups. Perfused VCAs gained an average of 15.5% weight after 24-h SNMP. The weight of cold-stored VCA remained stable with a mean loss of 0.94% weight after 24-h SCS, demonstrating no significant difference with the SNMP group (*p* = 0.10, non-parametric Mann-Whitney test).

Perfusion Outcomes

Perfusion parameters showed successful preservation by SNMP in all cases (SNMP-1, SNMP-2, SNMP-3). Flowrates (**Figure 4A**) remained stable in all cases between 35 and 50 mL/min with arterial resistances that approximated 1 mmHg/mL/min at the end of 24-h SNMP (**Figure 4B**). Weight gain was observed in all perfused VCAs (**Figure 4C**), staying below 25% after *ex vivo* perfusion. Potassium concentrations were measured within the physiologic range (3.5–5.5 mmol/L) at all timepoints (**Figure 4D**) and lactate levels were similar in all 3 perfused VCAs (**Figure 4E**).

Allotransplantation of Partial Hindlimbs After 24-h *Ex Vivo* Preservation

Post-Transplantation Clinical Observations and Survival

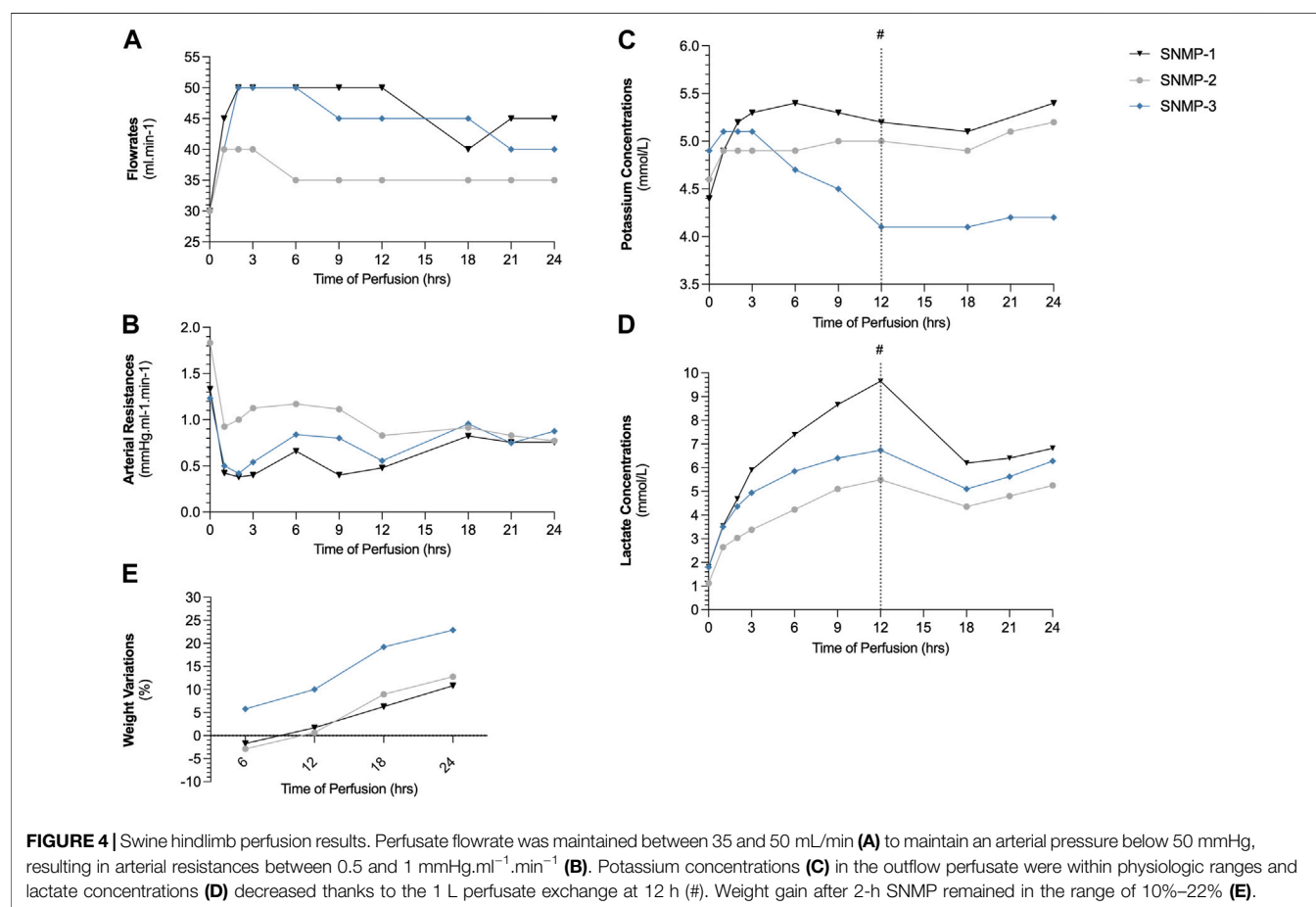
All animals from the SNMP group recovered from the allotransplantation surgery within 2 h, and were able to stand, walk and eat normally. None of the 3 recipient pigs of the SNMP group showed sign of pain or distress during follow-up and all remained on study through the end point on POD 14. In the SCS group, 2 out of 3 animals showed deteriorated general condition, loss of appetite and difficulty standing and walking. They were

TABLE 1 | Characteristics of VCA and ischemic conditions in each group.

	SCS group (N = 3)	SNMP group (N = 3)	p-Value
VCA weight after procurement (mean ± SD, g)	352.3 ± 26.6	350 ± 34	>0.99
VCA weight after ex vivo preservation (mean ± SD, g)	349 ± 25.7	403 ± 58.3	0.40
Weight variations with ex vivo preservation (mean ± SD, %)	-0.94 ± 0.66	+15.5 ± 6.48	0.10
Warm ischemia time before preservation (mean ± SD, min)	12.3 ± 4.1	14.7 ± 1.5	0.70
Warm ischemia time after ex vivo preservation ^a (mean ± SD, min)	105 ± 5.5	94.7 ± 10.4	0.20

There was no statistical difference between transplanted VCA in both groups ($p > 0.05$ using Mann-Whitney tests).

^aTime to perform inset of the allograft and vascular anastomoses.



subsequently euthanized at POD 5 and POD 9 after veterinarian examination (Figure 5A). There was no sign of allograft rejection during follow-up in either group under immunosuppressive therapy. In both groups, the VCA skin paddles appeared well vascularized for all recipients throughout the study (Figure 5B). No vascular failure of the VCA occurred during the study.

Biological Observations

The SCS group being reduced to only one surviving animal after POD 9, no statistical analysis was performed to compare the biological parameters between groups. In the SNMP group, the recipient pigs had stable hemoglobin level, potassium level and lactate level during follow-up (Figures 6A, C, D). Both

groups showed hyperleukocytosis immediately after transplantation that spontaneously resolved by POD 2 (Figure 6B). In the SCS group, abnormal values (low hemoglobin, increased potassium, and lactate levels) were observed on days of euthanasia, POD 5 and 9 (Figures 6A, C, D). Tacrolimus trough levels were in the targeted therapeutic range throughout the follow up period.

Macroscopic Observations and Histological Findings at End of Study

All SCS preserved allografts showed massive edema and seroma formation associated with macroscopically visible muscle degeneration even though vascular flow was respected. In this

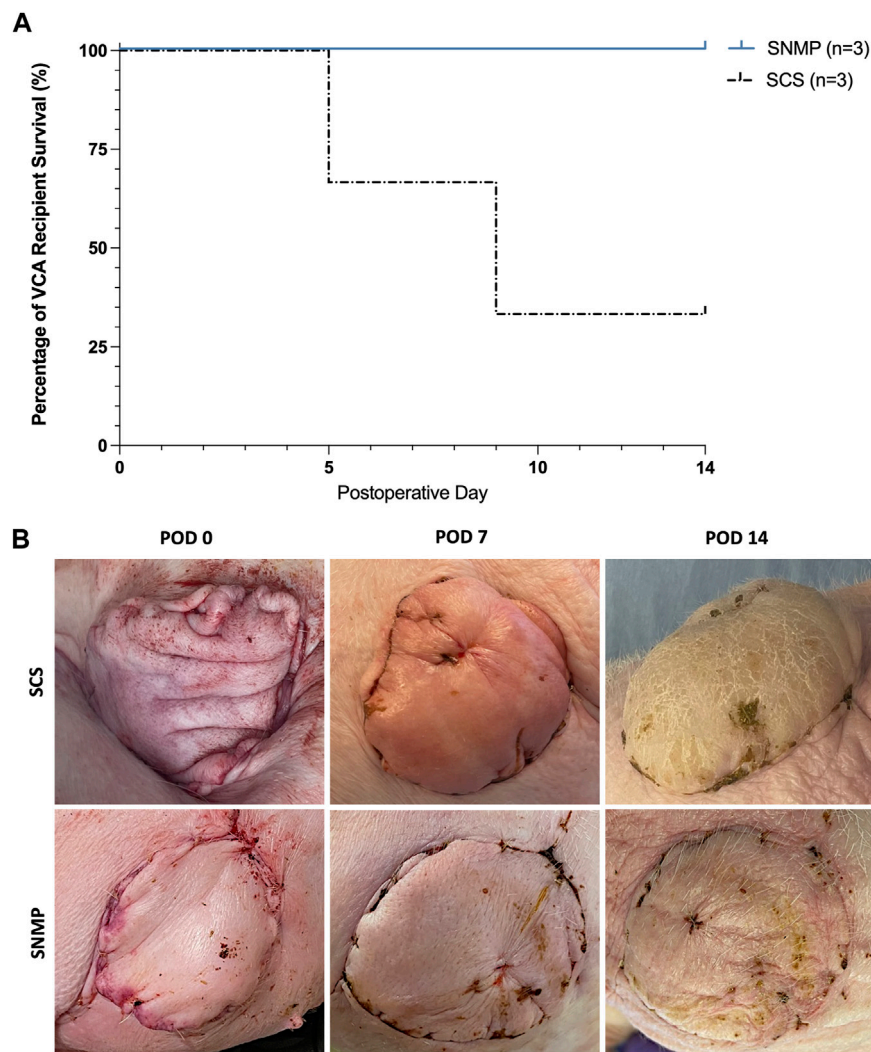


FIGURE 5 | 24 h SNMP leads to improved recipient outcomes compared to time-matched SCS. 2 out of 3 recipient animals were euthanized based on veterinary recommendation prior to the end of study in the SCS group, where all animals in the SNMP group were in normal condition **(A)**. Postoperative clinical aspect of the allografts was normal in the SNMP group whereas the SCS recipient that survived showed major allograft edema **(B)**.

group, allograft skin observations unveiled solely indirect signs of cutaneous suffering: mild epidermolysis and scarce hair regrowth. No ischemic change in skin color was observed. In the SNMP group, allograft skin were unremarkable and muscle aspect was macroscopically normal with good arterial inflow and venous outflow.

Skin biopsies performed after allotransplantation did not show significant rejection episodes, all samples were classified 0 on the Banff scale by the two blinded pathologists besides the SCS group at the euthanasia time point (Banff grade 1 [1, 2]). Detailed skin histology is displayed in **Supplementary Table S1**. End of study evaluation of the total histology score on muscle biopsies revealed a significant difference between groups (**Figures 7, 8**). A mean score of 3.167 was found in the SNMP group, ranking it in the no to minimal degeneration classification of the HISS, whereas a mean of 10.67 was obtained in the SCS group ($p = 0.034$ using a Sidak's multiple comparison test), marking it as severe degeneration outcome.

Histological subscores revealed differences in terms of inflammation, variations in myocytes shape and size, and damaged muscle fibers were always higher/worse in the SCS group (**Figure 8; Table 2**).

DISCUSSION

After several breakthrough clinical trials in solid organ preservation, machine perfusion is permeating the field of VCA, aiming to enhance preservation duration and allograft viability [7]. In this study, we demonstrated the superiority of preserving VCAs for 24 h with a SNMP protocol compared to conventional time-matched SCS in a porcine heterotopic limb allotransplantation model.

In previous reports, *in vivo* viability of VCA after machine perfusion preservation was assessed using replantation models with follow-up durations ranging from 12 h to 7 days. In 2017,

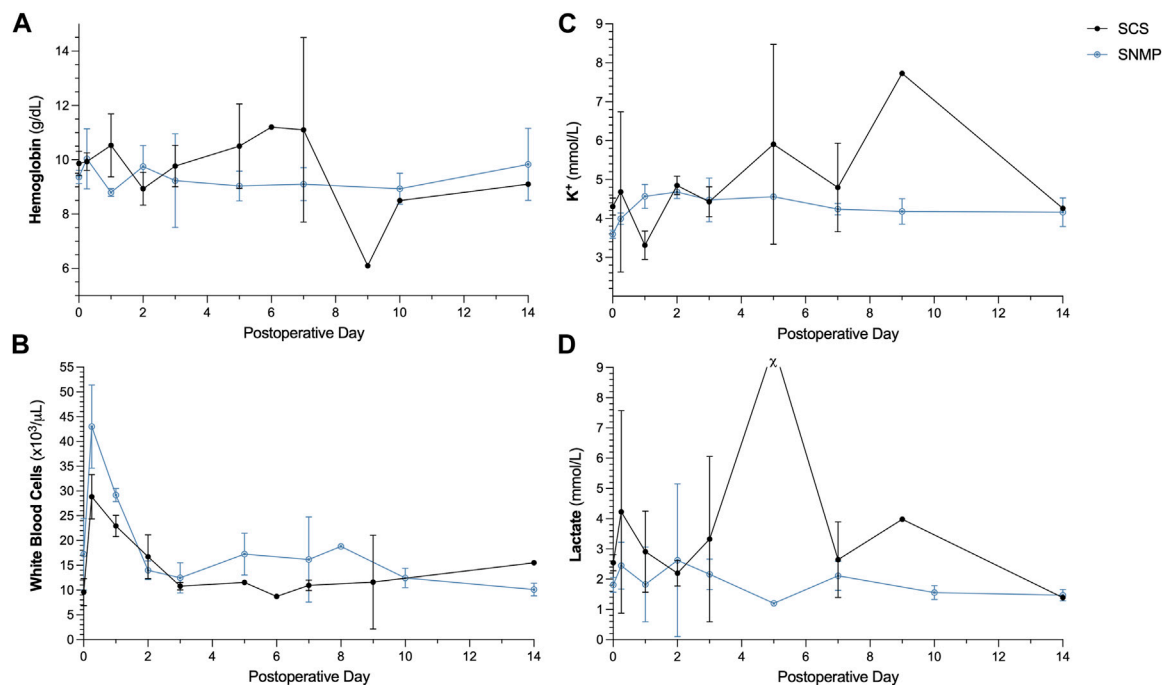


FIGURE 6 | Postoperative blood analyses in recipient animals. Hemoglobin (A), K⁺ (B) was similar in both groups. SNMP group displayed increased white blood cell count (C) in the immediate postoperative period which resolved spontaneously (B). In the SCS group a high lactate level (D) was observed before euthanasia of one the recipient animals on POD 5 (χ for value > 20 mmol/L which is beyond the range of the test).

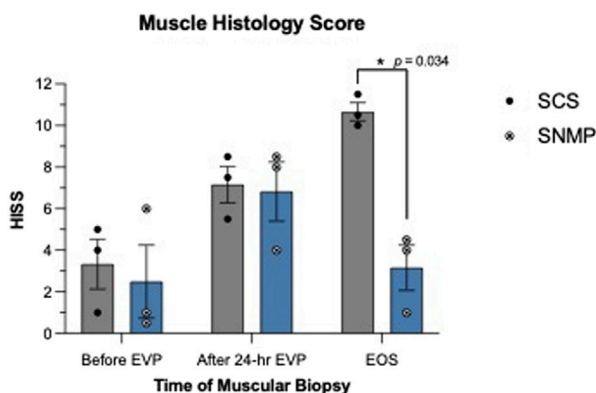


FIGURE 7 | Hypoxia-induced muscle injury score based on the Histology Injury Scoring System performed before and after *ex vivo* preservation. SNMP group was superior to SCS with minimal or no lesions.

Kueckelhaus et al. were the first to replant porcine forelimbs after 12 h of hypothermic perfusion [16]. They observed reduced ischemic injuries in perfused limbs compared to standard 4-h SCS, thus extending VCA preservation up to 3 times longer than the gold standard. Two years later, the same team extended the perfusion time to 24 h and validated *in vivo* viability with a similar replantation model [19]. More recently, Krut et al. replanted forelimbs after 18-h SNMP preservation and followed the recipient pig for 12 h to assess muscle function and histology compared to 4-h SCS preservation

[20]. Our findings were in line with these results, as we were able to preserve VCAs for 24 h in subnormothermic conditions with stable arterial resistances, moderate edema, and outflow potassium and lactate concentrations remaining in physiological values. Additionally, pigs that received perfused VCAs showed good clinical tolerance and did not suffer any systemic reaction, infection, or acute allograft rejection. In contrast, 2 out of 3 SCS control pigs developed poor general condition most likely due to graft failure (inflammation, rhabdomyolysis as suggested by high potassium levels). The lactate spike found in one of the SCS animals was considered resulting from hepatic and multi-organ failure resulting from graft failure. Pain management was considered optimal by experienced veterinarians. Complete necropsies were performed on all animals euthanized before POD14, and no alternative conditions such as sepsis or disseminated intravascular coagulation were found. Despite the lack of statistical significance, qualitative assessment of muscle histology in the SCS group showed major muscle degeneration, supporting the hypothesis of graft failure leading to the poor general condition of the recipient animals. However, graft failure was not clinically obvious given that there was no direct sign of graft ischemia and graft degeneration was concluded upon euthanasia dissection. Hence, delayed VCA failure can insidiously occur as late as POD9 with no or minimal skin lesions. This phenomenon was previously observed in rat hind limb transplantations after not 24 but 48 h of SCS. Interestingly, histological analyses of muscle biopsies showed no difference between SNMP and SCS immediately after *ex vivo* preservation but revealed a statistical difference at the end of study in favor of

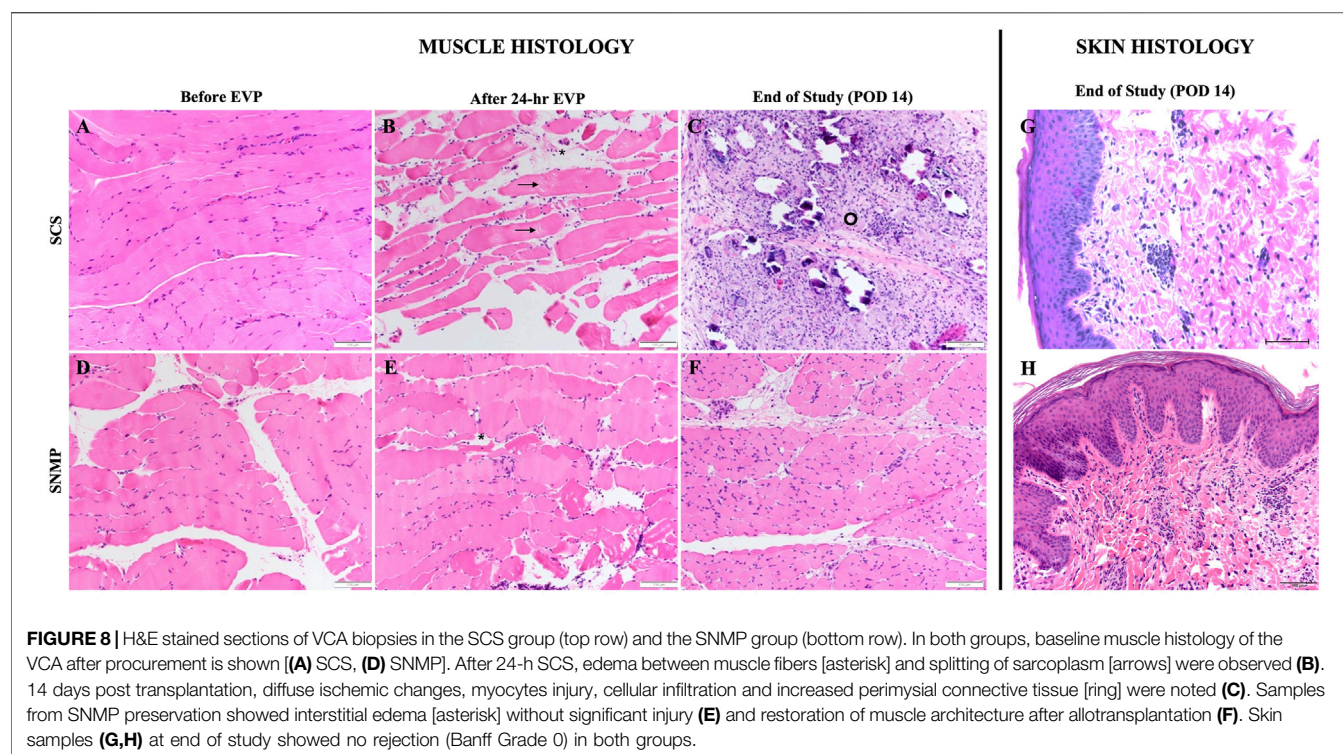


TABLE 2 | Detailed hypoxic-induced muscle injury score according to the Histology Injury Scoring System.

Hypoxic-induced muscle injury	Time of muscle biopsy	SCS group (n = 3)	SNMP group (n = 3)
Interstitial edema (median (min, max))	Before EVP	0 (0, 0)	0 (0, 3)
0. No significant increase	After 24-h EVP	3 (1, 3)	2 (2, 3)
1. Minimal	EOS	0 (0, 2)	0 (0, 1)
2. Intermediate			
3. Severe/diffuse			
Inflammation [median (min, max)]	Before EVP	0 (0, 1)	0 (0, 0)
0. Not significant	After 24-h EVP	0 (0, 0)	0 (0, 1)
1. Minimal	EOS	3 (2, 3)	0 (0, 0)
2. Intermediate			
3. Diffuse			
Variation in myocytes shape and size (median (min, max))	Before EVP	0 (0, 1)	0 (0, 1)
0. Homogeneous	After 24-h EVP	1 (1, 3)	2 (1, 3)
1. Mild heterogeneous	EOS	3 (3, 3)	0 (0, 1)
2. Intermediate heterogeneous			
3. Severe heterogeneous			
Damaged muscle fibers (median (min, max))	Before EVP	1 (0, 1)	0 (0, 2)
0. 0–5 myocytes/10 hpf (×20 magnification)	After 24-h EVP	1 (1, 3)	2 (2, 3)
1. 6–20 myocytes/10 hpf (×20 magnification)	EOS	3 (3, 3)	0 (0, 0)
2. 21–50 myocytes/10 hpf (×20 magnification)			
3. >51 myocytes/10 hpf (×20 magnification)			

EVP, Ex Vivo Preservation; EOS, End of the Study.

The bold values correspond to the values of most interest (End of the study time points).

SNMP, underlining the importance of *in vivo* analysis of the allograft in a longer follow up period than previously described. Our hypothesis is that reperfusion injuries slowly deteriorate muscular cells leading to extended myocyte apoptosis and microvascular damage, viciously killing the muscle tissue and releasing necrosis

marker in the systemic circulation although those markers have not been investigated in this study.

Protocols based on Subnormothermic perfusion appear to strike a balance in temperature. Hypothermic machine perfusion has generally led to satisfactory outcomes in VCA preservation [16,

20, 28] without additional oxygen carriers [28]. However, cooling VCAs down to 8°C–10°C appears to result in vascular injuries and subsequent higher interstitial edema (considered clinical revelation of vascular leakage induced by endothelial damage during *ex vivo* storage), especially after 24 h of *ex vivo* perfusion. Krezdorn et al. reported an average weight gain of +41% of after 24 h of hypothermic perfusion [19]. Similarly, Kruit et al. reported +18.6% of weight variation after 18 h of hypothermic perfusion [20], while we were able to limit edema formation to an average of +15% weight for all limbs after 24 h of SNMP. These results are also in line with previous studies on SNMP organ preservation [25, 29], where SNMP was used as an organ reconditioning phase after an ischemic period and prior to transplantation. However, we did not directly compare SNMP with different temperatures in this study. A direct comparison utilizing the same protocols and models are needed to draw further conclusions and develop a mechanistic understanding of how perfusion temperature effects this very complex tissue. Low-temperature preservation has the potential to mitigate cell metabolism, therefore suggesting longer preservation durations [30]. Subzero preservation techniques seem to be promising alternatives for multi-day preservation of solid organs [31–35], but solid evidence still needs to be demonstrated in VCA. In the realm of Normothermic Machine Perfusion (NMP), Fahradyan et al. [36] demonstrated 12-h preservation of porcine VCAs with preserved weight and muscle contractility. They suggested that extending normothermic red blood cell perfusion to 24 h could be feasible, albeit with limited significant evidence *versus* SCS controls. A pioneering work from Werner et al. [37] in fresh human arms demonstrated encouraging results of 24-h near-normothermic (30°C–33°C) perfusion. This sub-physiologic protocol however imposed using a blood-derived perfusate for adequate oxygen transportation. In contrast, the use of subnormothermic “room” temperatures (20°C–25°C) in machine perfusion offers several advantages. First, it simplifies the perfusion device setup, eliminating the need for a heating or cooling system [29, 38]. Second, it allows clinicians to reach a balance between oxygen needs and subdued tissue metabolism resulting in perfusate simplifications [28]. Perfusates that require red blood cells or oxygen carriers that are essential to normothermic “physiological” conditions are no longer needed to ensure adequate oxygenation of the composite tissue at +20°C, thus eliminating the potential immunogenicity of such additional components [39].

In our experiments, all limbs were perfused with a modified acellular Steen solution [25] to minimize edema. Fine-tuned control to maintain a low-pressure circuit (around 30 mmHg) was also needed to mitigate edema—likely due to excessive endothelial shear stress at high pressures - using a continuous flow perfusion pump. Whether non-linear or continuous flow provides better outcomes in VCA machine perfusion still remains unclear [40, 41]. Our previous work demonstrated that non-linear flow has some advantages over continuous flow with improved endothelial function and decreased ischemic injury, although the differences were limited [41]. In the present study we utilized continuous flow for practical reasons.

Allotransplantation is only rarely performed in machine perfusion studies, likely due to cost-related issues. To our knowledge, this study is the first to report allotransplantation of

perfused VCA in swine with a 2-week follow up. Our laboratory has been extensively working on VCA in miniature swine to implement new tolerance induction protocols in VCA, rising our expertise in immunosuppressants in swine [42, 43]. Consequently, our experience led us to use a bitherapy made of tacrolimus and steroids. As a matter of fact, on short term studies, the immunogenic risk is mostly confined to acute rejection episodes, which are usually mitigated with high-dose steroids [44].

A major limitation of this work was the small sample size ($n = 3$ per group). Performing allotransplantation instead of replantation to approximate clinical settings subsequently doubled the number of swine needed, thereby limiting the group sizes. While this limits the power of the statistical analysis, leading to increased risk of type-II errors, the SNMP group notably showed clear improved results including a higher recipient survival rate after transplantation, which in our opinion provided additional evidence to a growing VCA perfusion literature. We were also unable to assess limb function after transplantation with this surgical model. However, based on the positive results of this study, our SNMP protocol can be used in other swine VCA models to achieve this goal. Thus, one could argue that 24-h SCS is far more than what is usually the accepted limit for cold ischemia time in human clinical setting for VCA. Although, no studies have proven that swine VCA respond exactly as human tissues. Our previous experience in rat VCA preservation studies showed that rat VCA graft survival was similar after 6 and 24 h of SCS [25]. If graft injuries were evidently expected after 24 h SCS, it served as a negative control to our 24-h SNMP protocol. Since this work mainly aimed at testing the compatibility of 24-h SNMP with successful VCA allotransplantation, another limitation is the lack of focus on nerve histology and nerve conduction assessment, which should be specifically addressed in further studies.

Overall, we showed that a simplified SNMP protocol allowed 24-h VCA preservation in a swine allotransplantation model with confirmed tissue viability until 14 days after allotransplantation. Further studies should focus on assessing neuromuscular function in a long-term follow-up to match the clinical setting.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was approved by Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

Study design: MG, PT, YB, RdV, CP, KU, CC, MR, and AL. Performed study: MG, PT, YB, GR, VP, IF, and RdV. Collected data: MG, PT, and YB. Analyzed data: MG, PT, AA, HL, IH, and IR.

Important intelligent input: MG, PT, YB, RdV, AA, MR, NB, AL, CC, and KU. Manuscript preparation and corrections: all authors.

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AUTHOR DISCLAIMER

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the Department of Defense.

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CONFLICT OF INTEREST

KU served in the Scientific Advisory Board of Sylvatica Biotech Inc., which focuses on developing high subzero organ preservation technology. KU, CC, RJV and AL have patent applications relevant to this study. Competing interests for MGH investigators are managed by the MGH and MGB in accordance with their conflict of interest policies.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The work included in this manuscript was presented to the American Congress of Transplantation 2023 as an abstract.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontierspartnerships.org/articles/10.3389/ti.2024.12338/full#supplementary-material>

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Editorial Office

Avenue du Tribunal Fédéral 34
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Switzerland

Tel +41 (0)21 510 17 40
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