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Lung transplantation in the 21st Century: Innovative care for improved outcomes

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Lung transplantation in the 21st Century: innovative care for improved outcomes

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EDITORIAL
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Innovations in Lung Transplant Research and Practice: The Future is Now

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INTRODUCTION

Recent advancements in transplantation research and practice have focused on expanding the donor pool, developing novel biomarkers and diagnostic tools, and exploring innovative treatment opportunities. These efforts aim to address the persistent shortage of donor organs and improve long-term outcomes for transplant recipients.

With the coming of age of artificial intelligence (AI) in the 21st Century, it is expected that in the next decade medical transplant research - and its clinical implications - will exponentially lead to new discoveries, deepened insights, and better management of the logistical processes and disease mechanisms involved in transplantation. However, surprisingly none of the research papers included in our special issue of Transplant International on "*Lung transplantation in the 21st Century: innovative care for improved outcomes*" implemented the use of AI methodologies in their research. It is nevertheless expected that the "old-school" approach of performing scientific research is to be replaced by research practices incorporating AI tools to analyze, visualize, summarize, and present research findings. Is this a bad evolution? Probably - and hopefully - not, as proper use of AI may help to galvanize big data and complex research findings into more efficient logistical flows, better diagnostics, novel therapies, and more personalized treatment options in the field of transplantation.

The authors of the current editorial therefore opted to use a freely available AI tool (Perplexity AI, Inc., San Francisco, United States) to synthesize information regarding the sixteen scientific papers in the field of transplantation which were included in this special issue of Transplant International. The use of AI in medical reporting demonstrates its potential to efficiently aggregate and distill complex scientific information, potentially accelerating the dissemination of knowledge in rapidly evolving fields like transplantation medicine. However, it is crucial to note that AI-generated summaries should be reviewed and validated by subject matter experts to ensure scientific accuracy and contextual relevance. Post-generation, this summary was therefore manually checked and edited by the authors.

Editorial

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Abbreviations: A-NRP, abdominal normothermic regional perfusion; ACR, acute cellular rejection; AI, artificial intelligence; AMR, antibody-mediated rejection; cfDNA, cell-free DNA; CHS, controlled hypothermic storage; CSAI, clinically significant airway ischemia; CLAD, chronic lung allograft dysfunction; DBD, donation after brain death; DCD, donation after circulatory determination of death; dnDSAs, *de novo* donor-specific antibodies; DSBT, donor-specific blood transfusion; ECMO, extracorporeal membrane oxygenation; EVLP, *ex vivo* lung perfusion; PGD, primary graft dysfunction; TCMR, T-cell mediated rejection; UFH, unfractionated heparin.

Expanding the Donor Pool

Organ Preservation and Reconditioning

Considerable progress has been made in organ preservation techniques, moving beyond traditional static cold storage. Hoetzenecker et al. introduced the concept of semi-elective lung transplantation using prolonged static storage at 10°C, potentially expanding the geographical range for organ allocation [1]. The authors highlight that this method allows for prolonged cold ischemic times, up to 24 h, without compromising organ function or short-term outcomes. This technique has been validated through large animal experiments and a multi-center observational study. The 10°C preservation temperature has shown to cause less mitochondrial damage compared to traditional ice storage methods. This development could potentially transform lung transplantation logistics, enabling semi-elective procedures and improving organ sharing between distant regions.

Cenik et al. reviewed the principles of controlled hypothermic storage (CHS) for lung preservation in transplantation [2]. CHS allows preservation at temperatures higher than traditional ice storage, typically around 10°C. Animal experiments showed superior lung physiology after prolonged storage at 10°C compared to ≤4°C. Molecular analyses revealed better protection of mitochondrial health and higher levels of antioxidative metabolites with CHS. While initial clinical findings are promising and suggest a need for withdrawal from conventional ice-based method, further research is needed to draw more robust conclusions about the safety and efficacy of CHS in lung transplantation.

These studies highlight that novel organ preservation techniques may nevertheless help increase the number of viable donor organs and improve transplant outcomes soon.

Ex vivo lung perfusion (EVLP) has emerged as a promising technique for organ reconditioning. Chilvers et al. developed a split-lung *ex vivo* perfusion model, allowing for time- and cost-effective evaluation of therapeutic interventions in human donor lungs [3]. Their split-lung EVLP model that allows for the simultaneous perfusion and ventilation of two single lungs from the same donor. This offers several advantages: i) it provides a cost-effective and reliable platform for testing therapeutic interventions on human donor lungs, ii) the split-lung approach allows one lung to receive an intervention while the other serves as a control, eliminating inter-donor variation, iii) the model facilitates continuous monitoring of hemodynamic and airway parameters, as well as blood gas, perfusate, and tissue sampling, iv) pulmonary edema can be assessed directly using ultrasound and indirectly through lung tissue wet:dry ratio measurements, and v) this approach enables researchers to evaluate promising interventions more efficiently, potentially increasing the number of transplantable organs.

This new EVLP model could therefore accelerate research into organ preservation and reconditioning strategies.

Expanding Controlled Donation After Circulatory Determination of Death

Moreno et al. reported on lung transplantation in controlled donation after circulatory determination of death (cDCD) using abdominal normothermic regional perfusion (A-NRP) [4]. The authors present an update on cDCD lung donation, highlighting its potential to alleviate the shortage of transplantable lungs. They describe the Maastricht classification of DCD donors and emphasize that cDCD is the most accepted type for lung donation. The paper includes a step-by-step protocol for lung procurement using A-NRP, which is critical for achieving high retrieval rates. The authors also discuss donor selection criteria and the importance of adequate management in the intensive care unit.

This study highlights that increased use of cDCD with A-NRP may expand the donor pool.

Sandiumenge et al. compared systemic inflammation in brain dead (DBD) and DCD lung donors and its impact on lung transplant recipients [5]. The researchers measured plasma levels of cytokines IL-6, IL-8, IL-10, and TNF-α in 40 DBD and 40 DCD donors and their recipients. Results showed significantly higher levels of IL-6, IL-10, and IL-8 in DBD donors compared to DCD donors. Higher TNF-α levels in donors were associated with a higher incidence of primary graft dysfunction (PGD) in recipients.

The study highlights that DBD is associated with higher systemic inflammation than DCD, and higher donor TNF-α levels correlate with increased PGD incidence, which results may allow for tailored anti-inflammatory treatments to attenuate PGD, potentially improving patient outcomes.

Organ Allocation Practices

Shudo et al. evaluated the impact of the revised United Network for Organ Sharing heart allocation policy implemented in October 2018 on en-bloc heart-lung transplantation outcomes [6]. The researchers analyzed data from adult patients registered for heart-lung transplants before and after the policy change. Results showed significantly higher transplant rates, shorter waitlist times, and reduced waitlist mortality in the post-policy period. Despite higher-risk recipients in the post-period, short-term survival rates remained similar before and after the policy change.

The study highlights that the revised policy significantly improved access to en-bloc heart-lung allografts with better waitlist outcomes and similar post-transplant outcome.

Biomarkers and Diagnostic Tools

Donor-Derived Cell-Free DNA as Biomarker for Rejection

Novo et al. investigated the potential of cell-free DNA (cfDNA) as a non-invasive biomarker for predicting complications after lung transplantation [7]. The researchers analyzed 246 serum samples from 26 lung transplant recipients, focusing on chronic lung allograft dysfunction (CLAD). They used four different methods to measure donor fractions of cfDNA, including three digital droplet PCR applications and one method measuring absolute amounts of donor-derived cfDNA. The results showed statistically significant elevations of cfDNA in CLAD samples compared to non-CLAD samples across all four methods.

This study highlights the use of digital droplet PCR-detected cfDNA as a potential biomarker for predicting CLAD and

differentiating rejection from infection in lung transplant recipients.

Immunologic Biomarkers of Rejection

Auner et al. studied the clinical significance of transient and persistent *de novo* donor-specific antibodies (dnDSAs) in lung transplantation [8]. The researchers analyzed 405 lung transplant recipients, of whom 205 developed dnDSAs. They found that persistent, but not transient, dnDSAs were associated with CLAD and antibody-mediated rejection (AMR). Patients with persistent dnDSAs had significantly lower CLAD-free survival rates at 1-, 3-, and 5-year post-transplantation compared to those with transient dnDSAs.

The study highlights the importance of distinguishing between transient and persistent dnDSAs in predicting outcomes after lung transplantation and may guide future management, suggesting the need for prompt treatment of persistent dnDSAs.

Zajacova et al. compared histological and molecular diagnoses of lung transplant rejection, focusing on treatment responses [9]. The researchers analyzed 54 transbronchial biopsies from lung transplant recipients between 2015 and 2020. They found discrepancies between histological and molecular diagnoses in 54% of cases. Patients with molecular T-cell mediated rejection (TCMR) showed a significantly higher treatment response rate (50%) compared to those with no rejection (14%).

The study findings suggest that low-grade acute cellular rejection (ACR) may not always correspond with molecular TCMR, indicating that molecular diagnosis could better identify patients who would benefit from anti-rejection therapy.

Novysedlak et al. identified elevated PD-L1 and PECAM-1 as potential diagnostic biomarkers of ACR in lung transplantation [10]. The researchers observed a significant increase in PD-L1 tissue expression within the ACR group, suggesting an attempt to suppress immune responses. PECAM-1 levels were also elevated in cases of ACR. The findings indicate that both PD-L1 and PECAM-1 could serve as valuable markers for diagnosing ACR in lung transplant recipients.

This research may contribute to the development of more accurate diagnostic tools for identifying rejection in lung transplantation, potentially improving patient outcomes.

Treatment Opportunities

Peri-Operative Considerations

Vaiter et al. investigated the effects of lower doses of unfractionated heparin (UFH) for intraoperative extracorporeal membrane oxygenation (ECMO) anticoagulation [11]. The researchers analyzed 109 lung transplant patients who underwent central VA ECMO support between 2020 and 20223. They found that lower UFH doses led to reduced intraoperative blood derivative consumption and blood loss without increasing thrombotic complications. The study also suggests that lower UFH doses may decrease the incidence of surgical revision for hemothorax.

The study highlights that using lower doses of UFH for intraoperative ECMO anticoagulation during lung transplantation might reduce complications and lead to better outcomes.

Li et al. investigated risk factors, incidence, and outcomes associated with clinically significant airway ischemia (CSAI) in lung transplant recipients [12]. The researchers reviewed 217 lung transplants performed between 2016 and 2020, finding that 37.8% of patients developed CSAI. Risk factors for CSAI included recipient diabetes, intraoperative ECMO use, and single running suture technique. Patients with CSAI, particularly those who developed dehiscence or stenosis, had lower survival rates compared to those without CSAI.

The study highlights the importance of mitigating risk factors, identifying and managing CSAI to improve outcomes in lung transplant recipients.

Palleschi et al. reviewed the complex relationship between the diaphragm and lung transplantation [13]. The authors discuss how several factors before transplantation, including underlying respiratory diseases and comorbidities, can impact diaphragmatic function. They highlight that the surgical procedure itself can cause trauma to the diaphragm, potentially leading to morphological and functional alterations. Conversely, the diaphragm influences aspects of lung transplantation, from graft-to-chest cavity size matching to long-term postoperative respiratory performance.

The review emphasizes the need for careful dissection during the lung transplant procedure to avoid trauma to the phrenic nerve and diaphragm, but also the lack of standard criteria for evaluating and managing diaphragmatic dysfunction in lung transplantation, which hinders accurate assessment of outcomes.

Sempere et al. investigated systemic absorption of inhaled tobramycin in lung transplant recipients [14]. The researchers conducted a retrospective analysis of adult patients treated with inhaled tobramycin for at least 3 days. The primary indications for treatment were donor bronchial aspirate bacterial isolation (18 patients) and tracheobronchitis (15 patients). Key findings include: i) 82% of patients had detectable serum tobramycin levels, with 26% showing elevated levels (>2 μg/mL), ii) 26% of patients developed acute kidney injury during treatment, and iii) invasively mechanically ventilated patients had significantly higher median trough tobramycin levels compared to non-ventilated individuals.

The study concludes that inhaled tobramycin administration in lung transplant recipients, especially in those on invasive mechanical ventilation, may result in substantial systemic absorption, which is important to consider in the early post-transplant phase.

Immunomodulation and Tolerance Induction

Jin et al. reviewed the use of donor-specific blood transfusion (DSBT) in lung transplantation as a potential strategy for inducing immunological tolerance [15].

DSBT involves infusing fresh whole blood from the donor to the recipient before transplantation, aiming to improve graft acceptance and potentially induce donor-specific tolerance. The review summarizes existing knowledge on DSBT mechanisms and outcomes in solid organ transplants, including preclinical and clinical settings. It explores associations with regulatory T cells, mononuclear phagocytic cell modulation, and microchimerism. The authors also

discuss potential benefits and risks of DSBT in lung transplantation, offering insights for future research directions.

The review highlights that this approach, if successful, could help reduce the need for long-term immunosuppression and its associated complications.

Messika et al. reviewed the diagnosis and therapeutic armamentarium for AMR in lung transplantation [16]. The authors highlight the importance of identifying DSA and their association with various forms of rejection. The review explores current diagnostic methods and therapeutic approaches for AMR, including desensitization techniques and targeting the complement cascade. It also emphasizes the use of combined strategies such as immune cell depletion, immune pathway inhibition, and inflammatory cascade modulation.

The review highlights that these innovative techniques offer promising perspectives for lung transplant recipients facing this challenging complication.

Stem Cell Therapies, Regenerative Medicine, and Xenotransplantation

While not explicitly covered in the provided papers, stem cell therapies, regenerative medicine and xenotransplant approaches represent promising avenues for future research and treatment practices in transplantation, potentially offering new ways to repair or replace damaged organs.

CONCLUSION

In conclusion, recent advances in transplantation research and practice demonstrate a multifaceted approach to addressing the challenges of organ shortage and improving long-term outcomes, with promising developments in organ preservation, biomarker discovery, and immunomodulation strategies. Also, the use of AI in medical reporting has an immense potential to efficiently aggregate and distill complex scientific information, as demonstrated herein. Moreover, incorporation of AI tools in research methods may radically shape the 21st Century of (lung) transplant medicine, as is already evidenced by the steep increase in the number of scientific publications reporting on AI in transplant research since 2020 [17]. Hence, in the coming years AI is expected to truly transform our field by turning (pre-)clinical data into innovative care that results in improved outcomes of our transplant patients. So, let the future begin!

ETHICS STATEMENT

Ethical approval was not required for the studies involving humans because the current editorial summarizes individual studies, for which separate informed ethical approval was granted. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements because the current editorial summarizes individual studies, for which separate informed consent was granted.

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GENERATIVE AI STATEMENT

The author(s) declare that Generative AI was used in the creation of this manuscript. The authors of this editorial used a freely available AI tool (Perplexity AI, Inc., San Francisco, United States) to synthesize the information regarding sixteen scientific papers included in a special issue of Transplant International.

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Elevated PD-L1 and PECAM-1 as Diagnostic Biomarkers of Acute Rejection in Lung Transplantation

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Acute cellular rejection (ACR) frequently occurs following lung transplantation (LuTx) and represents a risk factor for the development of chronic lung allograft dysfunction (CLAD) as well as long-term survival. The histopathological diagnosis of ACR carries a burden of interobserver variability. The widespread utilization and cost-effectiveness of immunohistochemistry (IHC) was proven beneficial in diagnosing rejection in human kidney transplantations and LuTx rat models. However, its potential for ACR detection in patients remains unexplored. We analyzed surface markers (CD3, CD4, CD8, CD20, CD68, CD47, PD-1, PD-L1, and CD31/PECAM-1) on lung tissue cryobiopsy samples collected within 6 months post-LuTx from 60 LuTx recipients, 48 of whom were diagnosed with ACR. Additionally, serum samples from 51 patients were analyzed using a multiplex bead-based Luminex assay. The cytokines and markers included PD-L1, IL2, TNFα, IFNγ, and Granzyme B. We observed a significant increase in PD-L1 tissue expression within the rejection group, suggesting a concerted effort to suppress immune responses, especially those mediated by T-cells. Furthermore, we noted significant differences in PECAM-1 levels between ACR/non-ACR. Additionally, peripheral blood C-reactive-protein levels

Abbreviations: ACR, acute cellular rejection; AMR, acute humoral rejection; APCs, antigen presenting cells; BOS, bronchiolitis obliterans syndrome; CLAD, chronic lung allograft dysfunction; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein; DBD, donation after brain death; IHC, immunohistochemistry; IFNγ, interferon gamma; ILD, interstitial lung disease; ISHLT, the International Society for Heart and Lung Transplantation; PAH, pulmonary arterial hypertension; PD-1, programmed death-receptor 1; PD-L1, programmed death-ligand 1; LuTx, lung transplantation; RAS, restrictive allograft syndrome; ROC, receiver operating characteristics; TNFα, tumor necrosis factor alpha; WBC, white blood cells.

tended to be higher in the ACR group, while Luminex serum analyses did not reveal any significant differences between groups. In conclusion, our findings suggest the potential value of PECAM-1 and PD-L1 markers in diagnosing ACR.

Keywords: lung transplantation, acute cellular rejection, immunohistochemistry, luminex, checkpoint inhibitors

INTRODUCTION

Long-term allograft survival has always been significantly challenged by the persistent risk of transplantation rejection [1–4]. During transplantation, both ischemia-reperfusion and mechanical injury as well as inadequate organ storage conditions prompt immune system reactions through the local release of cytokines, chemokines, adhesion molecules, damage-associated molecular patterns, and other signaling molecules [5–7]. These events trigger an influx of innate immune cells to the graft, which is followed by the presentation of allogeneic antigens by antigen-presenting cells (APCs) to adaptive immune cells [8].

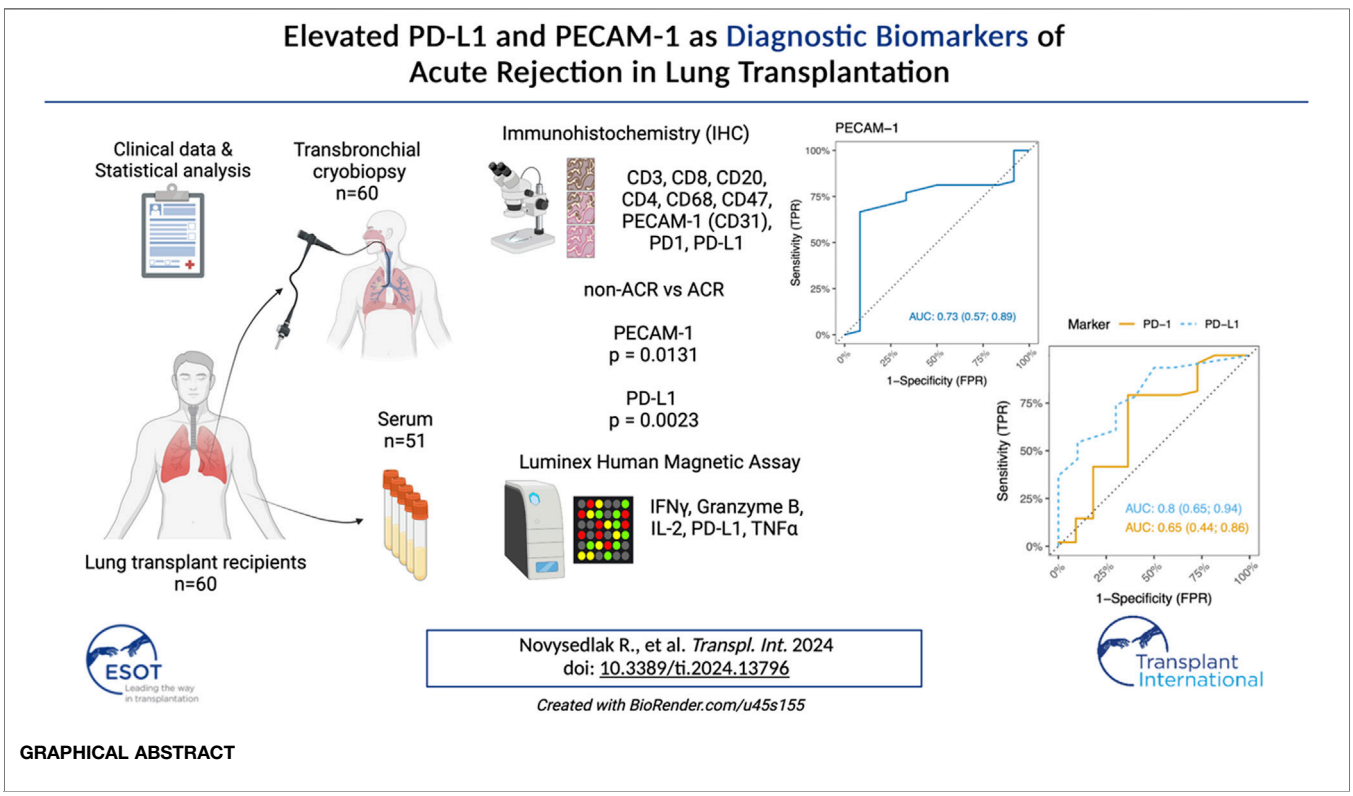
Acute organ rejection involves acute cellular rejection (ACR) orchestrated by T-cells and acute humoral rejection (AMR) driven by antibody-producing plasma cells [9].

Antibody-mediated rejection (AMR) is a profoundly studied phenomenon particularly in kidney transplants, leading to standardized nomenclature and diagnostic criteria. However, its applicability in lung transplants is limited, emphasizing the significance of T-cell-mediated rejection in lung allografts [10].

T-cell-mediated ACR in lung transplants, impacting small airways and vasculature, represents a significant clinical challenge [11–13]. The incidence of ACR is highest early post-lung transplantation, with 27% of adult patients experiencing at least one treated episode within the first year. ACR is associated with bronchiolitis obliterans syndrome (BOS), a main phenotype of chronic lung allograft dysfunction (CLAD), with late ACR episodes (after 180 days post-transplant) linked to an elevated risk of BOS [1, 14–18].

Diagnostic assessment of ACR faces interobserver variability, particularly in lower-grade rejection, and understanding of the specific traits and phenotypic patterns of infiltrating T-cells during ACR remains limited [11]. Therefore, ACR demands attention from researchers to pinpoint potential biomarkers that could help to understand immune responses and strengthen the diagnostic process and early detection of rejection.

Immune checkpoint molecules have been extensively studied in the oncological context [19]. However, their role and potential use in solid organ transplantation is far from being understood. Several studies have shown that the interaction between Programmed Death-Receptor 1 (PD-1) and Programmed Death-Ligand 1 (PD-L1) is essential for both initiating and sustaining tolerance to the graft [20].



GRAPHICAL ABSTRACT

PD-1 is a key inhibitory receptor involved in both adaptive and innate immune responses. It is expressed on various immune cells, including activated T cells, natural killer cells, B lymphocytes, macrophages, dendritic cells, and monocytes. PD-1 plays a crucial role in dampening autoimmune reactions and thus, preserving immune tolerance [21, 22]. As a PD-1 ligand, PD-L1 is typically found on macrophages, activated T and B cells, dendritic cells, and various epithelial cells, with its expression being elevated under inflammatory conditions. PD-L1 is often found in immune environments characterized by high loads of $CD8^+$ T cells and the production of Th1 cytokines and interferons [21].

In contrast to other costimulatory molecules, PD-L1 expression extends beyond hematopoietic cells, as it can also be detected on endothelial cells, placental trophoblasts, and even pancreatic islet cells [23].

In the context of transplantation, the PD-1/PD-L1 pathway has been primarily investigated in animal models, with limited research was conducted in humans, particularly among lung transplant recipients [24, 25].

PD-L1 expression was shown to be significantly upregulated following transplantation on endothelial cells within heart allografts [26].

This increased expression within the vasculature indicates that PD-L1 may play a crucial role at the interface between immune cells and the transplanted organ, highlighting its potential importance in regulating the alloimmune response. In this regard, molecules involved in endothelial-immune cell interactions warrant particular attention.

Platelet/endothelial cell adhesion molecule-1 (PECAM-1 or CD31) is a key regulator of leukocyte transmigration across the endothelium and has been shown to be essential for transendothelial migration. PECAM-1-mediated leukocyte migration can be effectively inhibited by PECAM-1-specific blocking antibodies or by downregulating PECAM-1 expression [27].

Thus, examining PD-1/PD-L1 coinhibitory signals could provide valuable insights into the regulation of the alloimmune response, while clarifying the involvement of PECAM-1 in transplant rejection could highlight its potential as a novel therapeutic target in transplantation.

Immunohistochemistry (IHC), a cost-effective technique, has proven useful in diagnosing ACR in human kidney transplants [28–30]. Although in rat models, IHC aimed specifically at $CD4^+$ and $CD8^+$ T-cell proportions and distribution, improved the sensitivity and specificity of lung rejection diagnosis and grading, the same approach in human lung ACR is insufficient [31].

To address these gaps, and to better understand the role of immune checkpoint molecules in transplantation, our study explores multiple IHC biomarkers, including CD3, CD4, CD8, CD20, CD68, CD47, PD-1, PD-L1, and CD31/PECAM-1 within a large cohort. We aimed to identify T-cell subtype proportions and phenotypes, assess immune exhaustion levels, understand immune system dynamics, examine leukocyte transendothelial migration patterns, evaluate "don't eat me" signals expression, and determine macrophage and B-cell proportions within lung allograft specimens.

Additionally, to obtain a detailed understanding of the immune landscape in LuTx recipients, we have extended our analyses by measuring T cell functionality via a multiplex assay. To provide a comprehensive profile of the immune status and functionality of T cells, which are critical in the context of transplantation, PD-L1, IL-2, Granzyme B, Tumor Necrosis Factor alpha (TNFα) and interferon gamma (IFNγ), were evaluated.

PD-L1 was included due to its role in immune regulation, whereas IL-2 provided insights into the activation status and responsiveness of T cells. Granzyme B, TNFα, and IFNγ are integral to the effector functions of T cells. Together, they provide comprehensive insights into T cells' cytotoxic potential, inflammatory responses, and the regulatory balance of immune activation, all of which are crucial for graft survival and effective immune defense.

Understanding not just the phenotype but also the function of T cells is vital for developing strategies to enhance graft survival and reduce the risk of rejection.

This comprehensive analysis aimed to provide crucial insights into immune events within lung allografts.

MATERIALS AND METHODS

Study Design

This retrospective study includes 171 adult patients (≥18 years) who underwent bilateral lung transplantation (LuTx) at Motol University Hospital in Prague between 1 January 2018, and 31 December 2021. Excluded were single, lobar, and multiorgan LuTx, as well as re-transplants. Patients without cryobiopsy within the initial 6 months post-transplant, lacking cryopreserved samples, or tissue samples for research were also excluded.

Routine and on-demand cryobiopsies were collectively analyzed, with routine samples taken after one, three, or six post-transplant months. Demographics and clinical data were obtained from patient files, and only laboratory results before ACR treatment initiation were considered. Serum samples taken prior to the initiation of potential rejection treatment were analyzed for selected cytokines using a customized Luminex Human Magnetic Assay. Tissue samples were evaluated using IHC. The study, approved by the Ethics Committee of Motol University Hospital (EK-530/21), received written informed consent from all patients at transplantation listing. Follow-up was censored on 24 September 2023.

Study Population

Donor and preservation variables included: age, gender, weight, height, BMI, donor type [donation after brain death (DBD) vs. donation after circulatory death], cytomegalovirus (CMV) status and times of ischemia for both lungs.

Recipient variables included: age, gender, weight, height, BMI, CMV status, underlying comorbidities, indication for transplant, immunosuppression regimen used, date of the first post-transplant lung tissue cryobiopsy, acute cellular rejection

TABLE 1 | Immunohistochemistry staining specifications.

Antibody	Clone; manufacturer	Dilution	Pre-treatment
anti-CD3	RBT-CD3 [BioSB]	1:20	Heating in a buffer solution of pH9 in a water bath
anti-CD8	C8/144B [Dako]	1:200	Heating in a buffer solution of pH9 in a water bath
anti-CD20	L26 [Dako]	1:300	Heating in a buffer solution of pH6 in a water bath
anti-CD4	4B12 [BioGenex]	1:250	Heating in a buffer solution of pH9 in a water bath
anti-CD68	PG-M1 [Dako]	1:100	Heating in a buffer solution of pH9 in a water bath
anti-CD47	PA5-80435 [Thermofisher Scientific]	1:200	Heating in a buffer solution of pH6 in a water bath
anti-CD31 (anti-PECAM-1)	JC70A [Dako]	1:40	Heating in a buffer solution of pH6 in a water bath
anti-PD1	polyclonal serum [Abd Serotec]	1:200	Without antigen retrieval
anti-PD-L1	22C3 [Dako]	Certified kit	Processed according to the certified Autostainer Link 48 protocol

(grades A and B), infection status, peripheral blood levels of C-reactive protein (mg/L), peripheral blood levels of white blood cells ($\times 10^9$/L) and percentage and count ($\times 10^9$/L) of its subtypes, namely, lymphocytes, monocytes, neutrophils, eosinophils, basophils and immature granulocytes.

Immunohistochemistry

Sixty formalin-fixed paraffin-embedded tissue samples were retrospectively analyzed, evaluating the expression of CD3, CD8, CD20, CD4, CD68, CD47, PECAM-1 (CD31), PD1, and PD-L1. Histologic sections (3 μm thick) underwent staining with specific antibodies, including Anti-CD3, Anti-CD8, Anti-CD20, Anti-CD4, Anti-CD68, Anti-CD47, Anti-CD31, Anti-PD1, and Anti-PD-L1. Staining protocols involved various pre-treatments and dilutions (**Table 1**), with detection using a micropolymeric non-biotin system, except for PD-L1. Manual scoring by an experienced pathologist assessed the absolute count of immune cells positive for selected markers per 1 mm^2, starting from "hot spots" within each sample. Our study focused on immune cells and evaluated cytoplasmic and membranous staining. Specifically, PECAM-1 showed membranous staining and PECAM-1-positive endothelial cells were excluded from scoring. Differentiation of PECAM-positive immune cells (specifically intraalveolar macrophages) from endothelial cells was enabled by distinct characteristics of the macrophages, such as their morphology, intra-alveolar location, and lower staining intensity, as shown in **Figure 4**. The nuclei were counterstained with hematoxylin.

Luminex Assay

Our sample preparation procedures were followed in accordance with the manufacturer's guidelines to ensure accuracy and reproducibility. Specifically, we focused on blood sera derived from 51 patients and analyzed a panel of cytokines and markers, including IFNγ, Granzyme B, IL-2, PD-L1 and TNFα. A customized Luminex Human Magnetic Assay, sourced from Biotechne, R&D Systems s.r.o. in Prague, was used. The assay enabled precise detection of cytokines and chemokines in serum from lung transplant samples. Data were acquired using the Bio-Plex 200 system.

Cryobiopsies

Transbronchial cryobiopsy was the method of lung tissue sample collection, facilitated through flexible bronchoscopy targeting primarily the left lower lobe when possible. This procedure, conducted under total anesthesia, adhered to standard medical protocols. Cryobiopsies were evaluated according to ISHLT guidelines and scored for acute cellular rejection (ACR) (Grade A) and lymphocytic bronchiolitis (Grade B) [32]. Both tissue samples and peripheral blood were meticulously preserved at a stable temperature of −80°C until the analysis was performed.

Statistical Analyses

Continuous variables were standardly reported as median (interquartile range) and categorical variables as number (percentage). Data were grouped into two main groups – control group (only grade A0 = non-ACR) and rejection group (ACR grade A1-3). Fisher's exact test was used to compare categorical variables between groups. Spearman correlations (ρ) and Mann–Whitney U tests were used to evaluate relations between clinical, IHC and Luminex variables and ACR. Kruskal-Wallis test was used to evaluate relations between all A0-A3 groups (**Supplementary Table S1**). Values falling below the lower limit of quantification were subjected to a halving procedure in the analytical process.

To evaluate the predictive capacity of IHC markers for graft acceptance or rejection (non-ACR vs. ACR), individual Receiver Operating Characteristic (ROC) curves were constructed for each marker, and the corresponding Area Under the Curve (AUC) was calculated. The Youden's Index and Euclidian distance were computed to find the ideal cut-off values. This part of the analysis was performed by an experienced biostatistician (A.B.).

RESULTS

Study Population and Baseline Characteristics

Immunohistochemical analyses were performed on a cohort of 60 tissue samples obtained from 60 bilateral lung transplant (LuTx) recipients. However, for subsequent Luminex analyses, samples from 9 patients were unavailable. Recipient age was 53 (42–60) years. Among recipients, 40 (67%) were male and 20 (33%) female. Indications for transplantation included chronic obstructive

TABLE 2 | Cohort donor, preservation, and recipient characteristics.

Cohort characteristics	Results
Donor and preservation	
Age at donation, years	44 (31–53)
Sex, n (%)	
Male	32 (53)
Female	28 (47)
Body mass index, kg/m^2	24 (22–26)
CMV status, n (%)	
Positive	39 (65)
Negative	21 (35)
Total ischemic time (longest time of two lungs), min	345 (294–390)
Total ischemic time (mean of two lungs), min	293 (245–330)
Recipient	
Age at transplant, years	53 (43–60)
Sex, n (%)	
Male	40 (67)
Female	20 (33)
Body mass index, kg/m^2	26 (19–28)
Indication for transplant, n (%)	
Chronic obstructive pulmonary disease	19 (32)
Interstitial lung disease	26 (43)
Cystic fibrosis	8 (13)
Pulmonary arterial hypertension	7 (12)
Time spent on waiting-list, days	183 (85–354)
CMV status, n (%)	
Positive	37 (62)
Negative	20 (33)

Data are expressed as median (interquartile range) if not otherwise indicated. Abbreviation: CMV, cytomegalovirus.

pulmonary disease (COPD) in 19 (32%) patients, interstitial lung disease (ILD) in 26 (43%), cystic fibrosis in 8 (13%) and pulmonary arterial hypertension (PAH) in 7 (12%) patients. Only DBD donors were reported in this cohort. Donor age was 44 (31–53) years. Two (3%) donors were older than 70 and one (2%) was younger than 18. Among donors, 32 (53%) were male, 28 (47%) female. **Tables 2**, **3** summarize the baseline characteristics of the study cohort. No differences in baseline characteristics were observed between control and rejection group. **Supplementary Table S1** presents the distribution of acute cellular rejection grades in the study cohort. Standard induction immunosuppression at our center consists of basiliximab, tacrolimus, mycophenolate, and corticosteroids. For selected patients, an alternative strategy to basiliximab is employed. **Supplementary Table S2** presents the percentage of patients in whom basiliximab and each alternative modality to it was used, either alone or in combination. For maintenance immunosuppression tacrolimus, mycophenolate, and corticosteroids are used. No significant difference was observed when comparing induction immunosuppressive regimens ($p = 0.3341$) and infection status ($p = 0.7191$) between the groups (**Supplementary Tables S2, S3**). A description of underlying immunological conditions is provided in **Supplementary Table S4**.

The Levels of C-Reactive Protein Were Associated With Acute Cellular Rejection

Our study cohort was initially stratified into two subgroups based on the presence or absence of ACR. These subgroups were subsequently compared in relation to differential white blood cell counts (WBC) and acute-phase proteins, specifically C-reactive protein (CRP). **Figure 1** shows scatter plots with median and interquartile range of the measured values. Interestingly, no difference was observed in total WBC count, and percentages and counts of neutrophils, monocytes, lymphocytes, and eosinophils (**Table 4**), suggesting the limited efficacy of basic leukocyte parameters in predicting ACR within this context.

On the other hand, CRP, an acute-phase protein synthesized in the liver due to interleukin-6 secretion by macrophages and T-cells, displayed variations between the observed groups. As shown in **Figure 1**, peripheral blood levels of CRP tended to be lower in the non-ACR group [8.15 (2.4–17.8) as compared to the ACR group 17.5 (8.45–36.83) $p = 0.055$].

TABLE 3 | Baseline characteristics of control and rejection group.

	no ACR (A0) n = 12	ACR (A1-3) n = 48	*p*-value
Donor age, years	45 (36–54)	43 (31–50)	0.54
Male donors	8	24	0.35
Female donors	4	24	
Donor BMI, kg/m^2	24 (23–26)	24 (22–27)	1.0
Total ischemic time (longest time of two lungs), min	299 (281–364)	351 (304–396)	0.12
Total ischemic time (mean of two lungs), min	255 (239–314)	297 (256–331)	0.13
Recipient age, years	58 (48–61)	52 (38–59)	0.39
Male recipients	9	31	0.73
Female recipients	3	17	
Recipient BMI, kg/m^2	26 (22–28)	26 (19–27)	0.64
Indication for transplant		—	
Chronic obstructive pulmonary disease	5	14	0.59
Interstitial lung disease	6	20	
Cystic fibrosis	1	7	
Pulmonary arterial hypertension	0	7	

Data are expressed as simple count (categorical variables) or median (range), respectively. Abbreviation: BMI, body mass index.

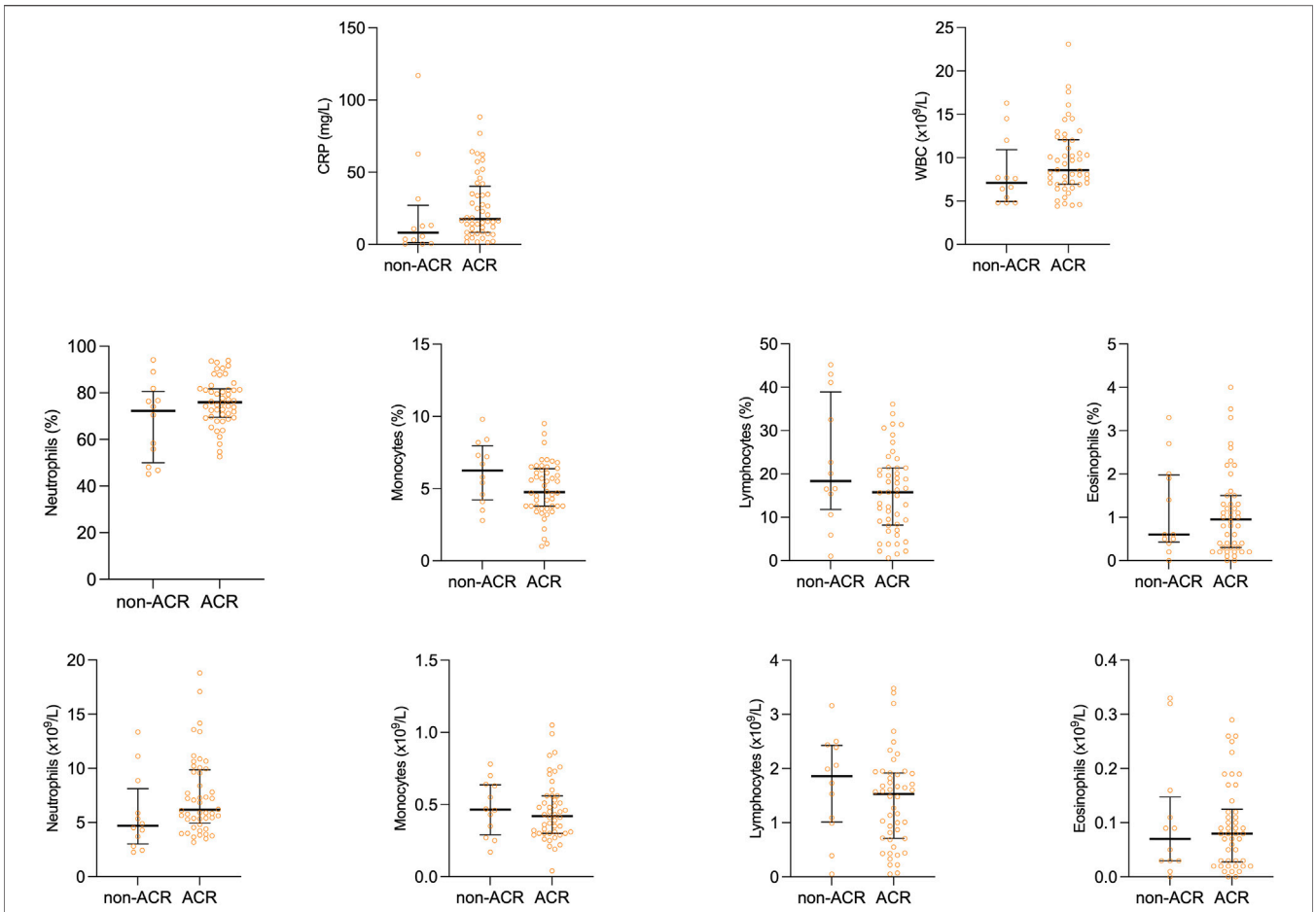


FIGURE 1 | Scatter plots of peripheral blood cell profile comparing rejection group to control group. Median and interquartile range is visualized.

TABLE 4 | Peripheral blood cell profile.

	no ACR (A0) n = 12	ACR (A1-3) n = 48	*p*-value
CRP (mg/L)	8.15 (2.4–17.8)	17.5 (8.45–36.83)	0.06
WBC count (×10^9/L)	7.1 (5.25–8.78)	8.55 (7.05–12.03)	0.15
Neutrophils (%)	72.25 (53.95–78)	75.95 (69.70–81.50)	0.19
Neutrophils (×10^9/L)	4.70 (3.48–6.63)	6.17 (5.17–9.73)	0.07
Monocytes (%)	6.25 (4.48–7.53)	4.75 (3.8–6.33)	0.09
Monocytes (×10^9/L)	0.46 (0.33–0.63)	0.42 (0.30–0.56)	0.70
Lymphocytes (%)	18.35 (14.20–34.65)	15.75 (8.33–21.4)	0.18
Lymphocytes (×10^9/L)	1.86 (1.06–2.40)	1.53 (0.72–1.91)	0.25
Eosinophils (%)	0.6 (0.48–1.93)	0.95 (0.3–1.45)	0.83
Eosinophils (×10^9/L)	0.07 (0.03–0.12)	0.08 (0.03–0.12)	0.97

Data are expressed as median (interquartile range). Abbreviations: CPR, C-reactive protein; WBC, white blood cells.

TABLE 5 | Positive immune cell counts per 1 mm^2 in lung tissue were determined for specific IHC markers in the study groups, excluding endothelial cells in PECAM-1 from the scoring system.

	no ACR (A0) n = 12	ACR (A1-3) n = 48	*p*-value
CD3	82 (38.5–97.75)	94.5 (56–136)	0.10
CD4	0 (0–5.25)	0 (0–6)	0.99
CD8	4.5 (0–30.25)	0 (0–19)	0.25
CD20	2 (0–10.25)	2.5 (0–18)	0.98
PD1	14 (6.5–42)	32 (19.75–59.75)	0.12
PD-L1	1.5 (0–5.25)	9.5 (3.25–18)	**0.0023**
CD68	37.5 (20.25–102.75)	77 (23.75–92.50)	0.61
PECAM-1	36.5 (35.25–43.25)	58 (40.75–84)	**0.0131**
CD47	275.5 (202–381.5)	338.5 (235.5–418)	0.29

Data are expressed as median (interquartile range). Two samples for PD-L1 were missing in both groups, and one sample for PD-1 was missing in the non-ACR group. Bold values indicate statistical significance at the level of $p < 0.05$.

Neither T-Cell Subsets Nor B-Cells and Macrophages Exhibited Significant Elevation in Patients With Acute Cellular Rejection

In the course of our investigations, our primary objective revolved around elucidating the potential impact of ACR on the proportions of critical immune cell types (T-cells, B-cells, and macrophages) and demonstrating whether ACR elicits substantial changes in the abundance or distribution of these cell populations. **Table 5** outlines the counts of positive immune cells per 1 mm^2 for selected IHC markers. We hypothesized that examining the specific surface markers, such as CD3, CD4, CD8,

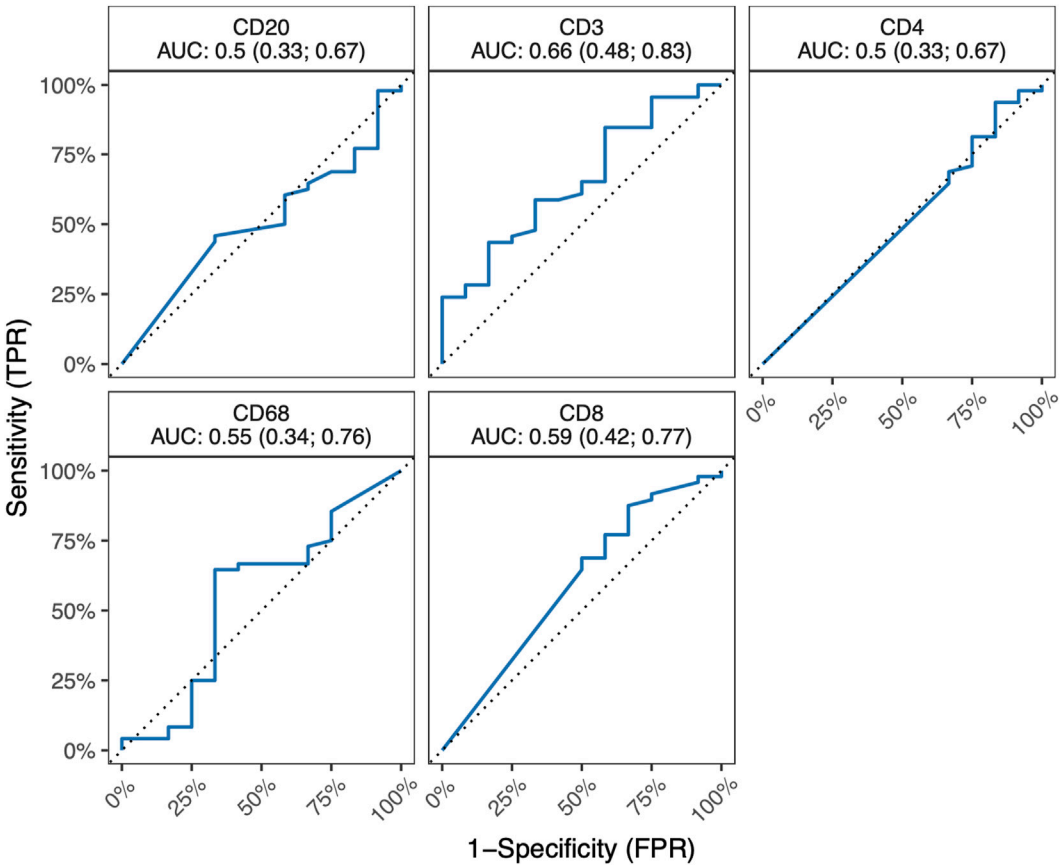


FIGURE 2 | ROC curves for IHC markers CD3, CD4, CD8, CD20 a CD68, along with corresponding AUC values and their 95% confidence interval. All 95% confidence intervals include 0.5, which shows that none of the markers are good predictors.

CD20, and CD68, might offer a viable means of detecting the initial stages of ACR. Unfortunately, these markers did not show any differences between the ACR and non-ACR groups. As depicted in **Figure 2**, neither T-cell subsets nor B-cells and macrophages exhibited significant elevation in patients with ACR.

PD-L1 Is Significantly Increased in Lung Transplant Recipients Exhibiting Acute Cellular Rejection

In the subsequent array of analyses, we examined immune checkpoints in lung tissue samples to understand the balance of activation/inhibition signals transmitted through immune receptors. Our primary focus was on the most prominent immune checkpoint pathway, predominantly occurring in T-cells, which involves the interaction between PD-1 and PD-L1 [33]. Following this, our attention shifted to exploring the novel potent macrophage checkpoint CD47, known as the "don't eat me" signal [34]. While CD47 displayed no significant variations between the ACR and non-ACR group, striking differences were observed when analyzing the PD-L1 expression within lung allografts.

As shown in **Figure 3A**, PD-L1 exhibited significant increase in the rejection group (PD-L1 $p = 0.0023$), indicating an ongoing attempt to inhibit immune responses, particularly those involving T-cells. On the other hand, while the increase in PD-L1 levels might imply an effort to foster peripheral immune tolerance through its interaction with the PD-1 receptor, there was no observed increase in PD-1 receptor within the ACR cohort when compared to the non-ACR group. **Figure 3B** shows areas under ROC curves, and associated 95% confidence intervals, based on marked values for PD-L1 (0.80 confidence interval [0.65; 0.94]) and PD-1 (0.65 confidence interval [0.44; 0.86]). PD-L1 does not include 0.5 in its 95% confidence interval, therefore it can be a good ACR predictor. **Supplementary Figure S1** shows Youden's Index and Euclidian distance for PD-L1. **Table 6**, **7** show confusion matrices for PD-L1. PD-L1 remained significant ($p = 0.0112$) when analyzed across all ACR A grades collectively, as shown in **Supplementary Table S5**.

In Human Lung Allografts, Leukocytes Exhibit Dynamic Transendothelial Migration

PECAM-1, also known as CD31, plays a crucial role in facilitating the movement of leukocytes across the

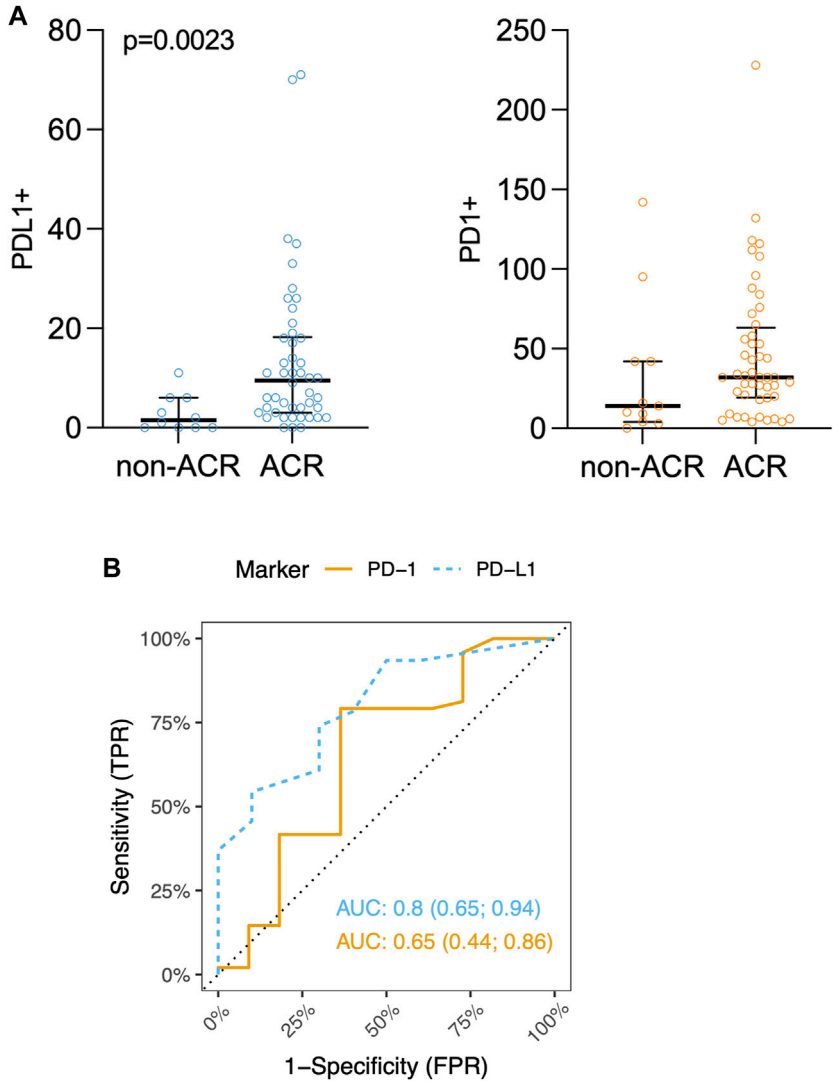


FIGURE 3 | (A) Scatter plots of PD-L1+ and PD-1+ immune cells counts (positive immune cells per 1 mm^2) in lung tissue biopsy. Median and interquartile range is visualized. **(B)** ROC curves for IHC markers PD-L1 and PD-1. Area under ROC-curves (AUC) (and associated 95% confidence interval) based on marked values for PD-L1 is 0.80 (0.65; 0.94) and for PD-1 is 0.65 (0.44; 0.86).

TABLE 6 | Confusion matrix showing Youden's Index for PD-L1 with cut-off value of 7 positive cells/mm^2.

PD-L1		
Actual	**Predicted**	
	Rejected	**Non-rejected**
Rejected	25	21
Non-rejected	1	9

TABLE 7 | Confusion matrix showing Euclidian distance for PD-L1 with cut-off value of 4 positive cells/mm^2.

PD-L1		
Actual	**Predicted**	
	Rejected	**Non-rejected**
Rejected	34	12
Non-rejected	3	7

intercellular junctions of vascular endothelial cells during the process of leukocyte transmigration [35]. Given the increased scientifical interest in anti-PECAM-1 therapies blocking transendothelial migration of leukocytes, our aim was to investigate the potential involvement of PECAM-1 in ACR of lung allografts. **Figures 4A–C** show the PECAM-1 IHC staining of the samples.

Interestingly, PECAM-1 expression, assessed via IHC, demonstrated a trend towards significance (p = 0.0874) when analyzed across all ACR A grades collectively (**Supplementary**

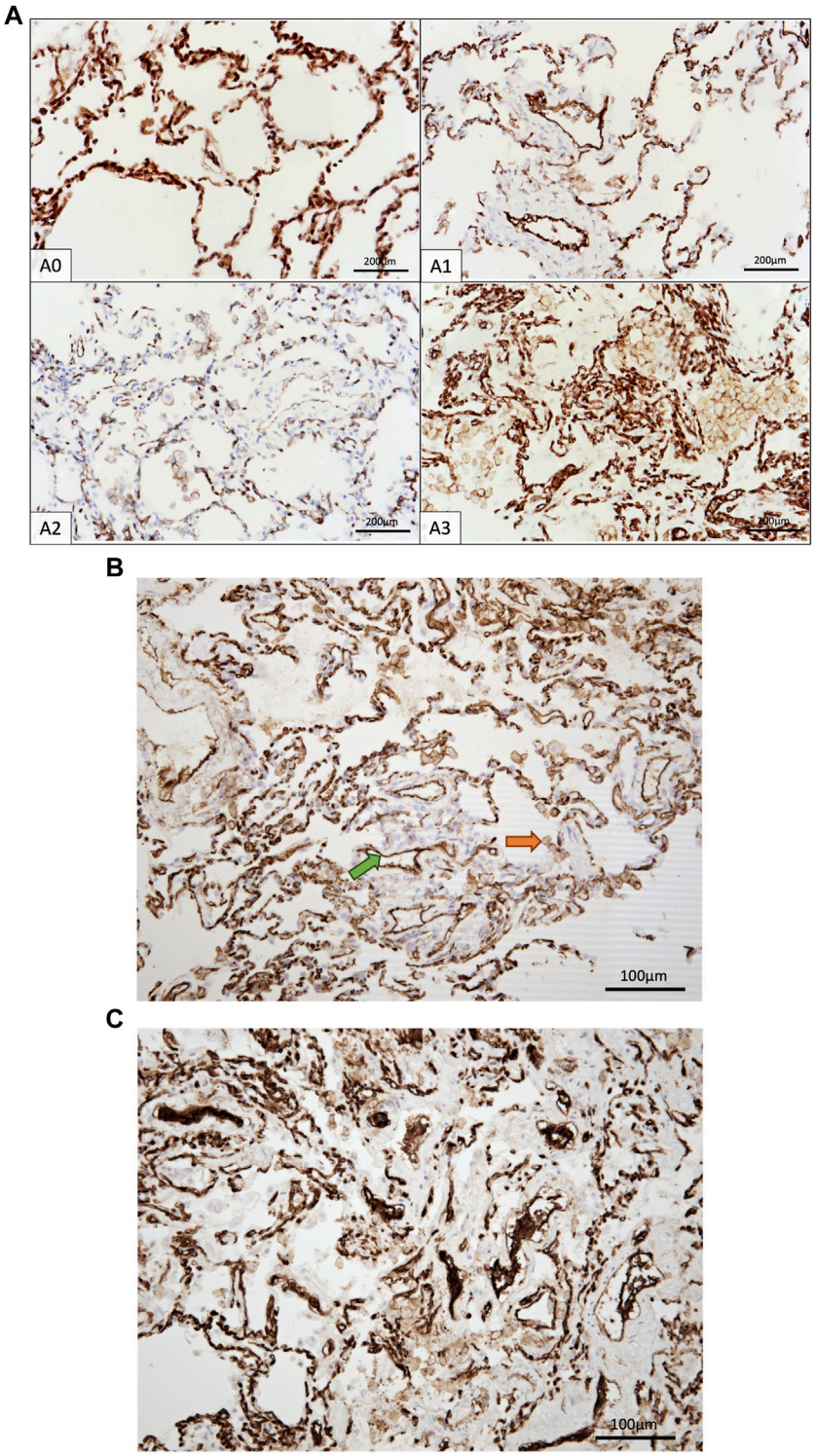


FIGURE 4 | (A) IHC staining of CD31$^+$ cells in control group and A1-A3 rejection groups. **(B)** CD31$^+$ immune cells (orange arrow) and endothelia cells (green arrow). Only immune cells were counted. **(C)** Endothelium exhibiting CD31 positivity, presumably indicative of endothelial swelling associated with endothelitis, a characteristic frequently observed in A3.

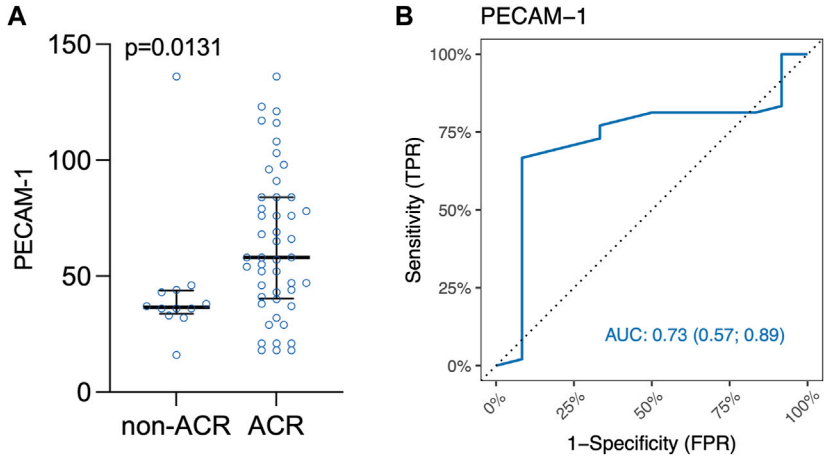


FIGURE 5 | (A) Scatter plot of PECAM-1/CD31 immune cells in lung tissue biopsy. Median and interquartile range is visualized. **(B)** ROC curve for IHC marker PECAM-1. Area under ROC curve based on marked values for PECAM-1 is 0.73.

TABLE 8 | Confusion matrix showing Youden's Index and Euclidian distance for PECAM-1/CD31 with cut-off value of 47 positive cells/mm^2.

PECAM-1		
Actual	**Predicted**	
	Rejected	**Non-rejected**
Rejected	32	16
Non-rejected	1	11

Table S5). PECAM-1 was significantly elevated in LuTx patients diagnosed with ACR (p = 0.0131) compared to those without ACR (**Figure 5A**). This finding suggests that PECAM-1 may have promising potential as a biomarker for ACR detection.**Figure 5B** shows area under ROC curve based on marked value for PECAM-1 (0.73).

Both Youden's Index and Euclidian distance cut-off point value based on the ranked values for PECAM-1 was 47 positive immune cells/mm^2 (**Supplementary Figure S2**) indicating a threshold for distinguishing between samples that are positive or negative for PECAM-1 expression. **Table 8** shows confusion matrix for PECAM-1.

T Cell Functional Capacities Were Unaffected in the Rejection Group

To gain a deeper understanding of T cell functionality in LuTx, we conducted a multiplex bead-based immunoassay using Luminex technology on serum samples to evaluate key molecules reflecting T cytotoxic and proliferative function. These molecules included PD-L1, IL-2, Granzyme B, TNFα, and IFNγ.

This multi-faceted approach allowed us to assess the cytotoxic capacities of T cells, and the inflammatory environment in the context of graft survival.

In the rejection group, our analysis revealed an increase in serum levels of IL-2, a cytokine that plays a critical role in T cell proliferation and immune regulation, alongside a decrease in PD-L1 levels. However, these changes did not reach statistical significance, with p-values of 0.8046 for IL-2 and 0.1224 for PD-L1, respectively. This suggests that while there may be a trend in these biomarkers, the observed variations are not strong enough to draw definitive conclusions about their roles in rejection processes.

Furthermore, no significant differences were detected in the levels of granzyme B, TNFα, and IFNγ, indicating that these immune markers may not be associated with rejection in this study population. **Figure 6** shows areas under ROC curves, and associated 95% confidence intervals, based on marked values for analyzed molecules. All 95% confidence intervals include 0.5, which shows that none of the markers are good predictors.

DISCUSSION

LuTx patients frequently face ACR complications, impacting lung function and contributing to CLAD. Our study analyzed ACR and non-ACR groups, focusing on variations in WBC counts and acute-phase proteins. Since these variables may be affected by immunosuppressive treatment, our study included only patients treated at our center, where the standard maintenance therapy consists of tacrolimus, mycophenolate, and corticosteroids. The induction immunosuppressive regimens varied among individual patients; however, there were no statistically significant differences between the ACR and non-ACR groups. Similar investigations by Vos et al. linked systemic inflammation, CRP levels, and graft failure, aligning with elevated CRP during acute heart rejection observed by Eisenberg et al [36–38]. In our study, despite no significant differences in total WBC count or cell percentages, CRP levels tended to be higher in the ACR group, indicating a potential association. Although the infection status of our patients did not statistically differ between ACR and non-ACR groups, changes in

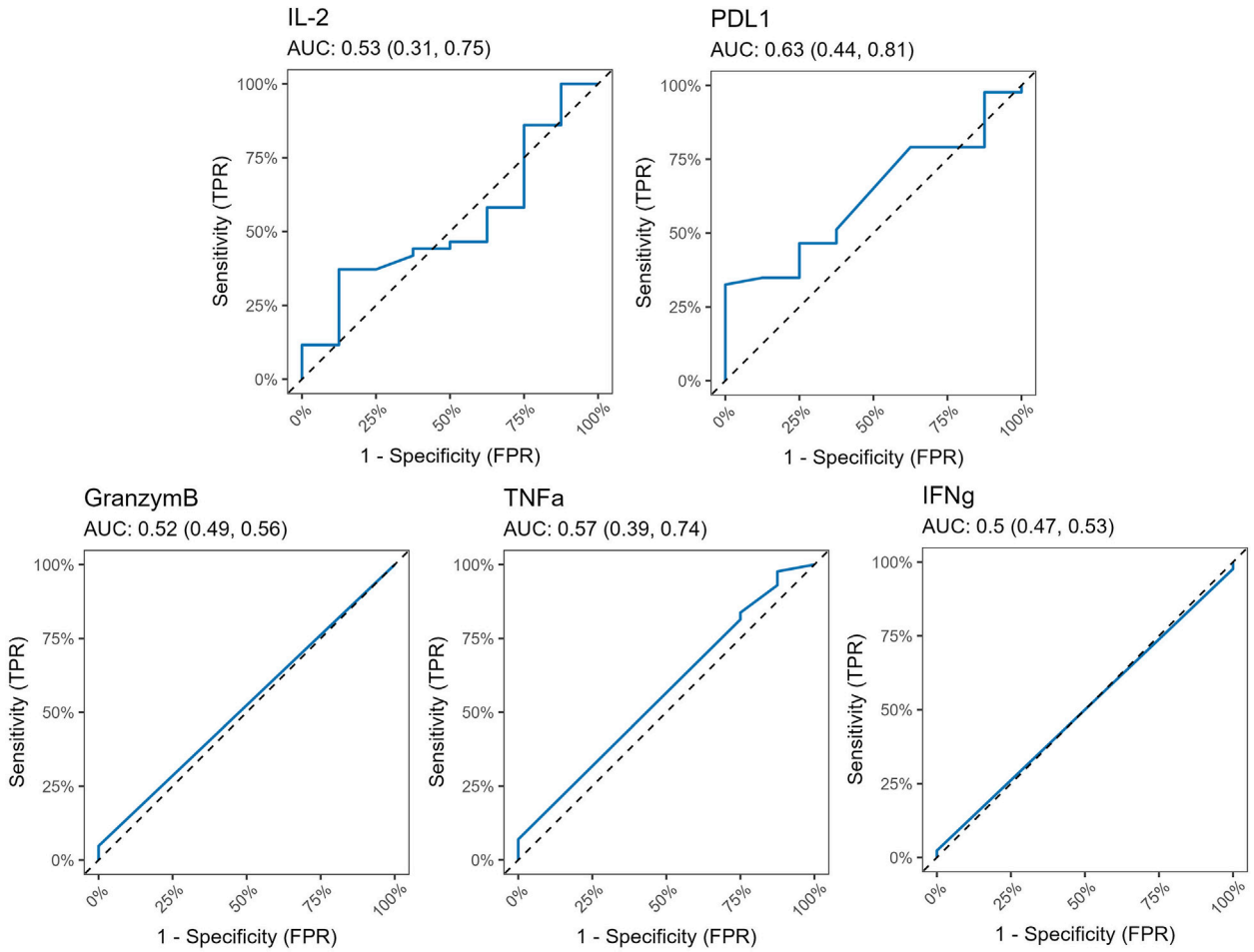


FIGURE 6 | ROC curves for Luminex markers PD-L1, IL-2, Granzym B, TNFα and IFNγ, along with corresponding AUC values and their 95% confidence interval. All 95% confidence intervals include 0.5, which shows that none of the markers are good predictors.

CRP levels should always be interpreted with caution, as not only accompanying infections, but also stress, inflammatory conditions, and other factors, may influence CRP [39, 40].

Next, we employed IHC to assess specific surface markers, aiming to uncover how ACR might influence the ratios of crucial immune cell (T-cells, B-cells, macrophages). Unfortunately, no significant differences were found between ACR and non-ACR groups.

Upon examining checkpoint molecules, it became apparent that CD47 did not seem a feasible marker for ACR. However, our focus shifted towards exploring the PD-1 and PD-L1 inhibitory pathway. To date, several studies have explored the functions of PD-1 and PD-L1 in transplantation. Wang et al. underscored their vital role in establishing cardiac allograft tolerance in mouse models [41]. Tanaka et al. highlighted PD-L1's pivotal role in both inducing and maintaining peripheral tolerance following heart transplantation by modulating the equilibrium among T-cell subsets [42]. Additionally, Choudhary et al. observed an upregulation of PD-L1 within cardiomyocytes, demonstrating a correlation with the severity of ACR after transplantation [43].

Righi et al., focusing on 24 LuTx patients, revealed the importance of PD-1 in acute rejection and its progression into CLAD. They proposed evaluating PD-1-expressing lymphocytes in transbronchial biopsies for prognostic monitoring [44]. Subsequently, Kaiho et al. investigated PD-1/PD-L1 in acute rejection using a mouse tracheal transplantation model, finding a PD-L1-mediated immune checkpoint association with rejection, suggesting a potential immunotherapy target in LuTx [45].

Our study contributes to the understanding of the involvement of the PD-1/PD-L1 axis in LuTx by demonstrating a significant increase of tissue PD-L1 levels within the ACR group. While it remains uncertain which cells produce PD-L1 in lung allografts *in vivo*, this increase indicates an active effort to suppress immune responses, particularly those associated with T-cells. However, we did not detect a concurrent rise in the PD-1 receptor among the ACR cohort when compared to the non-ACR group.

We hypothesize that this phenomenon may result from PD-L1 production by various non-immune cells within the lung tissue,

such as epithelial and endothelial cells, representing a localized immune suppression effort within the graft, primarily mediated by tissue-specific responses rather than T-cell-mediated modulation [46]. Furthermore, animal transplant models have shown that blocking PD-L1 leads to rejection, while blocking PD-1 and PD-L2 has no effect on graft survival. This indicates that PD-L1 and PD-L2 may play distinct roles in promoting tolerance, with PD-L1 expression, rather than PD-1 expression, emerging as the more reliable marker of immune regulation in transplantation [20].

Thus, the role of the PD-1/PD-L1 mechanism in acute rejection after lung transplantation has not yet been elucidated. These data, in accordance with previous studies, may imply the impairment of peripheral tolerance in LuTx recipients experiencing ACR.

In contemporary oncology, checkpoint molecules have emerged as pivotal targets in the therapeutic landscape, particularly within the realm of cancer treatment. This prominence arises from their capacity to modulate immune responses, a feature notably exploited to counteract the immunosuppressive microenvironment characteristics of malignancies [47]. Conversely, in the context of transplantation, the immune system often experiences heightened activation, resulting in the potential rejection of the transplanted organ. Hence, the contrasting immunological dynamics observed between cancer and transplantation underscore the likelihood of checkpoint molecules assuming a significant role in the latter scenario as well.

Khan et al. showed that the CTLA4, combined with the Fc portion of human immunoglobulin G1 (CTLA4-Ig) used as monotherapy immunosuppressant in mouse airway transplants promoted a favorable phase of immunotolerance, which facilitated microvascular and tissue repair [48].

The TIM family, notably TIM-1 and TIM-3, are pivotal regulators of the immune response and have been investigated in experimental transplant models. Murine studies reveal that inhibiting TIM-1 and boosting TIM-3 signaling enhances allograft outcomes [49]. The consistent findings across acute and chronic rejection models underscore the potential of TIM-3 interaction in mitigating detrimental immune responses [49]. The administration of stable galectin-9 in murine skin and cardiac transplants prolongs allograft survival by decreasing Th1 and Th17 cytokines and fostering Tregs [50–52]. To date, studies evaluating the role of LAG-3 in lung transplantation are lacking.

While there is speculation regarding the therapeutic utility of checkpoint molecules in modulating immune responses to prevent rejection, it is imperative to consider the potential adverse effects associated with such therapies. Of particular concern is the development of autoimmunity, a consequence that is undesirable across various clinical context. Moreover, according to Cui et al., immune checkpoint inhibitors were significantly associated with rejection in solid organ transplant recipients [53]. Therefore, any exploration of checkpoint inhibitor therapy in transplantation must carefully weigh the benefits of immune modulation against the risk of inducing autoimmune phenomena.

PECAM-1 plays a pivotal role in facilitating the migration of leukocytes across intercellular junctions within vascular endothelial cells during the transmigration process [35, 54]. The protective role of PECAM-1 in acute rejection has been demonstrated in various studies, yet its expression has not been previously analyzed by IHC in lung tissue during rejection episodes [55]. In 2022, Tran-Dinh et al. introduced an AI model evaluating CD31 cleavage for early ACR detection post LuTx [55]. We assessed the immunohistochemical surface expression of PECAM-1 in leukocytes from two distinct groups of LuTx recipients: individuals experiencing ACR and those without such complications. To our surprise, LuTx patients diagnosed with ACR showed a significant increase in PECAM-1 expression (IHC), prompting us to hypothesize that inhibiting transendothelial migration might represent a therapeutic approach for ACR.

In oncology, endothelial-immune cell interactions within the tumor microenvironment influence immune infiltration and function, highlighting the critical role of endothelial cells in immune response [56]. There is no reason to believe this would be any different in transplantation. Notably, endothelial cells in the donor lung are among the first to encounter the recipient's immune system.

PECAM-1 is involved in a wide array of processes related to inflammation, vascular biology, and various immune functions [57]. It has several splice variants, each capable of exhibiting distinct adhesive properties, which may subsequently impact its ligand-binding characteristics and functional role in leukocyte transmigration [58, 59]. The functional role of PECAM-1 is influenced by multiple factors, including the nature and tissue localization of the inflammatory response, as well as genetic determinants [57].

PECAM-1 possess both pro- and anti-inflammatory roles. Besides facilitating transendothelial leukocyte migration, it also plays a role in dampening leukocyte activation and reducing pro-inflammatory cytokine production [60]. In the context of ACR, macrophages expressing PECAM-1 may polarize into M2 subset which exhibit anti-inflammatory and graft-protective effects [61]. Thus, high PECAM-1 expression in the graft may reflect immune cell infiltration as well as active repair processes and endothelial resilience.

This study shows the potential of IHC in ACR diagnosis. Despite the additional cost, time, and effort required to perform IHC, its application could be advantageous in borderline cases as a supplementary technique to traditional histopathology. To improve assessments, the ISHLT recommends obtaining at least five adequate samples, reducing variability [32]. This is particularly crucial in cases of ACR, especially when confronting lower-grade rejection. This variability not only presents challenges in individual patient management but also hinders efforts to achieve standardization in multicenter trials [11, 62]. Identifying appropriate IHC markers could help tackle these issues and our data suggests that both PD-L1 and PECAM-1 need further exploration in ACR.

In order to better understand T cell functionality in LuTx, we also employed a multiplex bead-based immunoassay using Luminex technology to assess key molecules that reflect T cell

cytotoxicity and proliferation. The molecules analyzed were PD-L1, IL-2, Granzyme B, TNFα, and IFNγ. This approach aimed to provide insights into the cytotoxic potential of T cells and the surrounding inflammatory environment, which are crucial for improving graft survival, formulating targeted therapies, and enhancing outcomes for transplant patients.

The role of IL-2 in acute lung rejection has been previously reported [63–65]. Luminex analysis in our study cohort did not yield significant results. However, elevated levels of IL-2 in patients with ACR is in line with the older work of Jordan et al. [66] Our Luminex analyses were constrained by the small size of the study cohort. To this we also attribute the inconclusive results of other biomarkers investigated by the Luminex method. Future studies with larger sample sizes and the inclusion of more relevant biomarkers, such as PECAM-1, could provide more insightful findings.

In our center, cryobiopsies are the standard of care. The Zurich group has demonstrated that cryobiopsies offer a superior diagnostic yield for ACR compared to forceps biopsies, leading to reclassification and treatment strategy changes in 28.6% of cases [67]. Our findings show that nearly half of the samples in this cohort exhibit an A1 rejection grade. Notably, identifying A1 rejection in a clinically stable patient through biopsy may not necessitate therapeutic intervention.

This study adopts a retrospective design, encompassing solely double lung transplant patients with samples available in archives in a single high-volume transplant center. Observational design, limited cohort size and group size imbalances are notable. However, the cohorts, aside from size differences, exhibit consistent characteristics. Our selection might indeed introduce bias and therefore, a prospective study would be imperative to also ascertain an accurate representation of the prevalence within our patient cohort. Despite widespread use of IHC, interpretational variability also remains a challenge.

Conducting larger studies is essential for evaluating immunohistochemistry (IHC), with a specific focus on PD-L1/PECAM-1 markers, in the diagnosis of ACR.

CONCLUSION

IHC investigations of PECAM-1 and PD-L1 markers might be valuable for diagnosing ACR. Further research is required to enhance our understanding of the role of immune checkpoint inhibitors in lung transplantation.

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 studies involving humans were approved by the Ethics Committee of Motol University Hospital (EK-530/21). 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

Conceptualization: RN and ZOS; Methodology: RN, ZOS, JB, JT, VT, JS, AB, and MB; Formal analysis and investigation: RN, JB, JT, and ZOS; Data acquisition: RN, JB, VT, AS, KV, JS, and ZOS; Statistical analysis: AB, MB, and RN; Writing - original draft preparation: RN and ZOS; Writing - review and editing: RN, JB, JT, VT, AS, KV, JS, AB, JaV, MB, BV, RL, JiV, LC, and ZOS; Funding acquisition: RL, ZOS, and LC; Resources: RL, ZOS, and LC; Supervision: ZOS and LC. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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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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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.13796/full#supplementary-material

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Donor-Specific Blood Transfusion in Lung Transplantation

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Lung transplantation is still hindered by a high rate of chronic rejection necessitating profound immunosuppression with its associated complications. Donor-specific blood transfusion is a pre-transplant strategy aimed at improving graft acceptance. In contrast with standard stored blood or donor-specific regulatory T cells transfusions, this approach utilizes fresh whole blood from the donor prior to allograft transplantation, encompassing all cell types and plasma. The precise mechanisms underlying donor-specific blood transfusion-induced tolerance remain incompletely understood. Associations with regulatory/helper T cells, modulation of mononuclear phagocytic cells or microchimerism have been suggested. While numerous (pre-)clinical studies have explored its application in solid organ transplants like liver, kidney, and intestine, limited attention has been given to the setting of lung transplantation. This comprehensive review summarizes existing knowledge on the mechanisms and outcomes of donor-specific blood transfusion in solid organ transplants both in preclinical and clinical settings. We also address the potential benefits and risks associated with donor-specific blood transfusion in the field of lung transplantation, offering insights into future research directions.

Keywords: immunosuppression, lung transplantation, tolerance, transplant immunology, donor-specific blood transfusion

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INTRODUCTION

An important milestone was reached in 2022 with 70,000 adult lung transplantations (LTx) being performed over the past three decades according to the International Society for Heart and Lung Transplantation. LTx is a last resort for patients with end-stage pulmonary disease but the outcome remains limited with an internationally reported 5-year survival rate of 59% [1, 2]. After LTx, the recipient's immune system identifies the allograft as "non-self," activating a robust alloimmune response due to major histocompatibility complex (MHC) incompatibility between donor and recipient. Antigen-presenting cells (APC) trigger the maturation of upstream naïve immune cells into effector T or B cells. This intricate immunological process is also characterized the production of cytokines such as interleukin-2 (IL-2) and interferon-gamma (INF-γ) and mediated by regulatory

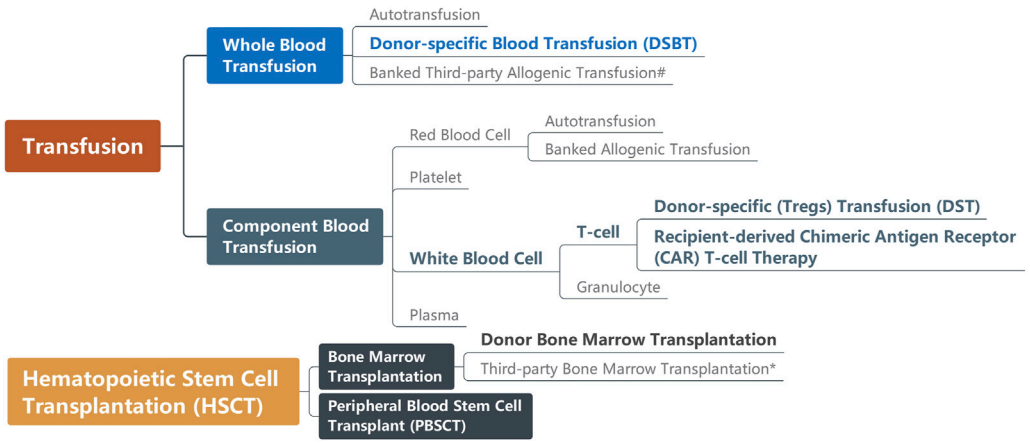

FIGURE 1 | Pan-transfusion techniques relevant to solid organ transplantation. Techniques (in bold) are reported as capable of improving graft acceptance. #Before transfusion, third-party whole blood usually is irradiated for leukoreduction; *Bone marrow transplantation causing graft-versus-host disease (GvHD) is the indication for some solid organ transplantation.

T cells (Treg) and/or B cells (Breg) population. Effective immunosuppression after LTx is crucial to prevent rejection and subsequent alloimmune injury to the lung [3–5].

However, chronic and profound immunosuppressive therapy induces drug toxicity (renal and cardiovascular toxicity, neurotoxicity, etc.) and increases susceptibility to infections and malignancies [6, 7]. Despite heavy immunosuppression, a higher rate of chronic lung allograft dysfunction (CLAD) is observed, compared to other transplantations such as liver and kidney. The future of LTx hinges on the prospect of widening the patient's therapeutic window improving graft acceptance without resorting to profound immunosuppression.

Before the era of modern calcineurin inhibitor-based immunosuppression, donor-specific blood transfusion (DSBT) has been used to facilitate graft acceptance [8, 9]. It involves the infusion of donor whole blood to recipients prior to transplantation, with the potential to improve graft acceptance or even induce donor-specific tolerance. In contrast to standard transfusion of blood products like red blood cells, platelets, or plasma, DSBT involves the use of whole blood directly obtained from the donor, containing all blood cell types and plasma proteins.

The definition of DSBT changed over time, leading to confusion about the concept in the literature. Initially, the research referred to this therapy was called donor-specific transfusion, abbreviated as DST [10]. However, advancements in blood apheresis techniques have narrowed the DST definition down to the transfusion of specific subpopulations of donor leukocytes (especially Tregs), resembling chimeric antigen receptor (CAR) T-cell immunotherapy in oncology, which has also been recently reported that the recipient-derived CAR-T cells targeting patients' B cells are capable of improving allograft acceptance after kidney transplantation [11–13]. Moreover, donor hematopoietic stem cell transplantation is also reported to permit solid organ allograft survival with preconditioning such as thymic irradiation, sublethal whole body irradiation and T cell depletion but without immunosuppression in several animal experiments and clinical trials [14–17]. Consequently, the crucial aspect of the original DSBT concept now lies in the transfusion of whole blood [DSBT(WB)]. Pan-transfusion techniques relevant to solid organ transplantation are summarized in **Figure 1**.

THE MECHANISM OF DSBT-INDUCED TOLERANCE IN TRANSPLANTATION

In current medical practice, the alloimmune response is non-specifically blocked to maintain graft acceptance by immunosuppressive drugs such as calcineurin inhibitors, antimetabolites, and anti-interleukin monoclonal antibodies (**Figure 2**) [18]. For example, corticosteroids inhibit pro-inflammatory gene expression and promote the expression of anti-inflammatory cytokines and transcription mediators [19, 20]. Posttransplant survival was hampered by their non-specific action and their severe metabolic adverse effects. To address this challenge, various "tolerogenic" approaches were explored. Tolerance refers to a state of acceptance without immunosuppression, while prope tolerance is reached with a limited amount of immunosuppression. For instance, the use of donor spleen cells, epidermal cells, skin extract, and whole blood were utilized in skin transplant experiments in an attempt to promote graft acceptance [21, 22].

In 1963, Halasz et al. noted that improving dog skin graft survival was achieved by subcutaneous injection of 2 mL donor blood 10 and 5 days prior to transplantation. This approach demonstrated superior outcomes compared to transplantation without prior blood injection or with blood from a third-party donor (26 days vs. 10 days and 16 days, respectively) [23]. Subsequently, in 1964 in a canine allogeneic kidney transplant model, they demonstrated that pre-treatment with subcutaneous injection of 2 mL donor blood 10 and 5 days before

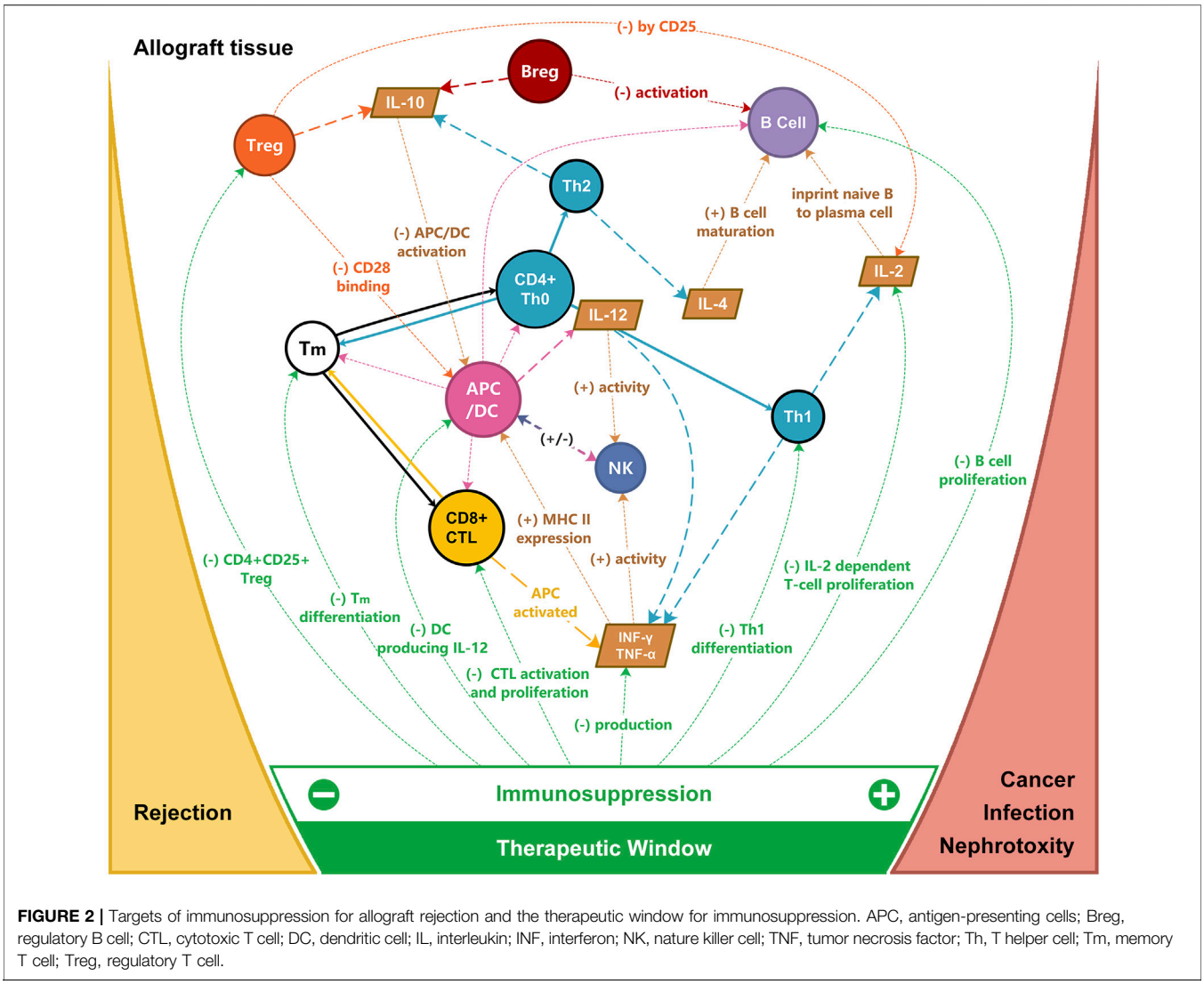

FIGURE 2 | Targets of immunosuppression for allograft rejection and the therapeutic window for immunosuppression. APC, antigen-presenting cells; Breg, regulatory B cell; CTL, cytotoxic T cell; DC, dendritic cell; IL, interleukin; INF, interferon; NK, nature killer cell; TNF, tumor necrosis factor; Th, T helper cell; Tm, memory T cell; Treg, regulatory T cell.

transplantation extended graft survival from 8 to 29 days. Of note, transfusion immediately after transplantation followed by repeated transfusion every 5th day prolonged survival albeit more modestly to 16 days [10]. Fabre and Morris later replicated these findings in a rat renal transplant model [(DA × Lewis)F1 → DA/Lewis] in 1972. Intravenous injection of 0.5 mL donor strain blood was given 1 or 7 days before transplantation or twice weekly for periods of 4 or more weeks. Longest survival was observed in the 7-day group [24]. Subsequent validation of irradiated DSBT in a rat pancreatic islet transplant model [Lewis ($RT1^l$) → ACI ($RT1^a$)] and non-irradiated DSBT with anti-CD28 antibody in a liver transplant model [DA ($RT1^a$) → Lewis($RT1^l$)] also confirmed the DSBT potential to improve graft acceptance and recipient survival [25, 26].

In 1980, Salvatierra et al. documented the first human application of DSBT in living donor renal transplantation with a one-haplotype match. A volume of 200cc of fresh (within 24 h) whole blood or equivalent packed cells (considering the regional blood bank preferences in subunit amount of blood and logistics of transfer or mailing blood from geographically distant donors) was administered three times at a two-week interval before living-donor transplantation. Immunosuppression was initiated 2 days before transplantation. No hyperacute or accelerated rejection was observed in 23 DSBT-treated patients who had lower 3-month rejection (44% vs. 82%), and higher 1-year graft survival (94% vs. 56%), compared to untreated patients with high mixed lymphocyte culture index. A total of 239 cases were monitored during 4 years. The graft and patient survival rate of recipients with 0 and 1 haplotype treated with DSBT were comparable to HLA-identical recipients without DSBT (graft survival: 82% vs. 84%; 4-year patient survival: 95% vs. 93%) [8, 9]. Our experience of the Leuven Immunomodulatory Protocol for human intestinal transplantation consists of the peritransplant administration of 400–600 mL of DSBT, along with a modified low immunosuppressive regimen and a series of maneuvers

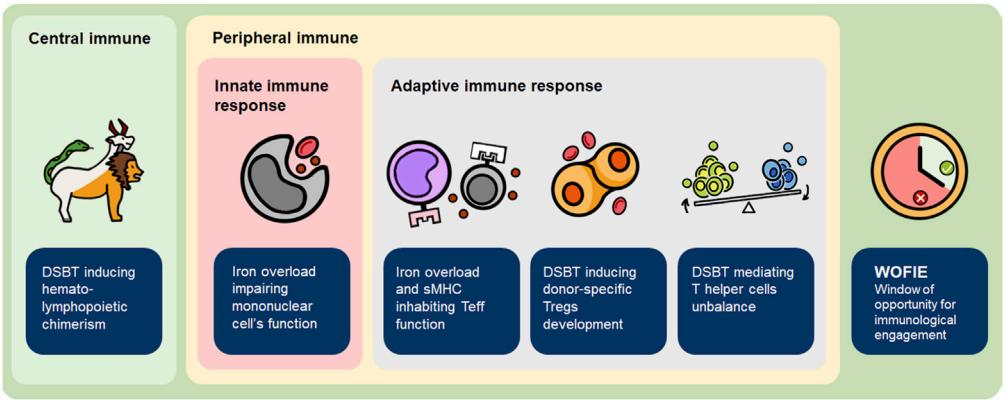

FIGURE 3 | Mechanism of how DSBT induces (donor-specific) tolerance. sMHC, soluble major histocompatibility complex molecules; Teff, effector T cell; Treg, regulatory T cell.

(ischemia and infection-free donor, selective bowel decontamination and glutamine administration, synchronizing donor and recipient surgery for a short ischemic time, etc.) aimed at promoting a low-inflammatory/pro-regulatory environment. No chronic rejection occurred in 13 treated intestinal transplant recipients with a 5-year graft/patient survival of 92% compared to a 5-year graft survival of 58% and patient survival of 61% [27, 28].

The potential benefits of DSBT have been demonstrated but the mechanisms by which DSBT operate remain unclear. Various hypotheses have been formulated (**Figure 3**):

DSBT With Transplantation Induces Hematolymphopoietic Chimerism

Chimerism refers to the stable persistence of a group of cells in another genetically distinct individual. In microchimerism (MC) the circulating cell population is below 5%. MC can be observed after non-leukoreduced and leukoreduced blood product transfusion and in transplant recipients, twins, and pregnant women [29, 30]. The lifespan of peripheral blood cells varies from hours to around 100 days [31]. Transfused leukocytes are expected to be completely cleared by alloimmune recognition and/or natural cell senescence. However, Lee et al. observed a transient proliferation of donor white blood cells in canine and human recipients circulation 3–5 days after unmanipulated packed RBC transfusion [32, 33]. Transfusion-associated microchimerism (TA-MC) was observed in 45% of severe trauma surgery patients and sometimes lasted for years [33, 34]. TA-MC may initially result from the proliferation of passenger leukocytes and, in the long term, from the differentiation of donor peripheral blood stem cells (PBSC), which can be present in the peripheral circulation at any time [35].

Like in trauma surgery, transplantation recipients may also suffer from peri-operative fluid loss, prolonged involuntary thermoregulation under anesthesia and/or extracorporeal life support, ischemia-reperfusion injury, and infection risks due to exposure to the environment (e.g., pathogens in recipient bronchus could contaminate the chest cavity during LTx). Their immune system is over-stressed, which creates a favourable environment for TA-MC to exist and continue to regulate recipient's immune system long-term after transplantation.

In a mouse allogenic femur transplant model, Bingaman et al. found that hematolymphopoietic chimerism (due to bone marrow transplantation) could lead to long-term donor-specific hyporesponsiveness [36]. In 1999, Spitzer et al. conducted a histocompatibility leukocyte antigen (HLA)-matched bone marrow and kidney transplant on a preoperatively induced female patient with multiple myeloma and end-stage renal disease. The low dose of cyclosporine monotherapy was completely withdrawn on Day 73. Renal function remained stable, with no evidence of acute or chronic rejection, and the patient survived over 5 years after transplantation [15, 16].

Starzl et al. proposed a two-way paradigm to explain how MC can induce tolerance. The outcome of transplantation is influenced by both host-versus-graft (HvG) and graft-versus-host (GvH) immune reactions, regulated by the migration and localization of the respective immunogenic leukocytes. If the donor antigen could primarily bypass or secondarily avoid collection by recipient lymphoid tissue, where the passenger leukocytes preferentially migrate to, the immune response could not be induced and the recipient could remain ignorant of the graft existence. This process is mediated by multiple cytokine and receptor pathways. For example, the persistence of both immune reactions could trigger mutual clonal exhaustion-deletion through FasL and tumor necrosis factor (TNF) pathways, which would be crucial for tolerance induction [37–40].

DSBT(WB) Impairs Mononuclear Phagocytic Cells and Effector T Cells Function

Following blood transfusion, a large amount of iron released from damaged red blood cells and present in plasma is phagocytized by monocytes. The iron homeostasis relevant intra-graft gene

expression can predict tolerance in liver transplantation. A higher serum level of hepcidin and ferritin and increased hepatocyte iron deposition were found in operationally tolerant liver transplant recipients [41]. The extracellular iron levels and the balance among ferritin generation and secretion and the primary form of ferrous iron storage play a crucial role in monocyte function [42, 43].

Excessive cellular iron level can impair transcription factor regulation, such as reducing activation of nuclear factor interleukin 6 (NF-IL6) and hypoxia-inducible factor-1 α (HIF-1α) and inhibiting phosphorylation of signal transducer and activator of transcription 1 (STAT1) [44]. NF-IL6 plays a central role in cytokine and iron-mediated regulation of nitric oxide synthase (NOS) expression. Reduced NF-IL6 can downregulate the expression of inflammatory cytokines (such as IL-1 and TNF) and granulocyte colony-stimulating factor (G-CSF) in mature macrophages [45, 46]. The absence of HIF-1α results in ATP level decreasing in macrophages further reducing phagocytosis and migration. HIF-1α deletion can cause a reduction of inflammatory cytokines such as IL-1β, IL-6, IL-10, TNF, and IFN-γ in macrophage and/or dendritic cells (DCs). HIF-1α is also essential for pro-inflammatory M1-type macrophage polarization and maturation of DCs [47–49]. STAT1 can regulate the number and phenotype of macrophages [50]. The STAT1 deficiency can abolish STAT1-dependent cellular response to both INF-α and -γ resulting in immunodeficiency [51, 52].

Iron overload can also directly affect the expansion and function of effector T cells. In patients with hereditary hemochromatosis, where iron overload is a prominent feature, the proliferative capacity, numbers, and activity of cytotoxic T cells ($CD8^+CD28^+$) were decreased, while the number of $CD8^+CD28^-$ T cells was increased [53–55]. Abundant $CD8^+CD28^-$ T cell numbers were associated with better graft function and reduced rejection by inhibiting antigen-presenting cell (APC) allo-stimulatory capacity in liver transplant patients [56]. Consequently, iron overload impairs phagocytosis and antigen presentation leading to decreased activation of effector T cells [57, 58].

Additionally, soluble MHC class I and II molecules (sMHC-I and sMHC-II) are distinctive components in DSBT(WB) compared to regularly stored red blood cell transfusions. sMHC molecule carries donor tolerogenic peptides that can bind to recipient T cells through T cell receptors (TCR). sMHC-TCR binding competing with recognition by APCs results in receptor blockade and apoptosis of recipient T cells due to the absence of co-stimulatory molecules [59–61]. Calne raised a concept of "the liver effect" to describe the immunosuppressive effect of liver transplantation on the other allograft [62]. Graeb et al. further showed in a rat [ACI($RT1^a$) → Lewis ($RT1^l$] model that donor liver-produced sMHC could suppress immune response in recipients and protect heart allograft from rejection [63]. The balance between sMHC-I and II has also been reported to be linked to immune homeostasis [64, 65].

Taken altogether, DSBT(WB) has the potential to block both the antigen presentation of mononuclear phagocytic cells and the activation of effector T cells. Transplantation in such an environment could therefore facilitate graft acceptance [66].

DSBT Induces the Development of Donor-Specific Tregs After Transplantation

Tregs account for 5%–10% of the T cell population in peripheral blood and contribute to immune homeostasis by regulating innate and adaptive immune responses [67]. Particularly $CD4^+$ Tregs expressing forkhead box protein 3 (Foxp3) have been shown to regulate alloimmune response after transplantation. In both animal studies and clinical research, it was found that Tregs interact with other effector T cells through inhibitory cytokines, cytotoxicity, and direct contact [68–71]. Compared to deleukocyted blood product, donor-Tregs could be transfused into recipients through DSBT(WB) and survive due to TA-MC, further mediating immune response after transplantation. Furthermore, it has been observed in a rat model [ACI ($RT1.A^aB^b$) → Lewis ($RT1.A^lB^l$)] that DSBT can also induce active expansion of $CD4^+$ T cells and donor-specific Tregs in the DSBT recipient's spleen by indirect allorecognition via residents DCs [72].

In the skin transplant model, CBA/Ca ($H\text{-}2^k$) mice received weekly intravenous transfusions of 0.25 mL whole blood from donor C57BL/10 ($H\text{-}2^b$) for five cycles. $CD4^+CD25^+$ T cells from the mesenteric lymph nodes and spleens of transfused mice, collected 1 week after the final transfusion, were co-transferred with naïve $CD45RB^{hi}$ cells (effector cells) into CBA-Rag deficient recipient mice 1 day before skin transplantation. This protocol induced long-term tolerance, with 100% skin allograft survival after 100 days without immunosuppression [73]. Similar outcomes were observed in rat heart and intestinal transplants [RA ($RT1^p$-$RT1A^c$:B/D^c) → PVG ($RT1^c$-$RT1A^u$:B/D^l)], where DSBT induced the development of $CD4^+CD45RC^-$ Tregs in recipients from post-transplant Day 5. These Tregs were highly effective in transferring donor-specific tolerance, as confirmed by adoptive cell transfer experiments. In addition to DSBT, the generation of Tregs requires the presence of graft, thymus, and spleen. These Tregs can be found in secondary lymphoid tissues and in the graft itself, suggesting a local protective effect [73–75].

Human transplant studies also support the role of Tregs in graft acceptance. In Leuven Immunomodulatory Protocol-treated intestinal transplantation recipients, a high level (1.8%) of circulating $CD4^+CD45RA^-Foxp3^{hi}$ memory Tregs was detected in the graft, correlating with long-term reduction of rejection [27]. Furthermore, the $Foxp3^{hi}$ Tregs subset was associated with improved outcomes graft and patient survival after kidney transplantation in a cohort of donor-specific hypo-responders [76–78].

DSBT Can Mediate the T helper Cells Unbalance Against Acute Rejection

Secretion of pro-inflammatory cytokines (IL-2, IL-12, TNF-α, INF-γ, etc.) by type 1 $CD4^+$ T helper cells (Th1) promotes differentiation of cytotoxic $CD8^+$ T cells, natural killer (NK) cells and macrophages which are associated with acute rejection. Conversely, the function of type 2 $CD4^+$ T helper cells (Th2) is more complex and depends on cellular targets, timing, and Th2 cytokine-dependent Tregs. Th2 produce cytokines such as IL-4, IL-6, IL-10 and IL-13 with both pro- and anti-inflammatory functions and hereby facilitate Tregs, mediate macrophage

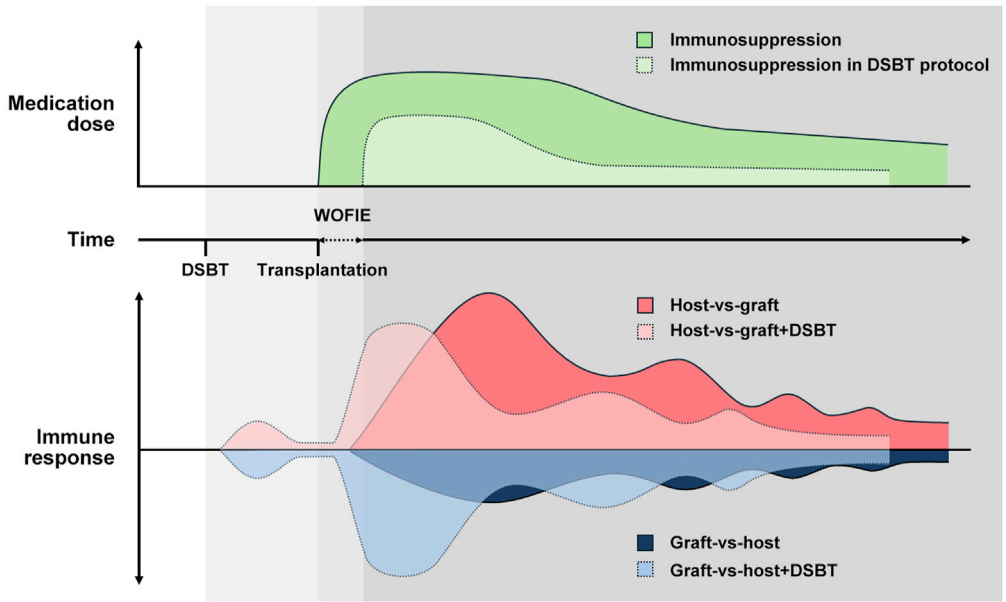

FIGURE 4 | How DSBT benefits from WOFIE and stimulates the regulatory immunologic mechanism according to the two-way paradigm hypothesis by Calne and Starzl. Despite the potential for HLA sensitization, DSBT does not provoke a strong or sustained immune response but creates a window of opportunity for immune engagement by promoting a balanced interaction between GvH and HvG responses. Non-specific excessive immunosuppression given before and immediately after the transplant can block this naturally occurring well-balanced tolerogenic response. On the longer-term lower levels of maintenance immunosuppression can prevent an overactive HvG response, thereby maintaining the balance and tolerogenic interaction.

polarization to M2 phenotype, and trigger B cells humoral immune response. Th2 are considered to be more linked to chronic rejection [79–84]. The balance of Th1/Th2 is implicated in the level of immune response post-transplantation.

In the rat model of DSBT [RA ($RT1^{p}$-$RT1A^{c}$:B/D^{c}) → PVG ($RT1^{c}$-$RT1A^{u}$:B/D^{l})], the Th1/Th2 cytokine profile differed, and a Th2 bias was observed after heart transplantation. On post-transplant day (POD) 5, INF-γ and IL-10 levels in allograft-rejecting rats peaked significantly higher than in DSBT-tolerized rats, whose levels did not peak until POD 9 and POD 30, respectively. IL-4 levels in DSBT-tolerized rats continued to rise until POD 30, while in allograft-rejecting rats, it peaked on POD 5 and was considerably lower than in DSBT-tolerized rats [85]. The mechanism by which DSBT modulates this Th1/Th2 balance remains unclear. A potential explanation is Tregs expansion after/by DSBT [86]. sMHC-I in DSBT may also regulate Th1/Th2 cytokine expression by decreasing IL-2 and granulocyte-macrophage colony-stimulating factor (GM-CSF) while increasing IL-4 and IL-15 [59].

DSBT May Stimulate the Regulatory Immunologic Mechanisms During "the Window of Opportunity for Immunological Engagement (WOFIE)" When the Immunosuppression is Postponed Initially for 72–96 h

The WOFIE theory is based on the two-way paradigm hypothesis of Calne and Starzl [38, 87–89]. Tolerance, like rejection, is an active immune response that relies on the balance between graft-versus-host (GvH) and host-versus-graft (HvG) reactions. This balance, crucial for graft survival, is influenced by the graft's immunogenicity, the recipient's immune system, and donor immune cells, resulting in graft rejection, tolerance, or GvHD. Despite the potential for HLA sensitization, DSBT does not provoke a strong or sustained immune response. Instead, DSBT creates a window of opportunity for immune engagement by promoting a balanced interaction between graft-versus-host (GvH) and host-versus-graft (HvG) responses.

Early after the allograft is exposed to the recipient's immune system, the GvH response is higher and HvG is lower in recipients who have received DSBT compared to those who have not. However, this increased GvH response remains balanced with decreased HvG, facilitating the development of immunological tolerance and potentially improving graft survival. Non-specific excessive immunosuppression given before and immediately after the transplant can block this naturally occurring well-balanced tolerogenic response. On the longer-term lower levels of maintenance immunosuppression can prevent an overactive HvG response, thereby maintaining the balance and tolerogenic interaction (**Figure 4**).

Calne et al. first introduced the concept of WOFIE in porcine renal transplant in 1994, administering irradiated leukocytes from the donor spleen to the recipient 6 h after transplantation, and creating a cyclosporine-free window of 48 h [88]. In 1998 they replaced leukocytes with DSBT on the day of transplantation and prolonged the cyclosporine-free window to 96 h in rhesus monkeys kidney transplantation

model [87]. In both models, this limited immunosuppression strategy was proved to be effective in improving graft acceptance. In rat intestinal transplantation [RA ($RT1^p$-$RT1A^c$:B/D^c) → PVG ($RT1^c$-$RT1A^u$:B/D^l)], Pirenne et al. demonstrated that DSBT-induced tolerance could be disrupted by high doses of methylprednisolone [90]. They further observed in rat heart transplantation [RA ($RT1^p$-$RT1A^c$:B/D^c) → PVG ($RT1^c$-$RT1A^u$:B/D^l)] that a low level of cyclosporine (10 mg/kg) given peri-transplant could lead to tolerance whereas a high level (50 mg/kg) compromised graft acceptance and recipient survival, by blocking the development of T regs. Additionally, administering 10 mg/kg cyclosporine on POD 0–4 failed to induce tolerance, but proved effective when given on POD 5–9 [91].

NEED TO EXPLORE THE POTENTIAL PROTECTIVE EFFECT OF DSBT IN LTX

In contrast to animal and clinical research in kidney, liver, heart and intestinal transplantation, the role of DSBT in LTx has not been explored. However, DSBT holds an important potential in addressing the shortcomings of postoperative immunosuppression after LTx. The conventional immunosuppressive regimen following lung transplantation typically consists of induction immunosuppression (anti-thymocyte globulin or Basiliximab) and a triple-drug combination of maintenance immunosuppression comprising a calcineurin inhibitor (cyclosporine or tacrolimus), an antiproliferative agent (azathioprine, mycophenolate, sirolimus, or everolimus), and corticosteroids (methylprednisolone and prednisone) [92, 93].

The lung is highly susceptible to rejection. The lung is a lymphoid organ exposed to the outside environment, accounting in part for its immunogenicity. In addition, epithelial cells can function as antigen-presenting cells and directly activate $CD4^+$ cells [94]. The immunogenicity of the lung renders LTx recipient dependent upon heavier chronic immunosuppression, compared to liver and kidney transplants, where patients may more easily transit to dual- or monotherapy with lower drug levels [95, 96]. Moreover, liver and kidney transplants can be performed with living donors. However, LTx is almost exclusively performed with organs from deceased donors, and therefore, the preoperative induction window is limited. LTx's prognosis is significantly inferior to other organs [97]. New strategies are necessary to overcome the issue.

Compared to DST, which faces limitations such as the unpredictable selection of donor cells, extended *ex vivo* proliferation time, and an elevated risk of combining with monoclonal antibodies, DSBT has the potential to be a more applicable and practical option in the setting of LTx for reducing postoperative immunosuppression with fewer technical and ethical constraints.

To translate the experience of DSBT in other solid organ transplants and further understand its mechanism, we need to first verify the safety of DSBT in LTx and rule out three severe complications:

Transfusion-Related Acute Lung Injury (TRALI)

TRALI is characterized by the onset of new acute lung injury within 6 hours of a blood transfusion, with no identifiable other risk factors. TRALI can occur in all kinds of blood products transfusion but most frequently in products with >60 mL of plasma [98]. The incidence of TRALI is 0.2‰, making it a leading cause of mortality associated with plasma-containing transfusions in the United States [99, 100]. Diagnosing TRALI can be challenging, particularly in distinguishing it from primary graft dysfunction (PGD) following LTx, as both complications present similar symptoms of hypoxemia and bilateral infiltrates on chest X-ray [99, 101]. Despite this similarity, TRALI is strongly linked to blood transfusions, while PGD may occur up to 72 h after LTx.

The exact mechanism of TRALI remains unclear, but it is believed to be triggered by donor leukocyte antibodies present in blood products [102]. Risk factors for TRALI include major surgery within 72 h, active infection, massive transfusion, and cytokine administration, which primes circulating hematopoietic cells before encountering antibodies, thereby increasing the risk of TRALI [99, 103–105]. Currently, there is no published data on TRALI in the DSBT animal model. In our own preclinical experience of DSBT in mice LTx, we did not observe any event of TRALI after iso- or allo-blood transfusion.

Hyperacute and Acute Rejections Due to "Transfusion-Related Sensitization" in Recipients

Hyperacute rejection rarely occurs after LTx, clinically featuring sudden hypoxemia, widespread pulmonary infiltration, and newly developed pulmonary hypertension within hours after reperfusion [106, 107]. Acute rejection is more common in about 10% of all adult LTx recipients within 1 year posttransplant [108]. In both rejections, preformed and *de novo* donor-specific antibodies (DSA) are the risk factors [109]. It has been confirmed in a rat model [ACI ($RT1.A^aB^b$) → Lewis ($RT1.A^lB^l$)] that DSBT can induce antibody-forming cells to produce DSA in the spleen [72]. Therefore, DSBT may sensitize recipients resulting in increased risks of hyperacute and acute rejection.

However, Ueta et al. found in the rat model that the *de novo* DSA were not detectable until Day 5 and reached a peak concentration on Day 7. These DSA targeted MHC-I on donor passenger T cells and suppressed acute GvHD [72]. While it is the anti-DQ (HLA-II) DSA that is more often considered associated with antibody-mediated rejection and worse prognosis in LTx [110–112]. Pirenne et al. also reported no hyperacute or acute rejection events in their DSBT-treated intestinal transplant patients [27]. Whether DSBT-induced DSA can sensitize LTx recipients and cause hyperacute and acute rejections remains controversial and should be closely monitored.

Transfusion-Associated Graft Versus Host Disease (TA-GvHD)

TA-GvHD is a rare fatal complication after blood transfusions. It is characterized by pancytopenia and multiple organ failure, likely

TABLE 1 | Clinical DSBT applications in different solid transplantations.

Year	Title	Donor	Organ	DSBT		Immunosuppression		Survival
				Volume	Time	Plan	Time	
1985 [9]	A seven-year experience with donor-specific blood transfusions. Results and considerations for maximum efficacy	Living	Kidney	200 mL/time	3 sperate occasions at approximately 2 week intervals	Azathioprine, prednisone (ATGAM for steroid resistant rejection) and cyclosporine (after introducing to clinics)	Since 2 days before transplantation	Patient 4-Year: 95%
2011 [125]	Beneficial effects of donor-specific transfusion on renal allograft outcome	Living	Kidney	150 mL/time	3 sperate occasions at approximately 2 week intervals	Azathioprine, methyl-prednisolone,cyclosporine	Since 2 days before transplantation	Patient 5-Year: 92.8% 8-Year: 81.5%
2016 [27]	The Leuven Immunomodulatory Protocol Promotes T-Regulatory Cells and Substantially Prolongs Survival After First Intestinal Transplantation	Deceased	Intestine	400–600 mL	One time collected during procurement, transfused perioperatively and finished before reperfusion	Induction: basiliximab or Thymoglobulin Maintenance[#]: tacrolimus, steroids, azathioprine and Thymoglobulin (refractory rejection)	Since POD 0	Patient 5-Year: 92%
2021 [127]	The use of organ donor blood in liver transplantation	Deceased	Liver	4 Units	One time collected during procurement, transfused perioperatively when needed for critical bleeding	Not mentioned	Not mentioned	Patient 5-Year: 87% 10-Year: 80%
2021 [126]	The Impact of Intraoperative Donor Blood on Packed Red Blood Cell Transfusion During Deceased Donor Liver Transplantation: A Retrospective Cohort Study	Deceased	Liver	500–900 mL	One time collected during procurement, transfused perioperatively when needed for critical bleeding	Not mentioned	Not mentioned	Graft Long-term*: 97.1%

*ATGAM, lymphocyte immune globulin; POD, postoperative day; # dose-decreased protocol; * which is defined as no retransplantation after 30 postoperative days.*

triggered by the proliferation of donor T cells in the circulation. These cells not only engraft but also attack host tissues, mirroring the pattern of GvHD [113, 114]. While case reports of TA-GvHD have been observed after liver, lung, and kidney transplantation [115–117], the underlying mechanisms remain inadequately explored.

TA-GvHD is particularly problematic in immune immature recipients, such as infants, due to their inability to recognize and eliminate foreign donor cells, coupled with the presence of shared HLA antigens, which are identified as primary risk factors [118–120]. In the DSBT protocol, the fresh whole blood is not irradiated and deleukocyted, which is an effective preventive measure against TA-GvHD [121]. For transplantation patients, partial HLA matching, especially when the donor is homozygous for an HLA haplotype while the recipient is heterozygous, results in the situation that the recipient's immune system fails to identify and clear the donor-specific leukocytes. In contrast, the donor leukocytes are activated to target the recipient tissue [122]. Transplant recipients could also be immunodeficient due to poor preoperative status and induction immunosuppression during the window of DSBT, which raises the risk of TA-GvHD. Retrospective analysis suggests that the dose of lymphocytes, approximately 10^7 lymphocytes/kg of recipient weight, correlates with the risk of susceptible TA-GVHD cases [123]. It suggests that the volume of DSBT is not "the more, the better," and the preoperative evaluation should be more cautious based on the HLA haplotype status.

An animal model of DSBT in LTx could provide essential insight into determining the best timing and dose of DSBT, immune cell differentiation after DSBT and the role of TA-MC in immune regulation. A previously published review has discussed the pros and cons of animal LTx models [124], and we propose that the rodent model stands out as a more suitable choice for DSBT research in LTx, considering factors such as cost, surgical complexity, and availability of analysis techniques.

FEASIBILITY OF DSBT IN LTX

Although it requires days before transplantation for DSBT's induction and after transplantation for WOFIE to induce tolerance in animal models, It has been proven in clinical liver, kidney and intestinal transplantations that DSBT is a feasible method combined with revised immunosuppression plan to improve graft/patient survival (summarized in **Table 1**) [9, 27, 125–127]. New hypothermic storage equipment permits preoperative organ preservation for a longer time up to a

maximum of 24 h, ensuring an adequate WOFIE, starting before transplantation and finishing at least before reperfusion or even earlier, for DSBT treatment in LTx [128, 129].

Our recent published data has already proven that DSBT is feasible and safe within the mice model with the same species setting as our mice LTx model [Balb/c ($H2^d$) → C57BL6/N($H2^b$)]. We observed no histological changes in mice's lung tissue or complications including fluid overload after a single DSBT, but the ratio of circulatory lymphocytes dropped after allo-transfusion compared to iso-transfused mice [C57BL6/N($H2^b$)→ C57BL6/N($H2^b$)] [130]. The ongoing pilot study observed a sequential hematological evolution and potential of immunoregulatory modulation with different DSBT protocols in mice LTx.

In conclusion, DSBT has demonstrated improved graft outcomes following solid organ transplantation (including liver, kidney, heart, and intestine) in various animal models and clinical studies. These findings could be applicable to LTx as well. Therefore, establishing a new animal model and protocol for LTx, along with further investigation into the underlying mechanisms, is essential.

AUTHOR CONTRIBUTIONS

Conceptualization: JP, LC, and XJ. Funding acquisition: XJ, RV, and LC. Methodology: XJ, JP, RV, CH, JK, JV, BV, and LC. Resources: JP and LC. Supervision: JP, RV, BV, and LC. Visualization: XJ. Writing-original draft: XJ. Writing-review and editing: 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.

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REVIEW
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Antibody-Mediated Rejection in Lung Transplantation: Diagnosis and Therapeutic Armamentarium in a 21st Century Perspective

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Humoral immunity is a major waypoint towards chronic allograft dysfunction in lung transplantation (LT) recipients. Though allo-immunization and antibody-mediated rejection (AMR) are well-known entities, some diagnostic gaps need to be addressed. Morphological analysis could be enhanced by digital pathology and artificial intelligence-based companion tools. Graft transcriptomics can help to identify graft failure phenotypes or endotypes. Donor-derived cell free DNA is being evaluated for graft-loss risk stratification and tailored surveillance. Preventative therapies should be tailored according to risk. The donor pool can be enlarged for candidates with HLA sensitization, with strategies combining plasma exchange, intravenous immunoglobulin and immune cell depletion, or with emerging or innovative therapies such as imlifidase or immunoadsorption. In cases of insufficient pre-transplant desensitization, the effects of antibodies on the allograft can be prevented by targeting the complement cascade, although evidence for this strategy in LT is limited. In LT recipients with a humoral response, strategies are combined, including depletion of immune cells (plasmapheresis or immunoadsorption), inhibition of immune pathways, or modulation of the inflammatory cascade, which can be achieved with photopheresis. Altogether, these innovative techniques offer promising perspectives for LT recipients and shape the 21st century's armamentarium against AMR.

Keywords: lung transplantation, antibody-mediated rejection, diagnosis, diagnosis biomarker, therapeutic approaches

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INTRODUCTION

Humoral immunity has been found to be a major waypoint towards acute and chronic lung allograft dysfunction (CLAD) [1–3], with the human leukocyte antigen (HLA) system being the tip of the iceberg. Alloimmunization and subsequent antibody-mediated rejection (AMR) are now well-known entities in solid-organ transplantation. Nevertheless, although the knowledge around AMR has been growing during the last two decades, several questions still arise in the clinical field of lung transplantation (LT). Difficulties in AMR diagnosis, which cannot rely solely on donor-specific antibody testing, are a significant challenge in LT, where the diagnosis of definite, clinical AMR is relatively rare event [4–6]. The presence of circulating antibody in the absence of clinical

graft dysfunction may be of no importance - or signify chronic, smoldering AMR that ultimately accelerates the onset of chronic lung allograft dysfunction and graft loss despite the absence of an acute syndrome. The biggest challenge in AMR diagnosis is to differentiate between these two types of apparently subclinical events.

Other challenges include treatment of alloimmunization in the pre-transplant period, in order to expand the donor pool for highly sensitized candidates. After transplantation, therapeutic challenges include the initiation of pre-emptive treatment of AMR in at-risk recipients, the actual treatment of clinical AMR and, in-between, the opportunity to treat subclinical AMR. These strategies have caveats, and the existing armamentarium is being enriched by therapies currently under investigation.

AMR IN LUNG TRANSPLANTATION: FROM MYTH TO REALITY

The earliest description of a harmful effect from antibodies directed against a graft was published in 1969 [7], describing increased frequency of immediate graft failure in kidney transplants with a positive crossmatch. The first case attributing hyperacute rejection in lung transplant to donor-specific anti-HLA antibodies (DSA) was published in 1996 [8]. However, the existence of AMR as a clinical entity in LT remained a matter of some debate even into the early 21st century [9]. A series of publications in the 2000s [10–13] describing histological patterns associated with HLA antibodies, and both acute and chronic graft dysfunction, contributed to the growing recognition of LT AMR, but the 2007 revision of lung rejection diagnosis and nomenclature [14] held back from making recommendations on AMR diagnosis. The authors cited insufficient knowledge, at the time of publication, of the true sensitivity and specificity of different diagnostic tools [14].

A multidimensional consensus statement aimed at standardizing AMR diagnosis was finally published in 2016 [15], and recommended diagnosis based on a combination of circulating DSA, evidence of complement activation, histological pattern, graft dysfunction and exclusion of other diagnoses, such as acute cellular rejection (ACR) or infection. The recommendations included three levels of diagnostic certainty (definite, probable and possible AMR) as well as definitions for clinical and sub-clinical AMR. The consensus statement allows a thorough classification and is the best tool currently available. A revision of these guidelines is ongoing and should be available in the next few months. However, several diagnostic gaps still need to be addressed. Emerging diagnostic strategies and novel molecular and digital techniques can improve the precision of AMR diagnosis and classification.

ADDRESSING DIAGNOSTIC GAPS OF AMR IN LUNG TRANSPLANTATION

Graft Dysfunction

Graft dysfunction is a major component of the diagnosis of clinical AMR in LT in the current guidelines. However, few studies clearly describe how graft dysfunction is diagnosed. As this is the sole feature distinguishing clinical and subclinical AMR [15] and it has prognostic implications [6], a clear definition is essential.

The ISHLT consensus statement defines allograft dysfunction as “alterations in pulmonary physiology, gas exchange properties, radiologic features or deteriorating functional performance” [15]. This description leaves much room for interpretation. Broadly, a 10% decline in the forced expiratory volume in 1 s (FEV_1) is frequently quoted as a threshold to intervene/investigate, but does this really indicate “graft dysfunction”? And how rapidly should the drop have occurred – intuitively, it seems that a 10% drop over 5 days has different implications to the same drop observed over a year. Furthermore, in the first 6–12 months post-transplant, when FEV_1 values are expected to improve until the patient achieves their baseline, even a stable FEV_1, as opposed to an increasing one, can be indicative of underlying abnormalities and graft damage. On the other hand, acute respiratory distress syndrome is obviously considered as graft dysfunction, even though the patient is unlikely to be able to perform pulmonary function testing in this context. Further studies should evaluate different dimensions of graft dysfunction and how these dimensions affect clinical outcome.

One potential avenue is enhanced assessment of CT images with the aid of quantitative image analysis and machine learning. The current AMR guidelines do not mention radiology, and its value currently lies in the assessment for other causes of acute graft dysfunction, such as infection or pulmonary edema, as well as guiding transbronchial biopsy. ACR and AMR may both present with ground-glass opacities, pleural effusions and interlobular septal thickening [16, 17]. However, machine learning techniques have already been shown to be capable of differentiating different phenotypes of CLAD and predict graft outcome [18, 19]. It is possible that a similar approach, applied in an acute setting, could help to differentiate different causes of acute graft dysfunction, including AMR.

Anti HLA Antibodies

Anti-HLA antibodies directed against the donor (DSA) have been associated with both acute and chronic rejection in kidney and heart transplantation. HLA antibodies are detected by single antigen bead (SAB) assays, in which latex beads coated with specific HLA antigens are mixed with the patient’s serum, and a secondary, fluorochorome-conjugated anti-human IgG antibody, is used to generate a fluorescent signal whose intensity corresponds to the amount of HLA-specific antibody bound to each bead. The mean fluorescence intensity (MFI) is what is usually reported to the clinician and used to guide management decisions; however, it is at best a semi-quantitative measure of circulating antibody concentration, with a measurement variability that may reach 25% [20]. Standardized cutoff values for positivity have not been established [21, 22]. Antibody binding sites, or epitopes, on each bead may be bound by more than one antibody, therefore it is possible to saturate the beads, and produce a high MFI even though the actual amount of any specific antibody present in the serum is relatively low; on the other hand, a single epitope might be present on several beads

(shared epitope), therefore reducing the MFI at the bead level. The antibody titer is a much more accurate measure of antibody concentration and is associated with graft outcomes.

Other antibody characteristics to consider include its specificity and complement activation capacity [23–26]. Class II DSA, particularly those against the DQ locus, carry a higher risk of CLAD and mortality compared to Class I DSA, or non-donor-specific HLA antibodies [2, 4]. Risk stratification according to DSA specificity should be considered, in order to guide (or refrain from) intervention. It should be noted that while research tends to focus on DSA, pre-transplant sensitization even with non-donor specific antibodies carries an increased risk of developing *de novo* DSA after transplant [27]. Older studies show an overall association between the presence of anti-HLA antibodies and shorter graft survival [3, 28, 29], though improved immunosuppression techniques, approaches to the sensitized transplant candidate and the ability to identify DSA have blunted this overall effect in the modern transplant era [3].

Patients can exhibit high titers of DSA without demonstrating any clinical graft dysfunction, while other patients can demonstrate graft dysfunction and other features of AMR without any detectable circulating DSA [30]. The existence of a clinical syndrome suggestive of AMR in the absence of circulating DSA is still debated, and explanations are speculative: missing donor HLA in the single antigen kit, intragraft DSA production with adsorption [31], epitope sharing [32], DSA targeting non HLA epitopes [33], and IgM DSA [25] are among the possibilities raised in DSA-negative cases otherwise suspicious for AMR.

Non-HLA DSA

The implication of non-HLA antibodies as the trigger for AMR has emerged in some reports [34–36], as it has been suspected in kidney transplantation [37]. Non-HLA DSA include non-HLA alloantigen, with more than 1,000 possible targets [37]; or autoantigen (e.g., collagen V, collagen I, and K-α 1 Tubulin, endothelin, AT1R) [34, 35]. If non-HLA DSA was previously considered to be a simple graft injury bystander, their harmful effect has been demonstrated by Xu et al. [33]. In this retrospective study, an increased risk of CLAD was observed in patients who had non-HLA DSA; moreover, a synergic effect was noted for patients who had both HLA- and non-HLA DSA [33].

Complement Activation

The clinical relevance of DSA may also depend on its capacity to bind complement. C1q binding has been cited as a potential adjunct to determine the significance of a circulating DSA, however, it is not one of the diagnostic criteria in the 2016 ISHLT consensus statement [15]. C1q activation requires the presence of 6 antibody molecules in close proximity and is thus a consequence of high concentrations of DSA [20]. C1q fixation has been associated with shorter time to CLAD and reduced graft survival [24], but this is not consistently demonstrated across studies [38]. More recently, Roux et al. [23] demonstrated an association between C3d activation and poorer graft survival rates. Moreover, a strong C3d activation was associated with higher DSA titers.

Tissue complement activation is detectable with positive C4d staining on the transbronchial biopsy (TBB). However, C4d staining can be associated with other conditions (infection, ACR, diffuse alveolar damage), and should therefore always be interpreted in the clinical context [15]. Conversely, negative C4d staining may reflect technical limitations rather than the absence of complement activation: C4d deposition in alveolar capillaries may be patchy or granular (and therefore interpreted as negative); interobserver agreement between different pathologists and different staining techniques has also been shown to be suboptimal [39, 40]. Moreover, a variety of non-complement dependent mechanisms of AMR have been described [41].

Aguilar et al demonstrated that even in C4d-negative AMR, 67% of patients had C1q positivity, suggesting that C1q and C4d act as complementary biomarkers of complement involvement in a given AMR process. However, no survival difference was observed between patients with C4d positive and C4d negative AMR, reinforcing the idea that C4d staining is not a necessary criterion for AMR diagnosis [39]. The added value of TBB C4d staining or circulating DSA associated C3d and C1q activation may be primarily in the selection of therapeutic strategies [42–44].

Histological Features of AMR

Histological features on TBB that have been identified to be suggestive of AMR include neutrophilic capillaritis, neutrophilic septal margination, organizing pneumonia and high-grade ACR [15]. Aside from the overlap with ACR or another concurrent diagnosis (**Table 1**), these features suffer from additional challenges in their role as diagnostic markers, including poor interobserver reliability [45, 46] and difficulty in identifying the compartmentalization of neutrophils in capillaries. Furthermore, reporting practices vary from center to center, which is a particular challenge for retrospective studies. Efforts such as the Lung Allograft Standardized Histological Analysis (LASHA) [47], are underway to standardize reporting practices.

The availability of high-throughput digital pathology slide scanners and advances in computing hardware, network spends and artificial intelligence (AI) tools, now allow the processing of large quantities of image data and have opened new avenues for AI to improve the accuracy and reproducibility of histopathological assessment. The success of such methods has been demonstrated in the oncology field, where deep learning algorithms were able to identify tumor features on pathology slides, and were associated with patient outcomes in lung cancer [48]; furthermore, they have even been shown to out-perform pathologists in detection of lymph node metastases on whole-slide images [49]. Studies are underway in heart, kidney and liver transplant [50]. In LT, the Duke University group [51] used AI to assess digitalized TBB slides annotated by LT pathologists to identify areas of normal lung tissue and A1/A2 grade ACR lesions. The algorithm was able to distinguish the vascular component of ACR with 95% accuracy. It is hoped that similar methods can be applied to the diagnosis of AMR to assist in identification of AMR-associated lesions.

TABLE 1 | Histological patterns suggestive of antibody-mediated rejection and its associated differential diagnosis.

Histological pattern	Differential diagnosis
Neutrophilic margination	Infection
	Ischemia-reperfusion injury (in a compatible timeline)
Neutrophilic capillaritis	Infection
	Ischemia-reperfusion injury (in a compatible timeline)
Acute lung injury pattern/diffuse alveolar damage	Infection
	Toxic inhalation
	Ischemia-reperfusion injury (in a compatible timeline)
Persistent/recurrent acute cellular rejection (any A grade)	Persistent/recurrent acute cellular rejection without AMR component
High-grade acute cellular rejection (≥A3)	High-grade acute cellular rejection without AMR component
	Infection
Persistent low-grade lymphocytic bronchiolitis (grade B1R)	Infection
	Gastroesophageal reflux disease
	Low-grade lymphocytic bronchiolitis without AMR component
High-grade lymphocytic bronchiolitis (grade B2R)	Infection
	Gastroesophageal reflux disease
	High-grade lymphocytic bronchiolitis without AMR component
Obliterative bronchiolitis (grade C1)	Chronic rejection
Arteritis	Infection
	Acute cellular rejection without AMR component
Any histologic findings in setting of DSA positivity (e.g., AFOP)	Infection

All diagnosis should be considered with consideration of clinical presentation, results of other investigations (bronchoalveolar lavage microbiology, DSA. . .) and response to therapies. AFOP, acute fibrinous and organizing pneumonia; AMR, antibody-mediated rejection; BAL, bronchoalveolar lavage; DSA, donor specific antibody.

Novel staining approaches are also being investigated. For instance, the phosphorylated S6 ribosomal protein was found to have a higher expression in TBB sampling of patients with AMR compared with controls [52]. These promising results should be confirmed in prospective studies.

EMERGING DIAGNOSTIC STRATEGIES

Transcriptomic Analysis

Transcriptomic analysis is increasingly being utilized in kidney and heart transplantation, in conjunction with a thorough clinical contextualization, to identify patterns of gene expression associated with rejection phenotypes. In LT settings, Halloran et al. [53] used microarray technology in TBB sampling, and identified archetypes associated with ACR, but not with AMR. The microarray approach has limitations, such as the need forof a dedicated lung sample, which is therefore unavailable for histological analysis, or the lack of accurate clinical contextualization, preventing the inclusion of transcriptomic results in a multidimensional approach. Transcriptomic analysis with alternative techniques, such as nanostring technology [54, 55], is under investigation for the diagnosis of AMR and other rejection phenotypes.

Donor-Derived Cell-Free DNA

Donor-derived cell-free DNA (dd-cfDNA) has recently gained a lot of attention as a marker of graft injury. Higher levels of dd-cfDNA have been observed in patients with clinical dysfunction associated with various injuries, including AMR compared to patients with stable function. Furthermore, a rise in dd-cfDNA preceded diagnosis of clinical AMR, but not ACR, by 2.8 months [5]. Another prospective multicentric cohort study reported a negative predictive value to rule out AMR or ACR ranging from 90% to 96% [56]. Although promising, these results deserve further validation. Keller et al. reported an independent association of dd-cfDNA level with CLAD or death. Unfortunately, the analysis suffers from a relatively small numbers of patients and the lack of assessment of the clinical severity of rejection episode, precluding any definitive conclusion on clinical utility in real life settings [57]. In our opinion, the lack of clinical contextualization would jeopardize this promising tool. Moreover, the severity of graft dysfunction should not rely on a biomarker but on clinical assessment.

EXISTING ARMAMENTARIUM FOR DESENSITIZATION, ALLOIMMUNIZATION, AND AMR

Anti-HLA antibodies can be found at all stages of the LT process and can impact the patient and the graft, on the waitlist or after transplantation. DSA is known to be associated with all forms of rejection, whether it be hyperacute [8], acute or chronic [10–13], thus leading to graft loss [1, 2, 58]. The identification of situations with risks for developing *de novo* DSA, and the calibration of the therapeutic response, are therefore vital in order to prevent—or limit—CLAD occurrence and evolution. Each clinical step corresponds to a specific scenario, but a shared armamentarium is used. A representation of AMR pathophysiology and the treatments discussed are depicted in **Figure 1**.

The preventive step aims at overcoming preexisting immunization in a candidate. In lung allografts, conflicting data have been reported with regards to the detrimental effects of pre-transplant sensitization. Several studies report an impaired

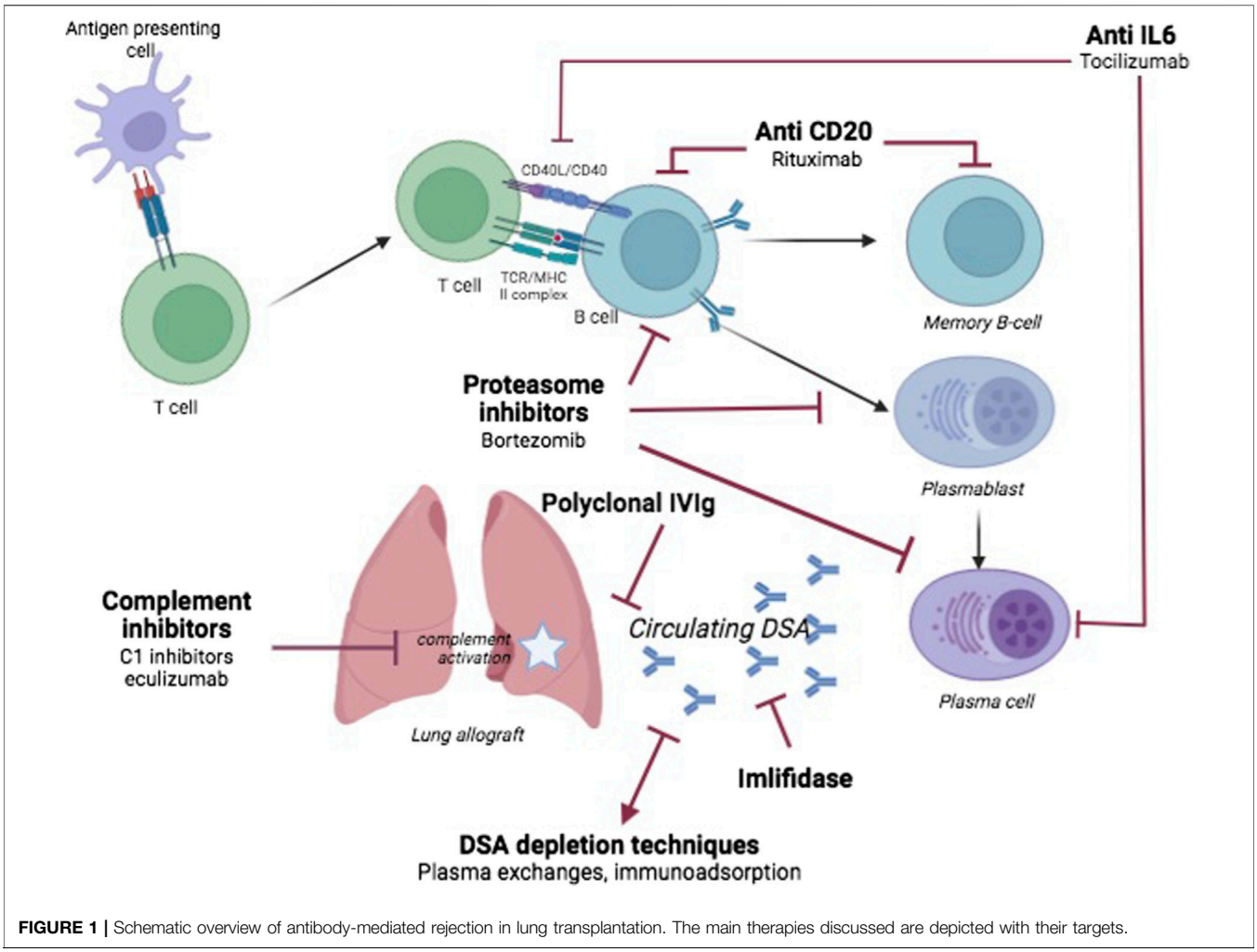

FIGURE 1 | Schematic overview of antibody-mediated rejection in lung transplantation. The main therapies discussed are depicted with their targets.

prognosis of LT recipients with a history of DSA [1, 2, 8, 11–13, 28, 58] but others did not identify any detrimental effect of pre-transplant sensitization [59] provided adequate screening is performed [60]. Regardless of the conflicting data, allosensitized candidates have a decreased likelihood of transplant compared with non-allosensitized, and higher odds of death on the waiting list [51, 52], with a significant association between elevated calculated panel-reactive antibody (cPRA), and decreased likelihood of transplant. In these cases, treatments administered before or during surgery aim to desensitize the recipient to anti-HLA antibodies and prevent the detrimental effects of pre-formed DSA at the time of transplantation [61–67]. A virtual crossmatch strategy based on historical immunization helps to mitigate the risks associated with the allocation of an allograft to a sensitized recipient. In our center, a virtual cross-match is systematically performed with the historical pre-formed DSAs detected on the date of listing. In most cases, allocation of a proposed graft to an immunized candidate with DSAs is forbidden if MFI is over 5,000. If the historical DSA MFI falls between 500 and 5,000, transplantation is allowed, but a peri-operative a desensitization protocol is applied [62].

After LT, the emergence of DSA in the absence of any clinical or histopathological pattern of AMR is still an issue, and the question of whether it should trigger early treatment or close monitoring remains unanswered.

The decision to initiate AMR treatment mandates the assessment of the clinical severity of the episode, the type of DSA and its epitope specificity, its titer (or mean fluorescence intensity - MFI), and the presence of possible complement activation. **Table 2** is a suggested proposal for a therapeutic approach to alloimmunization and AMR. Of note, it reflects only our center's approach, integrating bibliography, expert opinions, local experience, clinical constraints of specific patients, and logistic constraints of facilities. It is therefore open to discussion, including within our own team, and should be considered with caution, tailored to each patient, and adapted to the circumstances of each center and HLA laboratory.

Currently available treatments can target each step of the humoral response, usually in combination: antibody removal, inhibition of antibody production, inhibition of DSA effect and immunomodulation.

TABLE 2 | Therapeutic approaches to alloimmunization and antibody-mediated rejection including subclinical and clinical forms, with or without complement activation.

Mechanism and therapy	Indications	Schedule and duration	Effect onset	Side-effects
DSA Clearance				
Plasma exchanges Immunoadsoprtion	Alloimmunization with high MFI and anti-DQ Clinical AMR with or without complement activation	Plasma exchanges: daily, 5–7 days	Few hours	
Imlifidase	Pre-LT desensitization Rescue for clinical AMR with or without complement activation	Once	Few hours	Infection; transaminitis; Anaphylaxis, serum sickness
DSA production inhibition				
Anti CD20	Alloimmunization Subclinical or clinical AMR, with or without complement activation	375 mg/m^2 twice at D0 and D7 to D30; can be repeated at M6	72 h for B-cell depletion; weeks to months for antibody decrease	Anaphylaxis; neutropenia; hypogammaglobulinemia; infection
Proteasome inhibitors – Bortezomib	After anti CD20, in case of persisting DSA and subclinical or clinical AMR, with or without complement activation	1.3 mg/m^2, divided in 4 infusions between D1 and D11	Few hours for plasma cell depletion; weeks to months for antibody decrease	Peripheral neuropathy; neutropenia
Neutralization of intra graft DSA				
IVIG	Subclinical or clinical AMR, with or without complement activation	2 g/kg monthly for 6 months	Few hours	Renal impairment; hypervolemia; hyperviscosity
Complement inhibitors	Clinical AMR, with complement activation	C1-esterase inhibitor: 20 IU/kg twice weekly for 6 months	Few hours	Encapsulated infectious counterparts

It has to be underlined that existing evidence does not allow to draw definitive conclusions: different strategies and associations are reported, at different time-points of the LT recipient course, and in various situations of moving definitions. We therefore aim to list this existing armamentarium, and potential areas for future research.

Antibody Removal

Antibody removal is a cornerstone of the treatment of humoral response for both desensitization and AMR treatment. It can be achieved by plasmapheresis or immunoadsoption.

During plasmapheresis, plasma with its protein components is removed, and replaced by colloids, albumin, or fresh frozen plasma. Plasma exchange removes anti-HLA antibodies; but as it is a non-specific therapy, it also removes other large proteins, including coagulation factors, and anti-infectious immunoglobulins. Replacement by colloids, albumin, or fresh frozen plasma is essential to avoid coagulopathy [68]. In LT, the experience reported by Snyder et al. [64] is disappointing. They report the outcome of 18 highly sensitized candidates who underwent a desensitization protocol, including plasmapheresis, bortezomib, rituximab, and IVIg, among whom 9 received a LT. Their prognosis did not differ significantly than their non-desensitized alloimunised counterparts. The experience of the Toronto team, in a similar peri-operative situation, is more optimistic, and reported in both short-(65) and long-term [63] perspectives. Their strategy includes an assessment of the immunological risk, and a combination of plasma exchanges, IVIg, and thymoglobulin. Among 340 patients in their cohort, 53 had DSA. Four did not undergo any treatment. All of the remaining received plasma exchange, 43 received IVIg, and 23 of those received thymoglobulin. Interestingly, the DSA-positive patients were less likely to develop grade 2 ACR, similarly to those who had a cPRA above 30%, but no DSA. Respiratory function, 30-day survival and 1-year survival did not differ between groups (DSA-positive, cPRA>30% or neither) [65]. They extended the follow-up of these patients for a median of 6.7 years, and showed no differences in graft survival, CLAD-free survival, or overall survival [63]. In a study by the Foch Lung Transplant group [62], a perioperative desensitization protocol was applied in all recipients who had preformed DSA with MFI between 500 and 5,000. The protocol included one pre-operative plasma exchange followed by 5 plasma exchange sessions starting on postoperative day-1, a rituximab infusion, and finally 2 g/kg intravenous immunoglobulins. The mycophenolate mofetil dosage for maintenance immunosuppression was also increased if the MFI of the preformed DSA was above 1,000 on day 0. In this series, the 39 patients who had been desensitized because of high preformed DSA were compared to the 66 who had low preformed DSA, and the 216 who were not pre-sensitized. The outcome did not differ according to the presence of preformed DSA, in terms of freedom from CLAD, or 1 and 3-year graft survival. In contrast, these outcomes differed significantly according to successful clearance of the DSA. These data support an aggressive strategy of preformed DSA clearance in order to improve long-term outcomes.

Immunoadsorption has been developed in order to specifically remove IgG. As with plasma exchange, the plasma is separated from the whole blood, but instead of being discarded, it circulates through a column coated with a protein that binds the fixed region of immunoglobulins. Only the immunoglobulin antibodies are adsorbed, while other circulating proteins are reinjected into the patient, allowing a massive decrease in total IgG. Various immunoadsorption devices have been developed over time, in order to refine the removed proteins. Some use immobilized antibodies and deplete all subclasses of IgG. Others use immobilized staphylococcal protein A, and deplete IgG

autoantibodies, and circulating immune complexes containing IgG. Moreover, these devices are thought to carry beneficial immunosuppressive effects via B-cell apoptosis. Finally, some columns might carry immobilized antigens or synthetic epitopes in order to only extract the antibodies that are reactive with a single antigen [69]. The Duke University LT program team reported its experience with a desensitization regimen including IVIg and extracorporeal immunoadsorption [70]. In this center, during an 11-year period, 12 patients who had anti-HLA antibodies at the time of transplantation were desensitized, while 23 were not. Patients who underwent desensitization had fewer episodes of acute rejection and higher (although non-significant) freedom from BOS in the first 3 years. These data support the efficacy of a strategy which encompasses extracorporeal immunoadsorption, but, to our knowledge, no comparison between different antibody removal techniques has been performed.

Inhibition of DSA Effects

The use of intravenous immunoglobulins (IVIg) was first reported in kidney transplant [71, 72]. They are believed to neutralize the existing antibodies, blocking the effect of DSA on the allograft, and to downregulate B-cells. To date, most protocols reported in LT, whether it be for desensitization [64, 65], or treatment of alloimmunization with or without AMR, include IVIg in combination with other therapies [1, 58, 73–76].

Inhibition of Antibody Production: Administration of B-Lymphocyte or Plasma-Cell Depletion Treatments

Most of existing protocols consider the use of B-lymphocyte or plasma-cell depletion treatments [1, 58, 73–76], such as rituximab (anti-CD20 antibody) and bortezomib or carfilzomib (proteasome inhibitors), to be mandatory. Their administration aims at inhibiting the production of DSA and is a complementary step to DSA removal. Rituximab is widely used, either for desensitization [62], in alloimmunization [58, 73, 75] and AMR [1, 75]. It has been combined with bortezomib [64, 75, 76] or thymoglobulin [65]. One has to bear in mind the delay of action of rituximab, which depletes B cells within 72 h, but does not affect plasma cells or existing antibody levels. The effect on DSA is therefore seen only after a few months [77, 78]. Thymoglobulin, on the other hand, depletes B cells, T cells, NK cells and terminally-differentiated plasma cells, exerting a more immediate effect on antibody production [79, 80]. Proteasome inhibitors induce apoptosis of plasma cells, via the accumulation of ubiquitinated proteins. For instance, carfilzomib acts within 1 h of first administration and is thought to inhibit proteasome function for >48 h after each dose.

EMERGING THERAPIES

Other strategies are being used in case reports or small series. The following strategies are still being scrutinized and not routinely used in LT at the time of writing.

Imlifidase

Imlifidase is an IgG-degrading enzyme derived from *Streptoccous pyogenes*. It inactivates IgG antibodies by cleaving their lower hinge region. It has been successfully tested in highly immunized kidney transplant candidates [81], in spite of the risk of a secondary antibody rebound between day 3 and day 14 [81]; moreover, it has been reported to have an excellent post-transplant prognosis [82]. Such results had even led to a French consensus report on hypersensitized kidney transplant candidates [83], positioning imlifidase as an alternative to apheresis. In the LT literature, a single case has been published [66]. In this highly immunized candidate, LT was made possible after a dramatic decrease of anti HLA antibodies secondary to imlifidase administration and followed by an aggressive desensitization strategy with C1 esterase inhibitor, plasma exchange, alemtuzumab and IVIg.

Targeting of the Complement Cascade

The complement cascade is suspected to be an important pathway of AMR induced lung injury [15, 55], as it is in kidney [84], liver, and heart transplantation. Anti-complement drugs have therefore been used, in order to mitigate the local inflammatory response and thus the local effects of AMR. They are expected to be effective in cases of AMR with evidence of circulating complement-activating anti-HLA DSA [44].

Eculizumab, an anti-C5 monoclonal antibody, has been reported to be effective in various settings. For instance, in AMR occurring in the early course of LT, it has been used in combination with a more conventional strategy of AMR treatment [85, 86]. Both cases report the successful treatment of AMR in a LT recipient, either with hyperacute AMR on post-operative day-2, combined with bortezomib, rituximab, IVIg, and plasma exchange [85]; or acute AMR, occurring on post-operative day 7, and successfully treated with a combination of eculizumab, IVIg and rituximab [86]. Highly sensitized kidney [87] and heart [88] transplant recipients treated with eculizumab have been found to have a better prognosis than their non-treated counterparts. The integration of eculizumab in a desensitization strategy in highly sensitized candidates is an interesting possibility that should be investigated.

C1-esterase specific inhibitors are still being scrutinized in AMR, mostly in kidney recipients [42]. Data are scarce in LT. After being investigated in the very early course of LT in order to limit primary graft dysfunction [89, 90], the use of C1-esterase inhibitors has been reported in 2 LT recipients with AMR refractory to standard of care [43], with successful treatment. Both patients had acute respiratory failure, with DSAs, and histology pattern consistent with AMR. While one of the patients had very early respiratory failure (on post-operative day-2), the other had respiratory failure 3 years after LT. Both patients received the treatment for a prolonged course of 6 and 7 months respectively. The first patient improved, and the deterioration of the second plateaued, stabilizing the patient and allowing retransplantation. In both cases, IVIg was maintained along with C1-esterase inhibitors. No adverse effect has been described in these two cases. In spite of this encouraging case report, and of a strong pathophysiological rationale to use this therapeutic strategy, the research on C1-esterase inhibitors in pulmonary AMR remains sparse: to date, no trial is registered in the ClinicalTrials.gov database.

Immunomodulation by IL-6 Inhibitors

The use of the IL-6 inhibitor tocilizumab for AMR in LT is a matter of debate. Tocilizumab is a potent anti-inflammatory treatment and has been reported to treat kidney AMR [91, 92]. It has been reported in a single retrospective case series in LT [93]: the authors compared the outcome of 18 LT recipients who were diagnosed with AMR (Definite, n = 5; Probable, n = 12; Possible n = 1) receiving combination therapies, with 9 LT recipients with AMR (Definite, n = 2; Probable, n = 7) whose combination included tocilizumab. The results are encouraging, albeit non-significant: tocilizumab recipients had more DSA clearance, less DSA recurrence and less development of new DSA. Interestingly, whereas lung function did not differ either at AMR diagnosis or at follow-up, graft failure was significantly lower in the patients receiving tocilizumab. While this interesting paper has some limitations, it nevertheless provides valuable data, that could pave the way for a prospective trial. Clazakizumab is another IL-6 inhibitor, which is being investigated in a phase 3 trial in chronic AMR in kidney transplant recipients (NCT 03744910). To date, no trial in LT has been reported.

Extracorporeal Immunomodulation by External Chemo-Phototherapy (ECP)

ECP is a therapy used in various situations including in solid organ transplantation [94]. In LT, it has been mainly investigated in chronic lung allograft dysfunction [95–104]. It is based on the principle of isolation of white blood cells into an extracorporeal circuit, their sensitization to ultraviolet radiation by a photoactivable material (8-methoxypsoralen), and, after UV-A exposure, reinjection into the patient's circulation. It is thought to promote induction of lymphocyte apoptosis and production of T regulatory cells. A single paper by the Vienna team describes the use of ECP in AMR [105]. In this single center retrospective study, ECP was used as an add-on therapy in 16 of 41 LT recipients with AMR. The first-line treatment was immunoadsorption in 14 of these 16 patients, ATG + IVIg in one patient and ATG alone in one patient. Two of the immunoadsorption patients also received IVIg, and 2 others received ATG. The authors report a reduction of *de novo* DSA titers, a 1-year survival of 55% and a 1-year graft survival rate of 61%. This study provides encouraging results and demands ongoing investigation of this strategy. The study EXPORT-DSA, led by the Vienna LT team, is registered in the ClinicalTrials.gov database (NCT06112951). It is a prospective randomized trial of ECP in patients with persistent *de novo* DSA, without any sign of graft dysfunction. This study is not recruiting at the moment but should provide valuable insights on possibility of reducing DSA with ECP treatment.

CONCLUSION

As the pathophysiology of antibody-mediated rejection is better and better understood, the unmet needs in diagnosis and treatment progressively shrink. Several unanswered questions in AMR diagnosis may be addressed with the help of big data and novel diagnostic strategies. While there remains a great deal of heterogeneity in approaches to alloimmunization and AMR treatment, a tailored phenotypic characterization would allow a multimodal therapeutic approach, with innovative techniques and treatments, some of which are already in use in other organ transplantation fields. They provide promising perspectives for LT recipients and shape the 21st century's armamentarium against AMR.

AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH
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Treatment Responses in Histologic Versus Molecular Diagnoses of Lung Rejection

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Histologic evaluation of allograft biopsies after lung transplantation has several limitations, suggesting that molecular assessment using tissue transcriptomics could improve biopsy interpretation. This single-center, retrospective cohort study evaluated discrepancies between the histology of transbronchial biopsies (TBBs) with no rejection (NR) and T-cell mediated rejection (TCMR) by molecular diagnosis. The accuracy of diagnosis was assessed based on response to treatment. 54 TBBs from Prague Lung Transplant Program obtained between December 2015 and January 2020 were included. Patients with acute cellular rejection (ACR) grade ≥ 1 by histology received anti-rejection treatment. Response to therapy was defined as an increase in FEV1 of ≥ 10% 4 weeks post-biopsy compared to the pre-biopsy value. Among the 54 analyzed TBBs, 25 (46%) were concordant with histology, while 29 (54%) showed discrepancies. ACR grade 0 was found in 12 TBBs (22%) and grade A1 ≥ 1 in 42 TBBs (78%). Treatment response was present in 14% in the NR group and in 50% in the TCMR group ($p = 0.024$). Our findings suggest that low-grade acute cellular rejection is less likely to be associated with molecular TCMR, which might better identify lung transplant recipients who benefit from therapy.

Keywords: lung transplantation, acute cellular rejection, histopathology, gene expression, molecular biology

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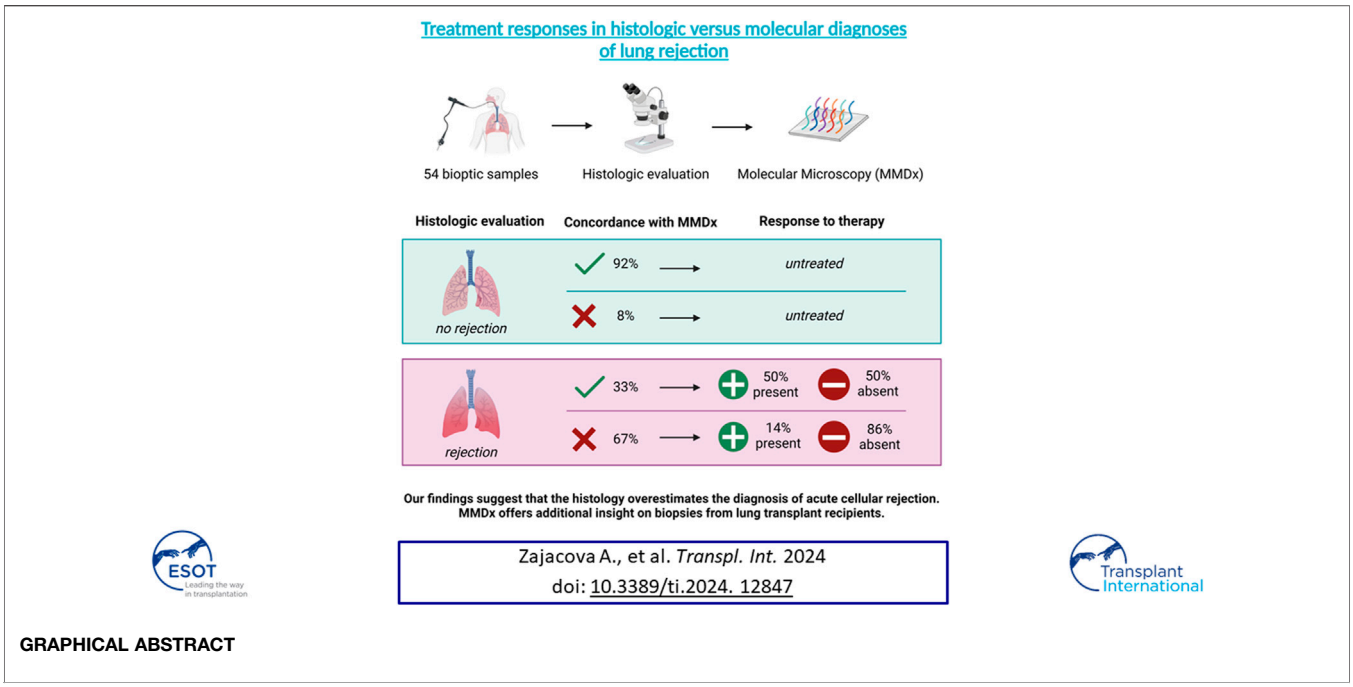
INTRODUCTION

Lung transplant recipients (LTRs) face the shortest long-term survival among all of the major solid organ transplant recipients, with median survival of 6.7 years [1]. Lungs, as an open system in constant communication with the environment, possess an efficient immune complexity that serves a beneficial purpose as a barrier to infections. On the other hand,

Abbreviations: ACR, acute cellular rejection; ATG, antithymocyte globulin; CF, cystic fibrosis; CLAD, chronic lung allograft dysfunction; COPD, chronic obstructive pulmonary disease; dd-cf DNA, donor-derived cell-free DNA; FEV1%, percent predicted forced expiratory volume in 1 second; ILD, interstitial lung disease; IPF, idiopathic pulmonary fibrosis; LuTx, lung transplantation; LTRs, lung transplant recipients; MMDx, Molecular Microscope Diagnostic System; NR, no rejection; TBB, transbronchial biopsy; TCMR, T-cell mediated rejection.



GRAPHICAL ABSTRACT

the potency of this system leads to a high rate of immune-mediated complications—of those, acute cellular rejection (ACR) is the most prevalent, affecting both morbidity and survival [2].

The management of ACR is limited by problems with the available diagnostic tools. The non-invasive tools routinely used for graft health monitoring, such as pulmonary function tests or radiological methods, lack both sensitivity and specificity for the diagnosis of ACR. Transbronchial biopsy (TBB) remains the gold standard for obtaining the diagnosis, despite its numerous limitations. Histologic evaluation is based solely on the abundance of perivascular and peribronchiolar lymphocytes, overlooking the composition and function of the immune cell subsets [3]. Obtained biopsy samples differ in size and quality and as demonstrated in the LARGO study, inter-pathologist interpretation of transbronchial biopsy for ACR is highly variable and limitedly reproducible [4].

Given the mentioned limitations, therapeutic strategies especially in minimal and mild ACR (grade 1 and 2) remain variable and often depend on the clinical condition of the patient, as well as on the preference of the physician [5].

Molecular analyses performed by the Molecular Microscope® Diagnostic System (MMDx) may allow us to overcome the limitations of histopathology by performing microarray analysis of numerous transcripts, followed by both unsupervised and supervised analysis. This approach, already established as a standard-of-care in heart and kidney biopsies, aims to differentiate between diverse pathophysiological pathways of both immune- and injury-mediated processes, offering promising precision in distinguishing ACR from histopathologically similar conditions, such as regulatory or reperfusion changes in LTRs.

Despite the promising results of the INTERLUNG study, demonstrating lower variability and higher accuracy in assessing T-cell mediated rejection (TCMR) by MMDx in comparison to histology [6, 7], to the best of our knowledge no study to date has compared the accuracy of MMDx to standard histologic diagnosis in relation to treatment response. In this cohort, we aimed to describe the discrepancies between TBBs classified as no rejection (NR) and TCMR by MMDx compared to conventional histopathological evaluation and to assess the diagnostic accuracy of both methods based on patient treatment responses. Our assumption was that the more accurate diagnosis of TCMR would correspond to a greater response to TCMR treatment.

MATERIALS AND METHODS

Study Population

We conducted a single-center retrospective cohort study based on prospectively collected transbronchial biopsies with all relevant clinical data obtained through in-depth review of the patients' medical records.

A total of 134 TBBs from patients transplanted between November 2004 and October 2018 were obtained between December 2015 and January 2020. All TBBs were examined by both histology and MMDx as a part of the multi-center INTERLUNG study (ClinicalTrials.gov #NCT02812290). For the purposes of our study, we selected TBBs that exhibited results of NR and definite TCMR according to MMDx with available pre- and 4 weeks post-biopsy spirometry. TBBs with rejection-like changes, inflammation, and injury detected by MMDx were excluded as our focus was on evaluating

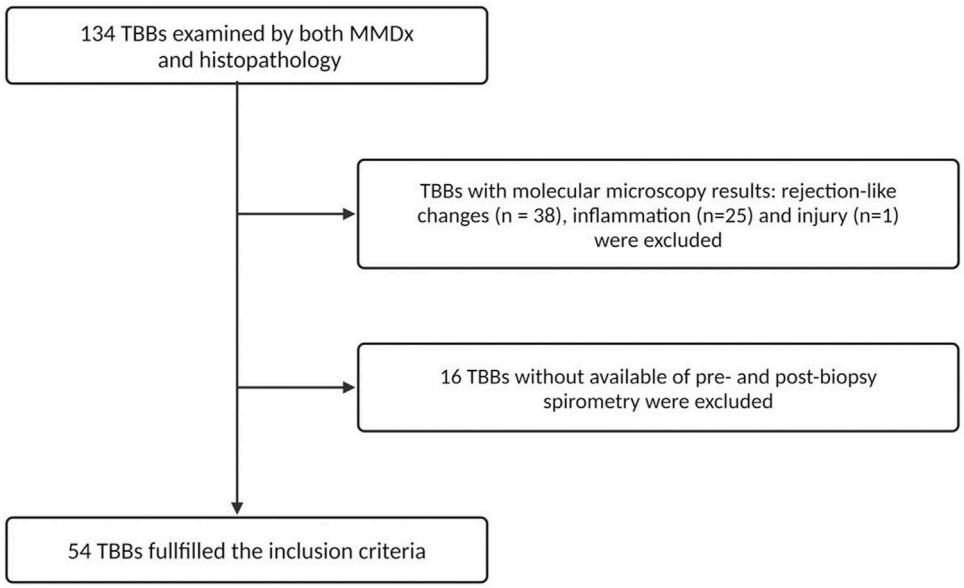


FIGURE 1 | Consort diagram of the TBBs included in the study.

discrepancies in TCMR detection between MMDx and histology (**Figure 1**).

All of the included LTRs diagnosed with ACR grade A1 or higher by histology received anti-rejection treatment (corticosteroids, antithymocyte globulin, or alemtuzumab). Response to treatment was assessed 4 weeks after the biopsy and defined as an increase in percent predicted forced expiratory volume in 1 s (FEV1%) ≥ 10% of FEV1% before biopsy. To assess the decline in function at the time of TBB, we calculated the FEV1 decline as the ratio of the FEV1% expected (the mean of the last two FEV1% measurements prior to the pre-biopsy value) to the pre-biopsy FEV1%.

Surveillance biopsies were performed at 1st, 3rd, 6th and 12th month after lung transplantation (LuTx). Biopsies for cause were indicated based on the clinician's decision, mainly due to a drop in lung function and/or radiological correlation. All patients with bioptically proven rejection received treatment. The center's therapeutic protocol consists of corticosteroids in the first line, with oral escalation to 50 mg of prednisone and subsequent tapering for asymptomatic A1, and high-dose methylprednisolone (MP) for ACR grade ≥ A2 and symptomatic A1 rejection. Second-line treatment options, including anti thymocyte globulin (ATG) or alemtuzumab, were administered when initial corticosteroid therapy failed to demonstrate benefit. This study was approved by the Motol University Hospital Ethics Board. A written consent was obtained from each patient whose TBB specimens were used.

Histology

TBBs for the histopathology evaluation were obtained during both surveillance and indication cryobiopsies. All histologic samples were fixed in neutral buffered 4% formaldehyde, postfixed and embedded in paraffin. The paraffin blocks were then sectioned into 4-μm-thick histological sections and stained with hematoxylin-eosin, Masson's trichrome, orcein, Prussian-blue and periodic acid-Schiff staining.

All TBBs were also examined using immunohistochemistry for the following markers: CD45RO, CD8, CD4, CD20 and C4d. Three μm thick histologic sections were used, and each sample was stained using the following antibodies and protocols: anti-CD45RO antibody (mouse monoclonal antibody, clone UCHL1 [Agilent–Dako, Santa Clara, CA, United States], dilution 1:300, pre-treatment by heating in a buffer solution of pH6 in a water bath), anti-CD8 antibody (mouse monoclonal antibody, clone: C8/144B [Dako], dilution 1:200, pre-treatment by heating in a buffer solution of pH9 in a water bath), anti-CD4 antibody (mouse monoclonal antibody, clone: 4B12 [BioGenex], dilution 1:250, pre-treatment by heating in a buffer solution of pH9 in a water bath), anti-CD20 antibody (mouse monoclonal antibody, clone: L26 [Dako], dilution 1:300, pre-treatment by heating in a buffer solution of pH6 in a water bath) and anti-C4d antibody (mouse monoclonal antibody, clone ZM78 [ZETA Corporation, Sierra Madre, CA, United States], dilution 1:150, pre-treatment by heating in a buffer solution of pH9 in a water bath). The detection was performed using a one-step micro polymeric non-biotin system (Bio SB—Bioscience for the World, Santa Barbara, CA, United States) with a peroxidase and 3,3′-diaminobenzidine tetrahydrochloride solution. The nuclei were counterstained with hematoxylin. TBBs were evaluated by three LuTx-focused pathologists.

MMDx

TBBs for the MMDx analysis were procured during a standard cryobiopsy procedure, during which two samples were collected. From the larger one, a small piece of tissue (2 mm × 2 mm × 2 mm) was excised and stored in RNA-later, which was followed by

immediate freezing down to −70°C, while the rest of the tissue was used for a histopathological examination. The frozen biopsy specimens were then shipped in batches on dry ice to the Alberta Transplant Applied Genomics Centre/TSI (Edmonton, AB, Canada) for RNA extraction, labeling (3' IVT plus labeling kit), and hybridization to PrimeView™ GeneChips® (Applied Biosystems, Thermo Fisher Scientific, United States) by MMDx diagnostic system. The data were preprocessed using robust multiarray averaging. Genome-wide mRNA measurements were used to assign each biopsy a molecular diagnosis. An ensemble of supervised and unsupervised machine-learning algorithms trained on gene expression data from a large reference set of lung transplant biopsies, including expanded dataset of 744 TBBs (all surfactant level) with a subset of 600 TBBs (high surfactant level), was used to classify biopsies into four archetypal groups—No Rejection (NR), TCMR, rejection-like and inflammation [6, 7]. Only TBBs with NR (absence of inflammation/rejection transcripts) and definite TCMR (effector T-cell transcripts and INFG effects) were further included in the study, as depicted in the consort diagram (**Figure 1**). TBBs with rejection-like and inflammation archetypes identified by a higher expression of injury/repair, macrophage and endothelial-associated transcripts, but lacking T-cell associated transcripts, were excluded. This exclusion criterion was applied to maintain the study focus on assessing TCMR detection through MMDx.

Statistical Analysis

All statistical analyses and visualizations were performed using GraphPad Prism 9.0 (San Diego, United States) and R version 4.1.3.[1] Wilcoxon-Mann-Whitney and Fisher's exact tests were used for continuous and categorical variables, respectively. The correlation between FEV1 decline and response to treatment (FEV1% change 4 weeks after biopsy) was evaluated using Spearman's correlation. Both univariate and multivariate logistic regression analyses were conducted to identify significant predictors of treatment response. Odds ratios (OR) with 95% confidence intervals (CI) and p-values were calculated to determine the statistical significance of each predictor. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Patient and Biopsy Characteristics

In this study, 134 TBBs were analyzed by both MMDx and histopathology. The MMDx results identified 17 cases of definite TCMR (12.7%), 53 NR (39.6%), 38 rejection-like changes (28.3%), 25 inflammation (18.7%) and 1 injury (0.8%). TBBs exhibiting rejection-like changes, inflammation and injury archetypes were excluded from further analysis as well as 2 TBBs with TCMR (11.8%) and 14 with NR (26.4%) due to the unavailability of either pre-biopsy spirometry or 4-week follow-up spirometry. 54 TBBs obtained from 41 LTRs fulfilled the inclusion criteria: 15 with TCMR (27.8%) and 39 with NR (72.2%) identified by MMDx. Histological examination revealed ACR grade A0 in 12 (22.2%), grade A1 in 24 (44.4%), grade A2 in 15 (27.8%), and grade A3 in 3 TBBs (5.6%).

Among the included patients, the median age at LuTx was 54.0 years (IQR 33.0–60.6), 18 were female (43.9%). The pre-biopsy spirometry was conducted on average 3 days prior to biopsy (SD 5.3 days) and the 4-week follow-up spirometry on average 29.9 days post-biopsy (SD 9.4 days). 36 of the LTRs underwent bilateral LuTx (87.8%), while 5 patients (12.2%) underwent unilateral transplantation. The primary diagnosis included COPD in 17 (41.5%), IPF in 11 (26.8%), ILD in 4 (9.8%) and CF in 9 (21.9%) patients (**Table 1**). The median time between LuTx and biopsy was 13.4 months (IQR 4.4–39.5). 21 TBBs were performed for surveillance purposes (38.9%) and 33 were indicated for a cause (61.1%). All LTRs who underwent surveillance TBB were asymptomatic at the time of biopsy. Among those biopsied for cause, a significant difference was observed: 90% of ACR/TCMR patients were symptomatic at the time of TBB, in contrast to 39% of ACR/NR patients (p = 0.016; see **Table 2**).

Agreement Between Histology Diagnosis and MMDx

Overall, the MMDx was concordant with the histologic diagnosis in 25 TBBs (46.3%), while discrepancies were observed in 29 (53.7%) of them. Among TBBs with ACR grade A0, agreement with the MMDx was present in 11 (91.7%; ACR-/NR) and only one biopsy showed a discrepancy (8.3%; ACR-/TCMR). Of the 42 patients with ACR ≥ A1 by histology, TCMR (ACR+/TCMR) was present in 14 of them (33.3%). For ACR grade A1, consistency was found in only 5 TBBs (20.8%; A1/TCMR), while discordance between histology and MMDx was observed in 19 TBBs (79.2%; A1/NR). Among the 15 TBBs with ACR grade A2, matching results were present in 6 (40%; A2/TCMR) and absent in 9 (60%) cases (A2/NR). All of the TBBs with ACR grade 3 agreed with the TCMR diagnosis by MMDx (A3/TCMR; **Figures 2**, **3**).

Response to Treatment

All patients with ACR ≥ A1 by histology (n = 42) received treatment—36 with corticosteroids (85.7%), 4 with ATG (9.5%) and 2 with alemtuzumab (4.8%). A response to treatment was observed in 11 LTRs (29.9%): 10 with corticosteroids (90.9%) and 1 with alemtuzumab (9.1%). 31 LTRs were unresponsive (73.8%).

Patients with ACR+/TCMR (n = 14) received therapy: 9 with MP pulses (64.3%), 2 with prednisone escalation (14.3%), 2 with ATG (14.3%), and 1 with alemtuzumab (7.1%). Seven patients (50%) responded to the treatment: 6 with MP pulses (85.7%) and 1 with alemtuzumab (14.3%).

In the ACR+/NR group, 19 patients received MP pulses (67.9%), 6 prednisone escalation (21.4%), 2 ATG (7.1%), and 1 alemtuzumab (3.6%), of whom 4 LTRs treated by MP pulses (14.3%) demonstrated a response. We found a significant difference when comparing the molecular NR and TCMR groups: 4 LTRs (21.1%) in the molecular NR group (ACR+/NR) and 7 LTRs (50.0%) in the molecular TCMR group (ACR+/TCMR) responded to the treatment (p = 0.024).

[1]www.r-project.org

TABLE 1 | Patient characteristics: TBBs with histological ACR grade ≥ A1 (ACR+) were divided into two groups, based on the presence of T-cell mediated rejection (TCMR) and no rejection (NR) by MMDx.

	ACR+/TCMR (n = 14)	ACR+/NR (n = 28)	*p*-value
Age at LuTx (years; median, IQR)	27.3 (20–57)	51.1 (21.3–59.5)	0.13
From LuTx to TBB (days; median, IQR)	517 (345–1,251)	486 (128–1,596)	0.44
Female (n, %)	7 (50)	15 (54)	0.99
Primary diagnosis			0.79
Chronic obstructive pulmonary disease (n, %)	4 (29)	9 (32)	
Interstitial lung disease (n, %)	1 (7)	1 (4)	
Idiopathic pulmonary fibrosis (n, %)	2 (14)	7 (25)	
Cystic fibrosis (n, %)	7 (50)	11 (39)	
Pulmonary hypertension (n, %)	0	0	
Other (n, %)	0	0	
LuTx type			0.28
Double (n, %)	14 (100)	24 (86)	
Single (n, %)	0	4 (14)	
Infection at TBB			0.35
Viral (n, %)	1 (7)	0	
Bacterial (n, %)	2 (14)	5 (18)	
Mycological (n, %)	0	0	
None (n, %)	11 (79)	23 (82)	
DSA at TBB			0.23
Class I (n, %)	0	1 (4)	
Class II (n, %)	3 (21)	1 (4)	
None (n, %)	10 (71)	20 (71)	
Not performed (n, %)	1 (7)	6 (21)	
High-dose corticosteroids within 3 months prior to TBB (n, %)	2 (14)	5 (18)	0.99
Chronic lung allograft dysfunction at TBB			0.19
Bronchiolitis obliterans syndrome (n, %)	5 (36)	6 (21)	
Restrictive allograft syndrome (n, %)	1 (7)	0	
Mixed (n, %)	0	0	
Undefined (n, %)	0	0	
None (n, %)	8 (57)	22 (79)	

Among the LTRs with ACR grade 1, 14 received MP pulses (58.3%), 8 prednisone escalation (33.3%), and 2 received ATG (8.3%). Only 4 (16.7%) responded to treatment, all treated with MP pulses, while 20 (83.3%) did not respond. Of the responders, 1 (20%) was from the TCMR group and 3 (15.8%) were from the NR group by MMDx. No significant difference in treatment response was found between A1/TCMR and A1/NR (**Figure 5**).

In the ACR grade 2 group, 14 LTRs (93.3%) received MP pulses and one (6.7%) received alemtuzumab. Six (40%) responded to the therapy: 5 (83.3%) from the TCMR group and 1 (11.1%) from the NR group by MMDx. There was a significant difference in treatment response between A2/TCMR and A2/NR ($p = 0.01$; see **Figure 5**).

Of the three patients with ACR grade A3, two received ATG without response (66.7%), and one received alemtuzumab with a positive response (33.3%). Both non-responders had CLAD at the time of biopsy. The first one was a patient with CLAD, grade 4 at time of biopsy, with persistent DSAs and received multiple anti-rejection therapies prior to included biopsy. The second one was a patient with a clinical diagnosis of steroid resistant ACR.

FEV1 decline is considered one of the major predictors of treatment response. Using Spearman correlation analysis, we found only a weak correlation between FEV1 decline and response to treatment, defined as FEV1% change 4 weeks after biopsy (r = 0.2, $p = 0.21$).

To evaluate TCMR as a predictor of treatment response, we performed logistic regression analyses adjusted for major potential confounders such as ACR grade and FEV1% decline at biopsy. Given the limited number of patients with ACR grade A3 (n = 3), for logistic regression analyses we combined ACR grades into two groups: ACR grade A1 (minimal) and ACR grades A2 + A3 (mild-to-moderate). This resulted in 24 patients in ACR grade A1 (57%) and 18 in ACR grades A2 + A3 (43%).

In the univariate logistic regression, both TCMR ($p = 0.018$) and FEV1% decline ($p = 0.018$) were significant predictors of treatment response, while the ACR grade was not ($p = 0.11$). In the multivariate analysis, none of the predictors reached statistical significance (**Table 3**). These results suggest that while TCMR and FEV1% decline are significant in univariate analysis, their effects are attenuated in the multivariate model, likely due to multicollinearity and the limited sample size.

To account for the expected increase in lung function within the first post transplant year, we compared the univariate logistic regression results for two different post-transplant periods. In the early period (0–365 days; n = 16, 38.1%), the effect of the FEV1 decline on treatment response had an OR of 1.23 (95% CI: 1.04–1.62, $p = 0.055$). In the late period (>365 days; n = 26, 61.9%), the OR was 1.08 (95% CI: 1.01–1.20, $p = 0.065$). We did not find a

TABLE 2 | Biopsy-related characteristics: TBBs with histological ACR grade ≥ A1 (ACR+) were divided into two groups, based on the presence of T-cell mediated rejection (TCMR) and no rejection (NR) by MMDx.

	ACR+/TCMR (n = 14)	ACR+/NR (n = 28)	*p*-value
From LuTx to TBB (days; median, IQR)	517 (345–1,251)	486 (128–1,596)	0.44
Reason for TBB			0.73
Surveillance (n, %)	4 (29)	10 (36)	1
Symptomatic (n, %)	0	0	
Asymptomatic (n, %)	4 (100)	10 (100)	
Indication (n, %)	10 (71)	18 (64)	0.05
Symptomatic (n, %)	9 (90)	9 (50)	
Asymptomatic (n, %)	1 (10)	9 (50)	
ACR grades			
A grade			0.02
Grade 1 (n, %)	5 (36)	19 (68)	
Grade 2 (n, %)	6 (43)	9 (32)	
Grade 3 (n, %)	3 (21)	0	
Grade 4 (n, %)	0	0	
B grade			0.25
Grade 0 (n, %)	1 (7)	6 (21)	
Grade IR (n, %)	3 (21)	9 (32)	
Grade IIR (n, %)	1 (7)	0	
Grade X (n, %)	9 (64)	13 (46)	
C grade			0.19
Grade 0 (n, %)	3 (21)	13 (46)	
Grade 1 (n, %)	2 (14)	1 (4)	
Grade X (n, %)	9 (64)	14 (50)	
Treatment			
Surveillance			1
Corticosteroids (n, %)	4 (100)	10 (100)	
Indication			0.71
Corticosteroids (n, %)	7 (70)	15 (83)	
Anti-thymocyte globulin (n, %)	2 (20)	2 (11)	
Alemtuzumab (n, %)	1 (10)	1 (6)	
Response to treatment	7 (50)	4 (14)	0.03
Surveillance (n, %)	0	0	
FEV1% decline at TBB (median, IQR)	9.3 (–2.2–14)	3.7 (0.4–8.5)	0.61
FEV1% before TBB (median, IQR)	52.7 (51.9–60.6)	94.9 (66.8–108.1)	0.04
FEV1% 4w after TBB (median, IQR)	56.1 (43.5–73.3)	91.4 (67.5–112.3)	0.05
FEV1% change (%)	0.8 (–18.5–6.6)	–0.5 [(–9.5)–6.6]	0.95
Indication (n, %)	7 (70)	4 (22)	0.02
FEV1% decline at TBB (median, IQR)	–22.1 [–30.8–(–15.8)]	–11.3 [–14.5–(–7.8)]	0.01
FEV1% before TBB (median, IQR)	37.6 (27.9–62.5)	65.5 (47.5–72.9)	0.03
FEV1% 4w after TBB (median, IQR)	54.0 (33.5–73.3)	62.9 (50.1–75.8)	0.41
FEV 1% change (median, IQR)	15.7 (–2.1–37.5)	2.1 (–6.5–7.9)	0.07

significant difference in the effect of the FEV1 decline on treatment response between the early and late post-transplant periods.

Regarding the presence of DSAs at the time of TBB, three patients (21.4%) in the ACR/TCMR group and two patients (7%) in the ACR/NR group had DSAs. Notably, none of these patients responded to therapy. For further details, see **Table 2** and **Figures 3–5**.

DISCUSSION

There is a need to develop novel, more sensitive biomarkers of graft alteration to guide patient management, given the limited survival of LTRs in comparison to other solid organ transplant recipients. Although data on some promising novel biomarkers for non-invasive monitoring have been published, such as donor-derived cell-free DNA (dd-cfDNA), Torque teno viral load, or exosomes, all of them lack specificity in identifying the underlying pathophysiological processes that lead to organ damage, failing to detect ACR specifically [8]. A molecular analysis of the tissue, despite its invasive nature, might offer a more specific understanding of the underlying graft pathology, providing a clear proof of TCMR, based on the presence of specific rejection-associated transcripts [6, 7]. MMDx has already been approved for use in clinical routine for kidney and heart transplant recipients [9, 10], but the MMDx approach in LTRs lacks more profound clinical data despite some very promising results in the INTERLUNG collaboration [6, 7, 11, 12]. In this retrospective study, we aimed to determine the accuracy of NR and TCMR diagnosis by MMDx in both surveillance and acute transbronchial biopsies based on treatment response and

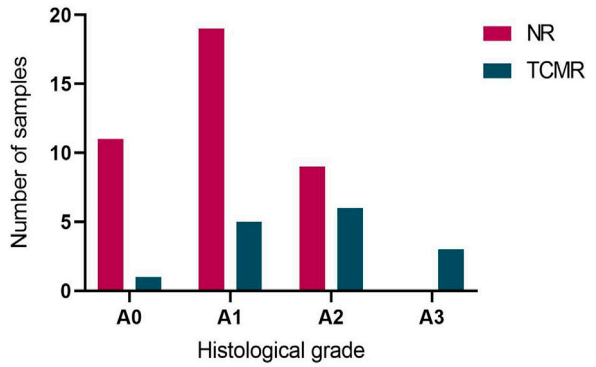


FIGURE 2 | Agreement between histological acute cellular rejection (ACR) grade A and diagnosis of no rejection (NR) and T-cell mediated rejection (TCMR) by MMDx. NR by MMDx was considered concordant with ACR grade A0 and TCMR concordant with ACR A grade ≥ A1.

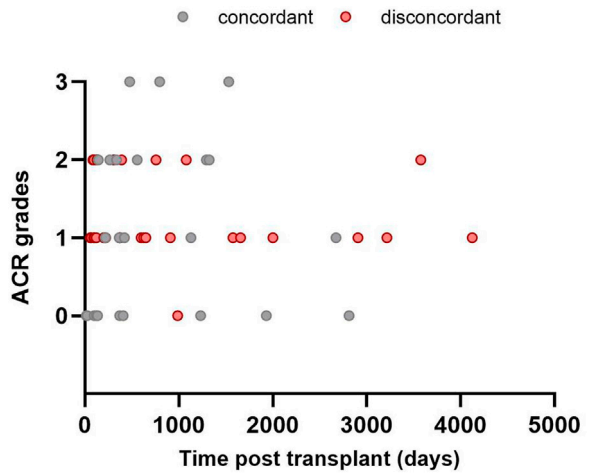


FIGURE 3 | Time distribution of included transbronchial biopsies: agreement between histology (ACR; grades A0–A3) and MMDx. No rejection (NR) by MMDx was considered concordant with ACR grade A0 and T-cell mediated rejection (TCMR) concordant with ACR A grade ≥ A1.

TABLE 3 | Univariate and multivariate logistic regression depicting predictors of treatment response—diagnosis of T-cell mediated rejection (TCMR), FEV1 decline at biopsy (ratio of the FEV1% expected to the pre-biopsy FEV1%), and grade of acute cellular rejection (ACR) with odds ratios (OR) with 95% confidence intervals (CI) and *p*-values determining the statistical significance of each predictor.

Univariate logistic regression

Predictor	Odds ratio (95% CI)	*p*-value
TCMR	6.00 (1.41–29.23)	0.018
FEV1 decline	1.08 (1.02–1.17)	0.018
ACR grade	2.27 (0.84–6.64)	0.11

Multivariate logistic regression

Predictor	Odds ratio (95% CI)	*p*-value
TCMR	2.96 (0.47–17.75)	0.23
FEV1 decline	1.07 (1.00–1.15)	0.07
ACR grade	1.69 (0.49–5.86)	0.39

compare the results of this novel approach to the standard histological evaluation.

The absence of perivascular and peribronchial lymphocytic infiltrates (histological grade A0) should provide clear information, equal to a healthy allograft, as per current diagnostic criteria [13]. Nevertheless, recent publications in kidney transplant demonstrated disagreements between no rejection in histology and rejection by MMDx, ranging around 20% for antibody-mediated rejection and 40% for TCMR [14]. The factors possibly affecting the accuracy of the diagnosis include inter-pathologist disagreement and sampling variations [15]. Although the latter might affect both histology and MMDx [16], when combined with dd-cfDNA levels, MMDx correlates with survival in kidney transplants better than histology [17, 18]. Another additional value of a molecular approach was depicted in the publication by Schachtner et al. [19] in kidney transplantation, demonstrating superiority of MMDx in TCMR borderline lesions that have not yet met histological criteria for ACR. These findings suggest the potential superiority of MMDx over traditional histological evaluation for guiding clinical decisions.

Our study demonstrated a very good concordance between the mentioned methods in non-rejecting biopsies. There was only one biopsy with a histological finding of no rejection and TCMR by MMDx diagnosis (ACR-/TCMR). The further clinical course of this patient was suggestive of rejection, highlighting the fact that ACR, as well as other immune- and infection-mediated pathways in the lung, might often present with heterogeneous, patchy distributions, that might not be fully represented in the bioptic sample. While a histological evaluation in these limited biopsies may not fulfill the diagnostic criteria for ACR, the presence of specific transcripts in the tissue could be detected by MMDx.

High consistency was also observed in moderate ACR (grade A3) TBBs by histology—MMDx demonstrated TCMR in all three of these biopsies. Thus, in our cohort, MMDx showed a strong concordance in lung biopsies with either absent or moderate rejection by histological assessment, but when it comes to minimal and mild rejection (grade A1 and A2, respectively), the results vary significantly between histology and MMDx, with an overall discordance rate of 72% (79% for ACR grade A1 and 60% for grade A2). We hypothesized that the presence of lymphocytic infiltrates, particularly in lower grades of rejection, might not necessarily indicate rejection, but could instead signify other pathological processes, especially if MMDx does not concurrently reveal the presence of TCMR-specific transcripts. In order to assess the accuracy of the diagnosis in the discrepant results, the presence of response to treatment was taken into consideration. In the ACR+/NR group, only 14% of patients presented with an improvement of lung function following an anti-rejection therapy, compared to 50% in the ACR+/TCMR group. These findings support the superiority of MMDx evaluations in a context of clinical decision-making. However, when analyzed for A1 and A2 grades separately, statistically significant difference in response to therapy was

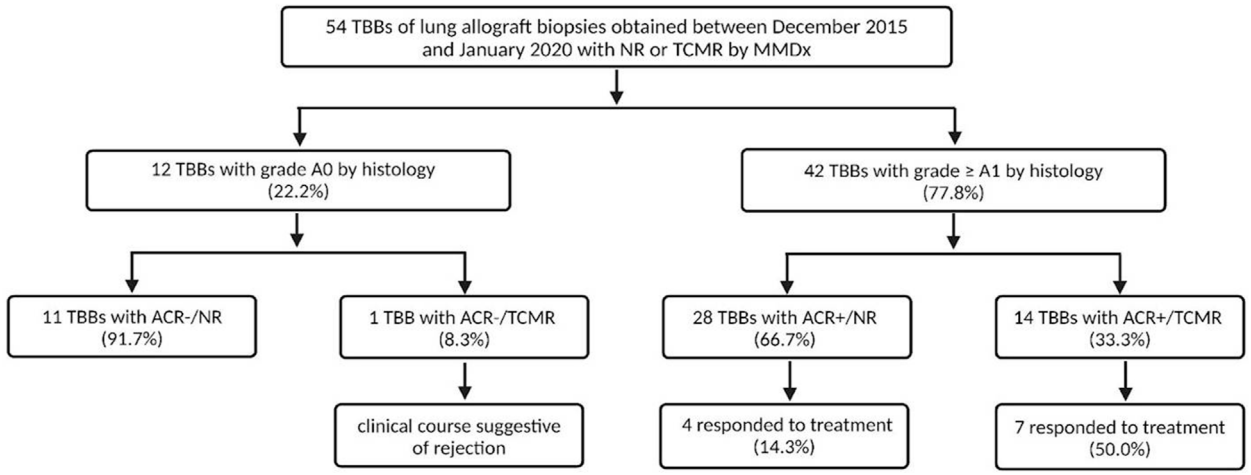


FIGURE 4 | Differences in responses to anti-rejection treatment of acute cellular rejection (ACR) in TBBs with no rejection (NR) and T-cell mediated rejection (TCMR) by MMDx for both ACR A0 and grade ≥ A1 groups. Response to treatment was defined as an increase in FEV1% ≥ 10% of FEV1% prior to biopsy.

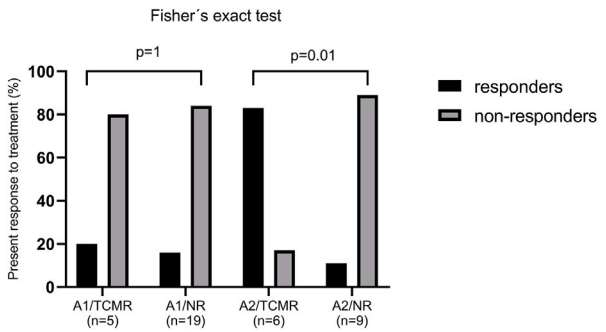


FIGURE 5 | Anti-rejection treatment response in patients with histological acute cellular rejection (ACR) grade A1 and A2 in patients with no rejection (NR) and T-cell mediated rejection (TCMR) by MMDx. Response to treatment was defined as an increase in FEV1% ≥ 10% of FEV1% prior to biopsy.

observed only in A2 samples (**Figure 5**). Moreover, none of the included LTRs treated with peroral prednisone escalation (treatment of choice for A1 grade in this cohort) showed response to treatment. Supporting this, Levy et al. demonstrated that the first untreated grade A1 rejection in spirometrically stable recipients within the first posttransplant year was not significantly associated with a risk for CLAD or death [3]. Especially in the early postoperative period, there is a wide variety of processes apart from rejection going on, including postischemic and reperfusion damage, as well as infectious complications. The absence of TCMR in biopsies showing minimal or mild ACR might be explained by the fact that the cell subpopulations and other immune components of similar-appearing ACR lesions may differ significantly between patients and may have different correlations with lung injury, even though the ISHLT criteria for ACR were met. The molecular approach provided by MMDx might overcome these limitations, however, its utility in our limited retrospective cohort was not demonstrated for samples with minimal rejection.

Within the ACR+/TCMR group, half of the LTRs (n = 7; 50%) responded to the therapy. Notably, 43% of the non-responders in ACR ≥ A1 by histology and TCMR by MMDx (ACR+/TCMR) had chronic lung allograft dysfunction (CLAD) present at the time of biopsy and received multiple courses of high-dose corticosteroids prior to biopsy. This observation raises the question of whether the failure to improve after corticoid therapy was in fact due to established CLAD changes. It is possible that patients with ongoing CLAD and TCMR might benefit from a more aggressive therapeutic approach. Further studies are required regarding this topic.

For lung transplant recipients, the incorporation of MMDx into the diagnostic routine might provide additional insights into the graft pathology, especially addressing the clinically challenging asymptomatic lower-grade acute cellular rejection, similarly to borderline findings in kidney transplants. Its utility might further clarify the necessity of treatment, especially in surveillance biopsies in the absence of other signs of rejection, preventing the unnecessary use of intensive anti-rejection protocols, and minimizing the risk of their significant adverse effects such as increased susceptibility to infections.

Our study has certain limitations, as it is a single-center retrospective study with a modest size of the cohort and the exclusion of TBBs with inflammation, rejection-like changes, and injury from the analysis. These archetypes, if incorporated into future studies on larger patient cohorts, might aid in clarifying the discrepancy of results in the case of a histological diagnosis of ACR with concurrent absence of TCMR-specific transcripts identified by MMDx.

In summary, our findings suggest that low-grade acute cellular rejection is less likely to be associated with molecular TCMR, which might better identify patients who benefit from therapy and offer additional insights into biopsies of lung allografts. While further research is required, our promising pilot data suggest that MMDx has the potential

to become a routinely used tool for diagnosing TCMR in lung transplant patients.

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 studies involving humans were approved by Ethics Committee for Multi-Centric Clinical Trias of the University Hospital Motol University Hospital V Uvalu 84, 150 06 Prague 5—Motol. 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

Concept and design: JH and AZ; Manuscript writing: AZ and JH; Acquisition, analysis and interpretation of data, manuscript reviewing: AZ, JH, MG, MM, KH, and PG; Biopsy collection, manuscript reviewing: DR, MS, JK, JV, JP, and JS; Histologic evaluation, manuscript reviewing: JB; Supervision: JH, LF, RL, and PH. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

PH holds shares in Transcriptome Sciences Inc. (TSI), a University of Alberta research company dedicated to developing molecular diagnostics, supported in part by a licensing agreement between TSI and Thermo Fisher Scientific, and by a research grant from Natera, Inc. PH is a consultant to Natera, 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.

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ORIGINAL RESEARCH
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Donor Fractions of Cell-Free DNA Are Elevated During CLAD But Not During Infectious Complications After Lung Transplantation

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During the last few years, cell-free DNA (cfDNA) has emerged as a possible non-invasive biomarker for prediction of complications after lung transplantation. We previously published a proof-of-concept study using a digital droplet polymerase chain reaction (ddPCR)-based method for detection of cfDNA. In the current study, we aimed to further evaluate the potential clinical usefulness of detecting chronic lung allograft dysfunction (CLAD) using three different ddPCR applications measuring and calculating the donor fraction (DF) of cfDNA as well as one method using the absolute amount of donor-derived cfDNA. We analyzed 246 serum samples collected from 26 lung transplant recipients. Nine of the patients had ongoing CLAD at some point during follow-up. All four methods showed statistically significant elevation of the measured variable in the CLAD samples compared to the non-CLAD samples. The results support the use of ddPCR-detected cfDNA as a potential biomarker for prediction of CLAD. These findings need to be validated in a subsequent prospective study.

Keywords: lung transplantation, allograft dysfunction, biomarker, cell-free DNA, droplet digital PCR

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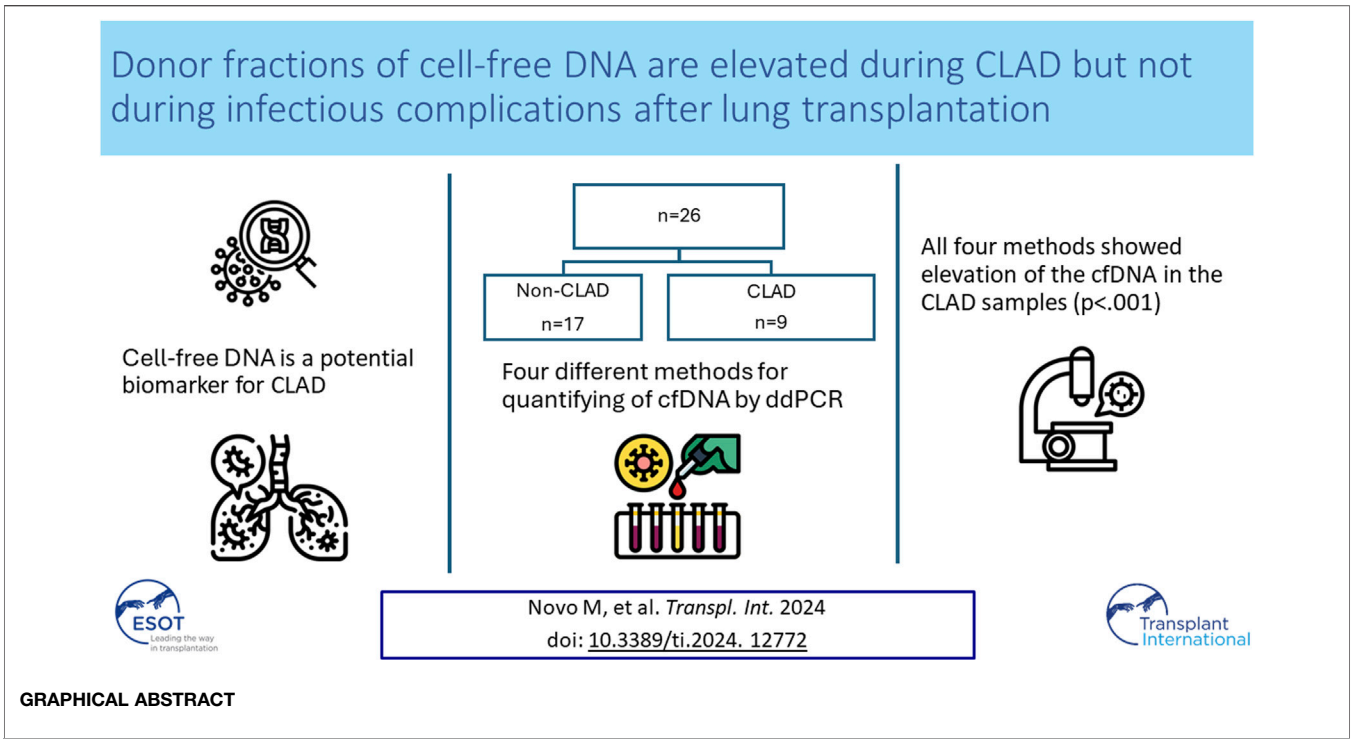
INTRODUCTION

Lung transplantation is a lifesaving treatment for patients with irreversible nonmalignant lung disease. During the last 30 years, approximately 70,000 adult lung transplant procedures have been performed worldwide [1]. Despite advances in organ procurement, improved surgical techniques and perioperative care, lung transplant patients have the shortest survival of all the major organ transplantation [2, 3] with a current median survival of 6.7 years [4].

The main limiting factor for survival is the high-rate development of chronic lung allograft dysfunction (CLAD) [4]. CLAD is currently defined as an irreversible decline of forced expiratory volume 1 s (FEV1) to ≤80% of a baseline FEV1.



GRAPHICAL ABSTRACT

Although several risk factors such as primary graft dysfunction [5], infections e.g., bacterial, viral and fungal [6], esophageal reflux [7], anti-HLA antibodies [8], acute cellular rejection [9] and choice of immunosuppression [10] have been proposed, the cause of CLAD remains elusive. Furthermore, the occurrence of risk factors does not adequately predict CLAD development. Several biomarkers associated with CLAD have been proposed, but their application in clinical practice has been limited due to insufficient specificity and sensitivity, as well as failure to detect early-stage disease [11, 12]. A reliable biomarker for isolated allograft damage would facilitate early detection of CLAD in a clinical setting and thus enable early therapeutic intervention [11], which would likely improve outcomes after LTx.

Cell-free DNA (cfDNA) can be released from injured cells into the bloodstream and detected in samples from bronchoalveolar lavage [13], urine, cerebrospinal fluid [14], as well as plasma and serum [15]. Quantification of cfDNA has been proven as a potential biomarker for the prediction of various diseases, including malignancy [16, 17], myocardial infarction [18], sepsis [19] and traumatic injuries [20]. After transplantation with a donated solid organ, two distinctly different sets of cfDNA may exist within the same individual, either donor-derived cfDNA (dd-cfDNA) or recipient-derived cfDNA (rd-cfDNA). Quantification of dd-cfDNA in transplant recipients has been shown to be useful for prediction of acute rejection in lung [21, 22], kidney [23], liver [24] and heart [25] transplantation.

We previously published a proof-of-concept study using droplet digital polymerase chain reaction (ddPCR) to quantify dd-cfDNA and rd-cfDNA in peripheral blood [26], showing potential to differentiate between CLAD and non-CLAD samples. Previously published methods, using various sequencing techniques, have solely reported the ratio between the two sources of cfDNA, referred to as donor fraction (DF) [21, 27, 28], which can still be calculated using our methodology [26]. The methodology also makes it possible to present donor and recipient cfDNA separately with quantification of the respective type of cfDNA [29, 30], which have already been proven in kidney [31] and liver transplant [32]. Moreover, ddPCR is practical in a clinical setting due to its fast turnaround time [31] and has the advantage to be both very sensitive and cost-efficient when compared to next-generation sequencing (NGS) based approaches [33, 34]. There are known variations in the total levels of cell-free DNA, both in pathological and physiological conditions [35, 36]. Donor fraction alone does not account for these fluctuations, and studies have shown absolute levels of dd-cfDNA perform better than DF after kidney transplantation [31, 37]. In this study, where we retrospectively used available samples from a prospective study, we found that the quantity and relative proportion of dd-cfDNA reflected several clinical effects, e.g., allograft damage. Samples were collected according to a fixed protocol. Samples collected 1 month after transplantation were consistently elevated, potentially confounding overall measurements. We also observed that any systemic affliction of the donor was associated with elevations of both the rd-cfDNA and dd-cfDNA, which might lead to a low DF despite CLAD.

This study aimed to evaluate our method further as a biomarker for CLAD, testing faster ways to process the PCR results and the impact of simplification on precision. In addition, the results in the proof-of-concept study also suggested that the

absolute amount of dd-cfDNA could possibly be correlated to CLAD which was also evaluated further in the current study.

MATERIALS AND METHODS

Patients and Study Design

Patients from a previously published cohort of patients undergoing lung transplantation between 2009 and 2011 at Sahlgrenska University Hospital were included [38, 39]. This cohort recorded and collected clinical status and samples at scheduled outpatient visits after LTx at 1, 2, 3, 4.5, 6, 9, 12, 18, 24, and 36 months. Furthermore, samples were also collected at every extra outpatient visit during this period. From this pool of previously collected serum samples, patients were selected based on serum availability. Patients with at least five samples from five separate time-points remaining were identified and included. Previously thawed samples were excluded. No samples from the proof-of-concept study [26] were used in the current study.

Induction therapy consisted of rabbit antithymocyte globulin, which was given for 1 to 3 consecutive days together with methylprednisolone IV. Post-transplantation immunosuppression included prednisone, 0.3 mg/kg/day and mycophenolate mofetil, 2 g/d. The patients then received either oral cyclosporine (CSA) (1-2 mg/kg) adjusted to maintain a serum level of 300–350 ng/mL or tacrolimus (TAC), 0.075 mg/kg given orally divided in 2 doses daily adjusted to maintain a serum level of 14–16 ng/mL. The dosage of immunosuppression was gradually lowered during follow up. Further changes in immunosuppressive therapy were based on clinical presentation [38]. For some patients, viral airway infections prompted a transient 1-to-3-week elevation of prednisone to approximately 0.3 mg/kg, according to local clinical deliberations. No other adjustments to base immunosuppression were made based on clinical events for any of the patients.

Respiratory viral agents were screened for at all outpatient visits. Bronchiolar lavage samples at 1, 3, and 12 months and for-cause were cultured for bacterial and fungal agents and airway viral agents. A previously described multiplex PCR, able to detect 17 viral agents [40], was used for respiratory viral agents. PCR-quantification was used for cytomegalovirus (CMV) and Epstein-Barr virus detection in all samples. All samples were processed at the hospital's routine clinical microbiological laboratory. If a positive sample constituted a clinically relevant infection, it was evaluated by an experienced clinician. Data regarding patient characteristics and clinical events was retrieved from electronic patient case report forms.

All serum samples were centrifuged at 3,000 × g after collection and aliquoted before frozen at −80°C within 24 h after sampling. The laboratory staff was blinded to all clinical and patient-related data. Serum samples were identified by serial numbers only during analysis and data management.

CLAD was defined as an irreversible loss of >20% of baseline FEV1, confirmed with at least two spirometries at least 3 weeks apart, where all other possible differential diagnoses such as infections, acute rejection, airway stenosis and antibody-mediated rejection had been excluded. At CLAD diagnosis, all patients with CLAD had been on Azithromycin 250 mg three times a week for more than 3 months at time of diagnosis. The CLAD diagnosis could be possible, probable or definite based on the time since initial loss of function (<3 weeks, 3 weeks-3 months or >3 months) without restitution or discovery of other more likely differential diagnoses. A loss of >10% of total lung capacity and restrictive allograft syndrome (RAS)-like opacities indicates the subtype RAS. The samples collected at the time of CLAD diagnosis 3 months before and after CLAD diagnosis were denominated as CLAD.

DNA Isolation and Genotyping

Whole blood samples were used for genotyping. Donor and recipient genomic DNA was extracted from EDTA-blood preparations using the DNeasy Blood & Tissue Kit (Qiagen).

A panel of 35 highly polymorphic SNP (single-nucleotide polymorphism) assay was used together with ddPCR (QX200 AutoDG Droplet digital PCR System, Bio-Rad) for genotyping and selection of informative assays to discriminate recipient DNA from donor DNA. Per recipient, 2-3 informative SNP assays were selected.

cfDNA Isolation, Target-Specific Preamplification and Analysis

Serum samples were used for longitudinal detection of cfDNA. cfDNA was extracted from 0.25 to 1.25 mL serum using the QIAamp® Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer's protocol. Concentrations of cfDNA were quantified with the Qubit® 3.0 Fluorometer (Thermo Fisher Scientific), fragment sizes were analyzed with the 4200 TapeStation (Agilent Technologies).

Absolute levels of donor and recipient cfDNA were quantified by ddPCR using one of the informative SNP assays. Calculations of copies were performed by Quant Soft (BioRad).

Target preamplification of cfDNA was performed using pooled primers for all 35 SNP [26]. The preamplified cfDNA was quantified by ddPCR using the informative SNP assays. The SNP assays were analyzed in triplicates, all experiments were included with no template controls. The copies generated by ddPCR for each allele at each SNP locus were calculated using Quanta Soft (Bio-Rad). The mean value from triplicate assays was used to calculate the levels of dd-cfDNA, rd-cfDNA, and DF.

At least five samples per patient must be adequately analyzed for the patient to be included in the final analysis. Samples were excluded due to sample hemolysis, insufficient plasma yield, high technical error rate and failed droplet generation.

Sample results were categorized by baseline groups but also by fungal, viral, and bacterial infectious events as well as CLAD, depending on analysis. Samples without the analyzed property or event at the time of sampling were used as controls. Events where no samples were available were not included.

Four distinct methodologies to analyze the results from the ddPCR were applied. DF calculated from each pre-amplified SNP individually was labelled Method 1 (M1). DF calculated from the mean of all pre-amplified SNPs per event was labelled method 2

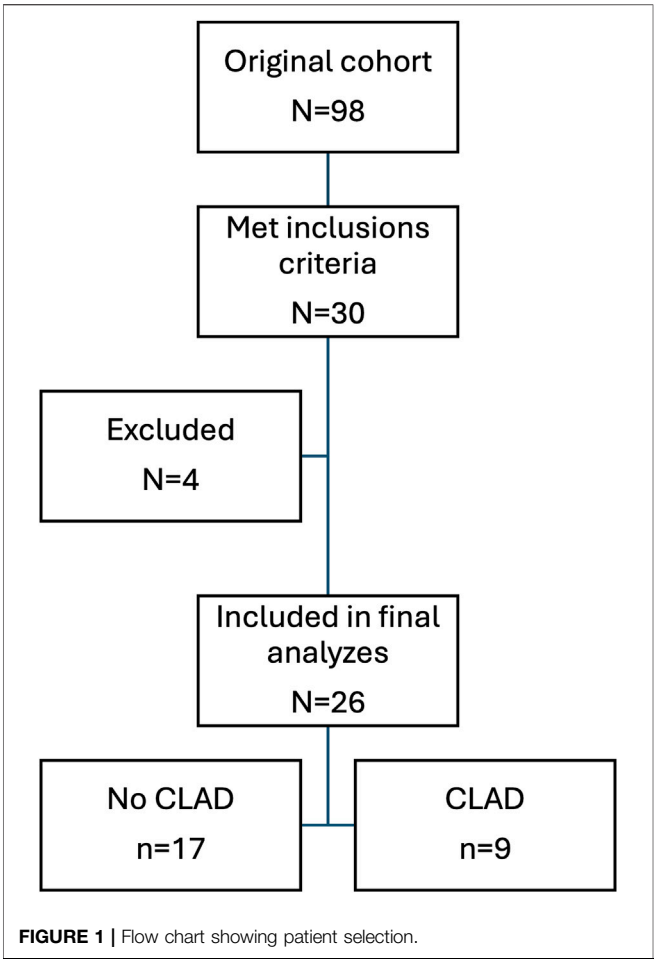


FIGURE 1 | Flow chart showing patient selection.

(M2). DF calculated from the first not pre-amplified SNP was labelled Method 3 (M3). Finally, using only the absolute value of dd-cfDNA quantified by the non-pre-amplified dd-PCR, was labelled Method 4 (M4). Means over all samples per individual were used for groupwise baseline comparisons. For infectious events and CLAD, comparisons were made between event and non-event samples.

Statistics

Data were analyzed by SPSS for macOS v 29.0. Values were presented as median and interquartile range (IQR). Comparisons at the group level were performed using the Mann–Whitney U test. $p < .05$ was considered statistically significant. ROC (receiver operating characteristic) curve and AUC (area under the curve) were used to calculate evaluation metrics of the different aspects of ddPCR-based cfDNA.

RESULTS

Thirty patients matched the inclusion criteria in the biobank. Samples from four patients (two male and two female) were excluded. Two were excluded due to fewer actual samples in the biobank than indicated, and two were excluded because of the high rate of technical errors in one sample each or fewer actual samples to analyze in the biobank than indicated (**Figure 1**). The technical errors in both samples did not show any difference compared to other signals for the PCR due to a high level of background genomic DNA, suggesting it to be the result of pre-analytical factors. Furthermore, in one of the two samples, one of the instruments failed and was unable to read one of the tested SNPs, and there was not sufficient remaining volume to re-do the test.

Twenty-six patients (15 female and 11 male) were included in the final analysis, of which nine (36.4%) developed CLAD at some point during follow-up with a max of 16 CLAD samples. The median age at the time of transplantation was 51 (IQR 42–63) years. Most of the patients were transplanted because of pulmonary fibrosis – 46.2% and chronic obstructive pulmonary disease (COPD) – 30.8%. Bilateral lung transplants were the most common (69.2%) (**Table 1**).

At the end of clinical follow-up, all the included CLAD patients had observed persistent graft dysfunction for more than 3 months and could, therefore, be defined as definite CLAD.

At baseline, we found no differences between patients who developed CLAD during follow-up and those who did not, nor any difference based on sex (**Supplementary Table S1**).

Females had significantly higher overall DF compared to males for M1 ($p = 0.011$), M2 ($p = 0.036$), M3 ($p = 0.036$), and M4 ($p = 0.047$). However, there were no differences in transplant type or CMV mismatch (**Table 2**).

The analysis of the dynamics over time showed that the samples available at one-month post-transplantation had significantly higher levels of M1 ($p < 0.001$), M2 ($p < 0.001$), M3 ($p = 0.005$), and M4 ($p = 0.007$) compared to all subsequent samples (**Supplementary Table S2**). There were too few samples after the CLAD diagnosis to perform any meaningful analysis on post-CLAD dynamics. At the end of follow-up, only two patients had developed the RAS subtype (3 samples), and there were no significant differences in any of M1-M4 ($p > 0.05$). Further analyses were performed with one-month samples excluded and no subdivision of CLAD samples.

The analysis of individual events showed that viral, bacterial or fungal infection M1, M2, M3 showed no significant difference between samples at the event and samples without the event. However, for M4, the test results for viral ($p = 0.034$) and fungal ($p = 0.021$) were significantly elevated whilst there were no significant differences for samples with bacterial infections. The DF levels and the dd-cfDNA level respectively, for samples with CLAD were elevated compared to samples without CLAD for M1 ($p < 0.001$), M2 ($p < 0.001$), M3 ($p < 0.001$) and M4 ($p < 0.001$) (**Table 3**). Only two patients developed acute rejection (AR) at any time during follow-up and none of these events had matching samples. No patient had a CLAD sample with a simultaneous infection of any kind.

ROC analyses by plotting sensitivity versus (1-sensitivity) for analysis of predictive accuracy for all methods are displayed in **Figure 2**. The AUC for M1= 0.709, for M2 AUC = 0.780, for M3 AUC =0.778 and for M4 AUC = 0.726.

TABLE 1 | Baseline characteristics of the study population (N = 26).

Variable		
Sex	Female, n (%)	15 (57.7%)
	Male, n (%)	11 (42.3%)
Age at time of transplantation, years	Median (IQR)	51 (41–63)
BMI (kg/m^2)	Median (IQR)	23.1 (19.7–21.7)
Indication for transplantation	Pulmonal fibrosis, n (%)	12 (46.2%)
	COPD, n (%)	8 (30.8%)
	Alpha-1 trypsin deficiency, n (%)	3 (11.5%)
	Other, n (%)	3 (11.5%)
Type of transplantation	Single, n (%)	8 (30.8%)
	Double, n (%)	18 (69.2%)
Mismatch	Cytomegalovirus, n (%)	6 (23.1%)
	Epstein-Barr virus, n (%)	1 (3.8%)
CLAD during follow-up	None, n (%)	17 (65.4%)
	CLAD, n (%)	9 (34.6%)

n, number; IQR, interquartile range; COPD, chronic obstructive pulmonary disease. Mismatch–seropositive donor and seronegative recipient. CLAD, chronic lung allograft dysfunction.

TABLE 2 | Characteristics of the study population regarding levels of DF calculated by four methods.

Variable	Median (IQR)	Variable	Median (IQR)	*p*-value
Sex				
Male		Female		
M 1	0.049 (0.030–0.120)	M 1	0.130 (0.070–0.200)	**0.011**
M 2	0.047 (0.030–0.122)	M 2	0.130 (0.061–0.202)	**0.036**
M 3	0.046 (0.018–0.161)	M 3	0.143 (0.095–0.344)	**0.036**
M 4	0.084 (0.037–0.288)	M 4	0.322 (0.143–1.204)	**0.047**
Type of transplantation				
Double		Single		
M 1	0.120 (0.040–0.180)	M 1	0.110 (0.040–0.150)	0.810
M 2	0.118 (0.04–0.168)	M 2	0.118 (0.040–0.180)	0.978
M 3	0.161 (0.053–0.229)	M 3	0.010 (0.022–0.195)	0.311
M 4	0.202 (0.046–1.04)	M 4	0.233 (0.077–0.390)	0.892
Mismatch CMV				
Yes		**No**		
M 1	0.080 (0.030–0.240)	M 1	0.110 (0.050–0.160)	0.930
M 2	0.082 (0.300–0.243)	M 2	0.120 (0.048–0.168)	0.790
M 3	0.107 (0.064–0.256)	M 3	0.118 (0.025–0.206)	0.882
M 4	0.305 (0.117–1.960)	M 4	0.214 (0.056–0.431)	0.295

M1 Method 1 DF calculated from each pre-amplified SNP, individually (n = 665).
M2 Method 2 DF calculated from mean of all pre-amplified SNPs, per event (n = 221).
M3 Method 3 DF Calculated from the first non-pre-amplified SNP (n = 198).
M4 Method 4 The absolute value of dd-cfDNA, quantified by the non-pre-amplified dd-PCR (n = 218).
Data are presented as median (Md) and interquartile range (IQR). Mismatch–seropositive donor and seronegative recipient. CMV, cytomegalovirus. The statistic calculations were done using Mann-Whitney U test. Significant p-values are highlighted in bold.

DISCUSSION

cfDNA as a prediction tool and possible biomarker for rejection after lung transplant was introduced in 2015 [22]. Most studies published have been analyzing the risk of acute rejection, antibody mediated rejection, or undefined rejection [33]. Only a few analyzed the risk of CLAD [41]. In the current study, we found that both DF and absolute levels of dd-cfDNA were significantly higher for CLAD samples than non-CLAD samples. For three of the analyzing methods, there were no statistical differences in test values between the presence and absence of any other clinical events included in the study. For the fourth method, using the absolute value of dd-cfDNA, both viral and fungal infections also had significantly higher values. Furthermore, the ROC AUC values show a fair ability of all methods to discriminate between CLAD and non-CLAD samples. The findings contribute to the pool of evidence for cfDNA as a useful biomarker for CLAD.

It has previously been shown that males generally have higher levels of cfDNA compared to females [42]. Surprisingly, in our series, we found that DF was higher among female than among male patients. Previous studies within the field of heart transplantation have not shown any sex difference in DF [43, 44]. To our knowledge this issue has not be studied in lung transplantation recipients and will warrant further studies.

Based on previous research, we expected higher levels of DF in double lung recipients compared to single ones because of donor lung mass [45]. However, we did not find any difference in DF levels regarding transplantation type. Our results were more in concordance with the findings of Kush et al. [46].

Samples collected at 1 month were observed to have an elevation of cfDNA compared to subsequent samples in the proof-of-concept study [26]. This observation was confirmed in the current study. The reason is likely lingering peri-operative injuries to the allograft. This finding suggests that it would be problematic to include samples drawn up to 1 month after LTx in pooled analyses and likely also in upcoming predictive modelling and establishing of a baseline value for dd-cfDNA from future prospective studies. Furthermore, this is a time point when CLAD can never be present due to its definition. However, these samples could possibly be of value for risk stratification post-transplant, as previously published by Agbor Enoch et al. [21].

There was no difference between the RAS and other subtypes of CLAD in our findings and thus we did not separate the subtypes in our analyses. However, the number of RAS patients were very few and the generalizability of this finding is low.

TABLE 3 | Levels of donor fraction DF of cfDNA obtained by four different methods with regard to different infections and CLAD.

Method 1 DF calculated from each amplified SNP individually (n = 665)					
	n	**No**	**n**	**Yes**	***p*-value**
		Median (IQR)		**Median (IQR)**	
Viral infection	382	0.060 (0.021–0.171)	283	0.052 (0.019–0.136)	0.118
Bacterial infection	611	0.060 (0.021–0.164)	54	0.038 (0.018–0.105)	0.125
Fungal infection	629	0.054 (0.021–0.154)	36	0.086 (0.035–0.157)	0.216
CLAD	616	0.050 (0.020–0.150)	49	0.120 (0.070–0.310)	<**0.001**
Method 2 DF calculated from mean of all amplified SNPs per event (n = 221)					
	n	**No**	**n**	**Yes**	***p*-value**
		Median (IQR)		**Median (IQR)**	
Viral infection	126	0.063 (0.026–0.151)	95	0.051 (0.021–0.153)	0.462
Bacterial infection	203	0.062 (0.026–0.159)	18	0.045 (0.020–0.130)	0.415
Fungal infection	209	0.059 (0.024–0.151)	12	0.101 (0.048–0.156)	0.200
CLAD	205	0.051 (0.022–0.140)	16	0.222 (0.089–0.329)	<**0.001**
Method 3 DF calculated from the first non-pre-amplified SNP (n = 198)					
	n	**No**	**n**	**Yes**	***p*-value**
		Median (IQR)		**Median (IQR)**	
Viral infection	117	0.059 (0.012–0.174)	81	0.087 (0.026–0.195)	0.177
Bacterial infection	181	0.070 (0.015–0.191)	17	0.061 (0.027–0.160)	0.981
Fungal infection	188	0.068 (0.015–0.182)	10	0.110 (0.049–0.269)	0.274
CLAD	183	0.059 (0.015–0.167)	15	0.388 (0.097–0.473)	<**0.001**
Method 4 The absolute value of dd-cfDNA quantified by the non-pre-amplified dd-PCR (n = 218)					
	n	**No**	**n**	**Yes**	***p*-value**
		Median (IQR)		**Median (IQR)**	
Viral infection	125	0.120 (0.000–0.260)	93	0.200 (0.060–0.475)	**0.034**
Bacterial infection	200	0.163 (0.000–0.390)	18	0.220 (0.058–0.438)	0.518
Fungal infection	207	0.133 (0.000–0.360)	11	0.520 (0.230–0.760)	**0.021**
CLAD	203	0.120 (0.000–0.330)	15	0.470 (0.238–0.840)	**0.001**

Data are presented as median (Md) and interquartile range (IQR). N–number. PCR, Polymerase Chain Reaction; SNP, Single-nucleotide polymorphism; CLAD, Chronic Lung Allograft Disfunction. The statistic calculations were done using Mann-Whitney U test. Significant p-values are highlighted in bold.

Although elevated levels of cfDNA have been found at the time points of viral [47] and other microbial [48] infections in transplanted patients, we did not find such an association with DF. We expected a slightly elevated DF in the infection suffers since plasma dd-cfDNA represents the allograft tissue injury. Our results were more in line with studies of Khush et al. [46] and Ju et al. [49], who did not find a significant difference in the plasma dd-cfDNA level between samples gathered with or without infectious events. One possibility is that allograft infection was not defined as only a confirmed serious invasive infection but also included milder forms of the presence of bacterial DNA in the airways with some clinical impact. This heterogeneity probably explains the lack of consistent elevated dd-cfDNA levels that would hypothetically be present in tissue injury [46]. Another possibility, when using DF, is that the inflammatory effect of infection is not isolated to the allograft leading to non-elevated quotas. When comparing the absolute levels of dd-cfDNA of bacterial infections, we see significantly higher values for viral infections which are disseminated, as well as for fungal infections, confirmed with directed bronchoscopy. This is a finding supporting these hypotheses, however it also introduces these conditions as confounders for CLAD.

The results of ROC analyses used to assess the sensitivity and specificity of DF to detect ongoing CLAD were 0.71–0.78, which may be considered acceptable in this context. Similar levels have been obtained in the study using the target-specific amplified ddPCR tests [43]. The AUC value is kept down by false negatives, which could be a result of the samples in the study not being collected in tubes that were not optimized for cfDNA extraction. However, it is not impossible that systemic affliction, in combination with CLAD, provided false low DFs. The sensitivity may be improved by further development of the methodology and using a standardized blood sample collection. Determining cut-off values would perhaps be possible in a further diagnostic system but beyond the scope of the current study. The best sensitivity was shown using M2, but M3 without pre-amplification performed almost as well and is a much faster method. There have been issues concerning the evaluation of cfDNA in clinical practice [27] in part due to different and complex technical approaches, with different

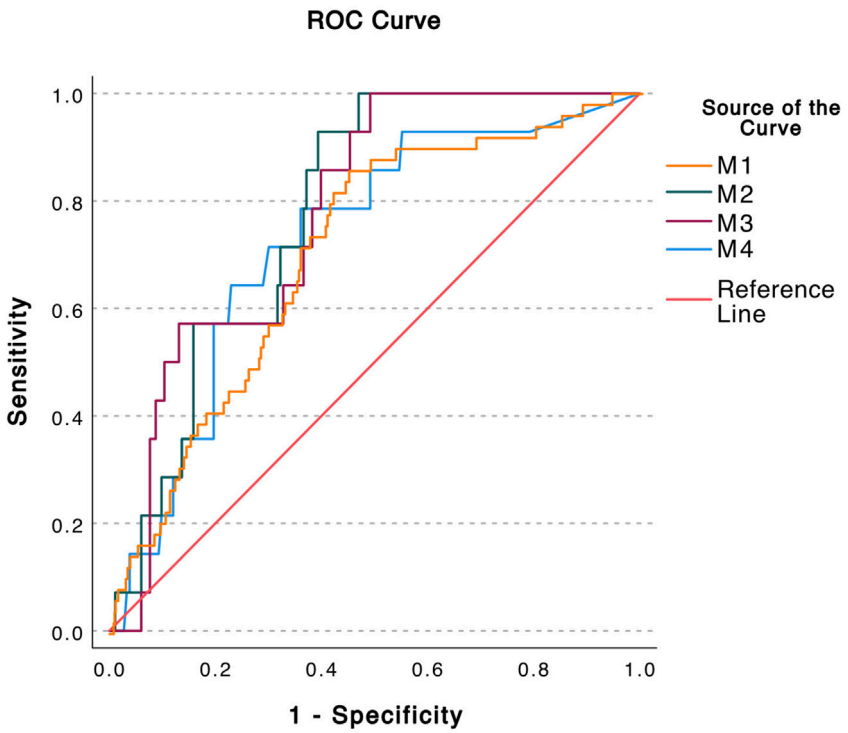


FIGURE 2 | Calculation of the predicative accuracy of the donor fraction (DF) obtained by four different methods by ROC. AUC = Area Under Curve. M1 Method 1 DF calculated from each pre-amplified SNP individually (AUC = 0.709) M2 Method 2 DF calculated from mean of all pre-amplified SNPs per event (AUC = 0.780) M3 Method 3 DF Calculated from the first non-pre-amplified SNP (AUC = 0.778) M4 Method 4 The absolute value of dd-cfDNA quantified by the non-pre-amplified dd-PCR (AUC = 0.726).

efficiencies in testing cfDNA. Using several averaged assays can alleviate this issue. The slightly improved AUC for CLAD discrimination when using averaged assay values compared to using multiple singulars provides some support for this assumption.

Interestingly a DF level ≥1% has been proven as a clinically relevant threshold [21, 22] for cfDNA and graft injury. In this study, even lower levels could be associated with CLAD. The difference is most likely due to higher levels of recipient genomic DNA in the samples in the current data set [50], which, in turn, is caused by preanalytical factors such as degree of hemolysis in collection tubes, transport times, and centrifugation procedures. However, the diverging methodologies preclude any definite conclusions from comparisons of absolute rd-cfDNA levels between studies.

The study is unique in using many long-term stored frozen samples for the detection of cfDNA in lung transplantation. Although all the samples used in the study had been frozen for more than 5 years in ordinary cryo-tubes and no cell-free DNA collection tubes had been used for blood sampling, the method still showed a remarkable quality of the samples. This suggests that secondary site sampling and freezing are possible, which would expand the options for sampling and storage of cfDNA. The rather complicated method can be set up in a limited number of laboratories to cover several transplant programs. However, it is very plausible that using standard sampling equipment and procedures would have rendered fewer negative samples.

The original study was performed several years ago, and follow-up routines and dominant immunosuppressive regimens have changed since. Furthermore, the collection of serum samples was not performed according to the protocol initially designed for the method [29]. Therefore, the results of this study must be interpreted with caution, awaiting further prospective studies using standardized sampling protocols. Also, no cases of antibody-mediated rejection were found when testing was prompted. However, at the time, no surveillance testing of anti-HLA antibodies was performed. Thus, no data on the effects of anti-HLA antibody dynamics in correlation to cfDNA dynamics was possible to extract.

The major strengths of the study include the long follow-up period, the standardized way in which the surveillance program was performed and how the collection of tests have been carried out and fairly high number of analyzed samples.

Future studies of the current method for cfDNA analysis in lung transplant patients need to be prospective with larger cohorts designed with the purpose of determining practical cut-off values for clinical application. For instance, this study was designed and initiated before the ISHLT consensus document for the standardization of definitions of infections in cardiothoracic transplant recipients [51]. However, infections in our study were deemed clinically relevant in the presence of microorganisms in the

airways and assessed by an experienced clinician as clinically relevant. Given the retrospective nature of the available data and our selection inclusion, neither CRP nor anti HLA-antibodies were prospectively collected, and this would be of great interest in future prospective settings. Furthermore, future studies need to define inter-patient variability and include to analysis of cfDNA response to different types of CLAD as well as dynamics of cfDNA after CLAD has been developed. The analysis of more clinical variables, for examples donor specific antibodies would be of great interest.

In conclusion, in this study we used combined methods for detecting and quantifying both dd-cfDNA and rd-cfDNA. We found that, regardless of the method to quantify DF, elevated levels of dd-cfDNA were associated with CLAD development. Further prospective research is warranted to validate the measurement of cfDNA, to predict and avoid complications in a clinical setting.

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 studies involving humans were approved by the ethical review board in Gothenburg (Diary nr: 791-08). 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

MN collected data, performed statistical analysis, and drafted the manuscript. RN conceptualized the study, oversaw sample handling, participated in data interpretation, and reviewed the manuscript. JW participated in data interpretation and reviewed the manuscript. GD participated in data interpretation and reviewed the manuscript. JB participated in data interpretation and reviewed the manuscript. AR conceptualized the study, oversaw performance and quality control of PCR analyses and reviewed the manuscript. JM conceptualized the study, collected the samples, performed statistical analysis, drafted and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

Unrelated to the current study JM discloses consulting fees from Boehringer Ingelheim, AstraZeneca, GlaxoSmithKline, Takeda Pharma, Vicore Pharma, and Mallinckrodt.

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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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.12772/full#supplementary-material

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REVIEW
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Diaphragm and Lung Transplantation

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Mutual interactions between the diaphragm and lung transplantation (LTx) are known to exist. Before LTx, many factors can exert notable impact on the diaphragmatic function, such as the underlying respiratory disease, the comorbidities, and the chronic treatments of the patient. In the post-LTx setting, even the surgical procedure itself can cause a stressful trauma to the diaphragm, potentially leading to morphological and functional alterations. Conversely, the diaphragm can significantly influence various aspects of the LTx process, ranging from graft-to-chest cavity size matching to the long-term postoperative respiratory performance of the recipient. Despite this, there are still no standard criteria for evaluating, defining, and managing diaphragmatic dysfunction in the context of LTx to date. This deficiency hampers the accurate assessment of those factors which affect the diaphragm and its reciprocal influence on LTx outcomes. The objective of this narrative review is to delve into the complex role the diaphragm plays in the different stages of LTx and into the modifications of this muscle following surgery.

Keywords: review, lung transplantation, diaphragm, diaphragm dysfunction, phrenic nerve

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INTRODUCTION

Lung transplantation (LTx) is a well-established treatment for benign end-stage pulmonary diseases in selected patients. During the entire LTx process, numerous factors can interfere with the function and the morphology of the diaphragm, the main respiratory muscle. Starting from the preoperative phase, the diaphragm may be influenced by patient-specific and disease-related variables, such as the underlying respiratory disease, the comorbidities and the chronic treatments [1, 2]. These interactions persist throughout the postoperative period, beginning with the surgical intervention, which itself can represent a stressful trauma for the diaphragm. Conversely, the diaphragm can influence the different phases of LTx. The presence of a diaphragmatic dysfunction before LTx, may require a different patient management. During surgery, the volume of the recipient's chest cavity can depend on the diaphragmatic morphology leading to a size-matching issue. In the post-LTx setting, diaphragmatic dysfunction may hamper respiratory weaning, and impact on the long-term respiratory function of the patient or on sleep-related disorders [3–6]. Despite all these relevant dynamics, little is known on the real effects of diaphragmatic function abnormalities on LTx patients and *vice versa*. Notably, a standardized framework for the evaluation, definition, and management of diaphragmatic dysfunction within the context of LTx has yet to be established. This

deficiency hampers the accurate assessment of those factors which affect the diaphragm and its reciprocal influence on LTx outcomes. The primary objective of this narrative review is to delve into the intricate role the diaphragm plays in the different stages of LTx and into the modifications of this muscle following surgery, along with an overview of fundamental aspects of diaphragmatic function.

THE DIAPHRAGM

The diaphragm is the main respiratory muscle. Its contraction, along with that of the other respiratory muscles, generates sub-atmospheric pressure in the pleural cavity, creating a pressure gradient for air entry into the lungs. At rest, expiration is a passive process relying on the elastic recoil of the inflated lungs, whereas during exercise, expiration becomes active [7].

The diaphragm is a dome-shaped muscle, composed of vertical muscular fibres originating from a central tendon. It serves as the anatomical division between the thoracic and the abdominal cavity and it is innervated by the phrenic nerve. The diaphragm accounts for about 70% of the inspired air volume during quiet breathing: the muscle contracts with a piston-like movement causing a flattening of the dome and a decrease in the intra-thoracic pressure, thus allowing lung inflation. The pressure is generated against resistive and elastic loads that depend on airway resistance and chest wall compliance [7–9].

The function of the diaphragm is affected by many pathological mechanisms and physiological variables (e.g., level of consciousness, posture, lung expansion, lung compliance). The diaphragm's inability to maintain adequate ventilation can be caused by interference with innervation, contraction, or mechanical coupling to the chest wall [10, 11].

Diaphragmatic Dysfunction: Definition and Presentation

A diaphragmatic dysfunction can be defined as a loss of the function of the diaphragm, namely, respiration. It can be uni- or bi-lateral, transient or permanent, partial (weakness) or complete (paralysis), and its clinical significance can be variable. The clinical spectrum of diaphragmatic dysfunction is diverse, spanning from asymptomatic individuals to those experiencing severe respiratory failure. This can be secondary to a wide variety of factors related both to the characteristics of the dysfunction (e.g., bilateral vs unilateral) and the patient (e.g., obesity, lung disease) [12, 13]. In bilateral dysfunction, symptoms are more commonly present and intense. Conversely, a unilateral diaphragm dysfunction may often result asymptomatic. When present, symptoms may include orthopnoea and dyspnoea during exertion. Additionally, a diaphragmatic dysfunction can be linked to sleep-related breathing disorders, particularly in obese individuals. In cases of more pronounced diaphragm paresis, it can lead to snoring, breath cessation, and daytime sleepiness [14]. In the LTx setting, the respiratory function of the patient is already compromised due to the underlying respiratory disease, and this may interfere with a clear understanding of the role of diaphragmatic function abnormalities.

Techniques for Assessing Diaphragmatic Function

The diaphragmatic function can be evaluated through specialized tests, although not all are routinely available in clinical practice. However, several common diagnostic tools can also be employed to suspect or explore a potential diaphragm dysfunction. Some of these tests are based on evidence that are not specifically conceived on LTx patients. The presence of an underlying respiratory disease should always be kept in mind because it could overshadow the interpretation of diaphragmatic function.

Standard respiratory function tests are routinely available and easily accessible, thus even if not specific, in case of abnormalities (e.g., reduced forced vital capacity - FVC) they may rise the suspicion of diaphragmatic dysfunction [15, 16].

Supine respiratory function tests are the only specific pulmonary function tests available for diaphragmatic evaluation [16, 17]. A supine reduction of more than 30% or 15% of FVC is consistent with bilateral or unilateral diaphragmatic weakness, respectively.

The polysomnography is not specific for the diaphragm; however, it has been shown that unilateral diaphragmatic dysfunction has been linked to a higher prevalence of OSAS compared to healthy subjects [14, 18–22]. Thus, in patients suffering from diaphragmatic dysfunction, it may reveal a diagnosis of OSAS. On the contrary, in a LTx patient with a recently diagnosed OSAS, further testing for diaphragmatic dysfunction may be appropriate.

Even if diaphragmatic dysfunction may relate with exertional dyspnoea, exercise testing may not be specific for its diagnosis. Peripheral muscle weakness might determine a reduction in maximum oxygen consumption, overshadowing the detrimental effect of diaphragmatic weakness, and the inspiratory reserve is predominantly impacted by the thoracic respiratory muscles rather than the diaphragm [16, 23].

Pressure measurements are not always available in routine clinical practice but are specific. These tests measure the trans-diaphragmatic pressure (Pdi) as the difference between oesophageal and gastric pressures [16]. It is achieved through insertion of two balloon-tipped catheters through the nasal passage. Pdi can be measured during voluntary respiratory manoeuvres (e.g., sniff) or by inducing muscle contraction through electrical or magnetic phrenic nerve stimulation (TwPdi). The volitional nature of this test renders its reliability contingent upon patient effort and motivation.

The maximal inspiratory and expiratory pressures (MIP, MEP) involve assessing respiratory pressure during maximal efforts against a closed mouthpiece and indicate global respiratory muscle strength, thus they are not specific [15]. However, when MIP or SNIP are less than 60% or 30% of predicted values, unilateral or bilateral diaphragm paralysis, respectively, can be suspected.

Electroneuromyography (EMG) is specific and records action potentials of diaphragmatic muscle cells contraction [15, 16]. It investigates the electrical activation capacity of the diaphragm by surface or trans-oesophageal electrodes. Surface diaphragmatic EMG has certain limitations, including electrode placement

accuracy, signal attenuation through interposing tissues, and potential crosstalk from adjacent muscles. Trans-diaphragmatic EMG, performed via a gastroesophageal catheter with an array of wire coils, offers a more precise assessment of diaphragmatic electrical activity. Both surface and trans-diaphragmatic EMG can be performed during volitional (i.e.,: sniff, maximal contraction) or non-volitional (i.e.,: transcutaneous electrical or magnetic nerve stimulation) tests. Possible abnormalities in diaphragmatic dysfunction may be a reduced motor output, an abnormal neuromechanical coupling during loaded breathing or an increased level of assistance during mechanical ventilation.

Optoelectronic plethysmography and respiratory inductance plethysmography are not routinely available diagnostic tools, and they are not necessarily employed to specifically detected a diaphragmatic dysfunction. These techniques involve the analysis of thoracic and abdominal surface motion [24, 25]. In case of bilateral diaphragm paralysis, an asynchronous motion (e.g., paradoxical breathing during inspiration) can be detected.

The chest X-Ray is one of the most employed tests to initially explore a suspected diaphragm dysfunction [26, 27]. In asymptomatic patient, it could commonly lead to an accidental diagnosis. It allows assessment of the shape and the position of the diaphragm. Several static parameters have been proposed to standardize the diaphragm evaluation. Most commonly, a right-sided dysfunction is defined when the hemidiaphragm is > 2–4 cm higher than the left side, whereas on the left side, the hemidiaphragm is at the same height or more than the right side. Another interesting measurement is the diaphragmatic height index (calculated as the ratio of the distance between the apexes of the two hemidiaphragms and the height of T10 vertebra) which may predict diaphragm paralysis effectively, demonstrating high sensitivity (>90%) and specificity (>85%) [28]. However, bilateral dysfunction is more complex to evaluate with a static chest X-ray.

Magnetic resonance imaging (MRI) is not commonly employed to assess diaphragmatic dysfunction in LTx. Static MRI studies provide data on muscle size and structure, which could reveal specific features of rare diseases [29]. It can also be employed for dynamic imaging of the thorax and diaphragm using non-contrast breath-hold sequences and free-breathing diffusion-weighted imaging. As an example, a relationship between craniocaudal diaphragmatic excursion, diaphragm fatty infiltration, pulmonary function tests, and abdominal volumes (as an index of diaphragm activity) was demonstrated in patients with Duchenne Muscular Dystrophy.

Ultrasonography is a cheap and accessible test that allows the analysis of diaphragm dome excursion, thickness (Tdi), and thickening fraction (TFdi) [4, 30]. The latter one is defined as the difference between end-inspiratory and end-expiratory thickness divided by end-expiratory thickness, expressed in percentage. Measurements can be made both in dynamic M-mode and B-mode. It should be noted that healthy individuals may frequently exhibit TFdi values exceeding 100%. Overall, a reduced thickening fraction to less than 20%–29% is considered significant for diaphragmatic paralysis.

Finally, fluoroscopy is considered the gold standard for diaphragmatic dysfunction diagnosis. It can be employed during sniff manoeuvres to assess diaphragm dysfunction [31]. A comparison between upright and supine fluoroscopy can also be useful. Findings of diaphragmatic dysfunction include a reduced or absent diaphragm excursion and a paradoxical motion (e.g., one hemidiaphragm ascending while the other descends).

The list of available diagnostic tests along with their interpretation is reported in **Table 1**.

THE DIAPHRAGM IN THE LUNG TRANSPLANTATION PROCESS

The intricate relationship between LTx and the diaphragm extends across various stages of the transplantation process. This interplay can be simplified in two main phases: before and after LTx.

The Diaphragm Waiting for Lung Transplantation

Several factors can influence diaphragmatic function in patients waiting for LTx (**Figure 1**) [10].

Patients suffering from obstructive respiratory disorders, such as chronic obstructive pulmonary disease (COPD) and cystic fibrosis, experience chronic overexertion of respiratory muscles due to the increased resistance necessary for adequate lung inflation [33, 34]. Furthermore, in the presence of hyperinflated lungs characterized by an increased functional residual capacity (FRC), the diaphragm is flattened and operates at suboptimal lengths, resulting in fatigue and dyspnoea during physical activity [35]. In addition, this may eventually lead to a paradoxical inward movement of the lower ribcage margin during inspiration, known as the “Hoover sign” [36]. Evidence indicate that COPD patients exhibit diminished voluntary and induced respiratory pressures, including MIP, Pdi and TwPdi [35]. Notably, MEP emerges as an independent risk factor for survival in COPD patients [37]. Studies have also revealed a correlation between abnormalities in diaphragmatic morphology, assessed via CT-scan, and the severity of COPD [38]. Chronic overexertion of the diaphragm is also found in restrictive respiratory disorders, such as idiopathic pulmonary fibrosis [39, 40]. In this case, the diaphragm may result elevated, due to reduced lung compliance. However, the overload following the increased stiffness of the lung may not only cause exhaustion, but also respiratory muscle training [40]. Some Authors speculate that the presence of chronic systemic inflammation may also play a role in respiratory muscles deterioration [40].

Diabetes is a well-known cause of neuropathic damage and can lead to a muscular deficit too [41]. Its prevalence is consistent even in the cohort of patients waiting for LTx, reaching up to 25% [42]. However, the relationship between diabetes and phrenic nerve or diaphragm dysfunction remains unclear [3].

Nutritional status plays a pivotal role both before and after LTx [43]. Obesity has been linked to increased post-LTx mortality [43, 44] and sparks debate about potential structural alterations in the human diaphragm [2, 45]. Obesity is also the most common

TABLE 1 | Available diagnostic tests to assess a diaphragmatic dysfunction.

Test	Details	Findings/interpretation
Standard respiratory function tests (non-specific) [15, 16]	Routinely available and easily accessible. Even if not specific, in case of abnormalities they may rise the suspicion of diaphragmatic dysfunction	• Reduced VC • Normal or increased RV • Decreased TLC • Decreased FVC • Decreased maximum expiratory flow • Normal DLCO
Supine respiratory function tests (specific) [16, 17, 32]	Only specific pulmonary function test available for diaphragmatic evaluation	Bilateral diaphragmatic weakness • Seated FVC <50% of predicted • Supine decrease of FVC >30% Unilateral diaphragmatic weakness • Seated FVC <80% of predicted • Supine decrease of VC higher than 15%
Polysomnography (non-specific) [14, 18–21]	Even if not specific, it has been shown that unilateral diaphragmatic dysfunction has been linked to a higher prevalence of OSAS compared to healthy subjects	• Sleep hypopnea in moderate to severe diaphragmatic weakness • Increased severity of sleep disorder breathing (particularly during the REM phase, where the diaphragm is the primary inspiratory muscle) • Reduced responsiveness to CPAP • Higher incidence of necessitating BPAP.
Exercise testing (non-specific) [16, 23]	Potential overshadowing from peripheral muscle weakness	Reduced exercise tolerance
Pressure measurements (specific) [16]	Trans-diaphragmatic pressure (Pdi) is the difference between esophageal and gastric pressures, measured through the insertion of two balloon-tipped catheters through the nasal passage. There are voluntary Pdi (e.g., sniff) or induced Pdi through nerve stimulation (TwPdi) Voluntary Pdi limit is its volitional nature	Unilateral diaphragm paralysis • TwPdi <10 cmH_2O with unilateral phrenic nerve stimulation Bilateral diaphragm paralysis • TwPdi <20 cmH_2O with bilateral phrenic nerve stimulation Clinically significant inspiratory muscle weakness can be excluded when sniff-Pdi or Pdimax exceed • 80 cmH2O for men • 70 cmH2O for women
Maximal inspiratory and expiratory pressures (MIP, MEP) (non-specific) [15]	It assesses respiratory pressure during maximal efforts against a closed mouthpiece and indicate global respiratory muscle strength	Unilateral diaphragm paralysis • MIP or SNIP <60% predicted values Bilateral diaphragm paralysis • MIP or SNIP <30% predicted values Clinically significant inspiratory muscle weakness can be excluded when SNIP exceed • 70 cmH2O for men • 60 cmH2O for women Or MIP exceed • 80 cmH2O for men • 70 cmH2O for women
Electroneuromyography (EMG) (specific) [15, 16]	EMG records action potentials of diaphragmatic muscle cells contraction by surface or trans-esophageal electrodes. Both EMG can be performed during volitional (i.e.,: sniff, maximal contraction) or non-volitional (i.e.,: transcutaneous electrical or magnetic nerve stimulation) tests	• Reduced motor output • Abnormal neuromechanical coupling during loaded breathing • Reduced efficacy of contraction (when associated to ventilation measurements) In the ICU • Increased level of assistance during mechanical ventilation • Increased effort to breathe
Optoelectronic plethysmography and respiratory inductance plethysmography (non-specific) [24, 25]	Not routinely available and not necessarily employed to specifically detected a diaphragmatic dysfunction. They analyse thoracic and abdominal surface motion	Bilateral diaphragm paralysis • Asynchronous motions (e.g., paradoxical breathing during inspiration) Unilateral diaphragm paralysis • Chest asymmetry
Chest X-Ray (specific) [26–28]	One of the most employed. Bilateral dysfunction is more complex to evaluate	• Elevated hemidiaphragm (frontal, end-inspiratory chest X-ray), in particular o on the right side, the hemidiaphragm is > 2–4 cm higher than the left side

(Continued on following page)

TABLE 1 | (*Continued*) Available diagnostic tests to assess a diaphragmatic dysfunction.

Test	Details	Findings/interpretation
		o on the left side, the hemidiaphragm is at the same height or more than the right side • Inspiratory-expiratory difference between the two hemidiaphragms
Magnetic resonance imaging (MRI) (non-specific) [29]	Not commonly employed. Both static and dynamic MRI can be performed	• Reduced craniocaudal excursion • Diaphragm fatty infiltration
Ultrasonography (specific) [4, 30]	Cheap and accessible. It shows diaphragm dome excursion, thickness (Tdi), and thickening fraction (TFdi). TFdi is the difference between end-inspiratory and end-expiratory thickness divided by end-expiratory thickness, expressed in percentage	• Reduced thickening fraction to less than 20%–29%
Fluoroscopy (specific) [31]	Gold standard. Upright and supine comparison can be useful	• Reduced or absent diaphragm excursion • Paradoxical motion (e.g., one hemidiaphragm ascending while the other descends)

VC, vital capacity; RV, residual volume; TLC, total lung capacity; FVC, forced vital capacity; DLCO, diffusing lung capacity of carbon monoxide; REM, rapid eye movement; CPAP, continuous positive airway pressure; BPAP, bi-level positive airway pressure; MIP, maximal inspiratory pressure; MEP, maximal expiratory pressure; SNIP, sniff nasal inspiratory pressure.

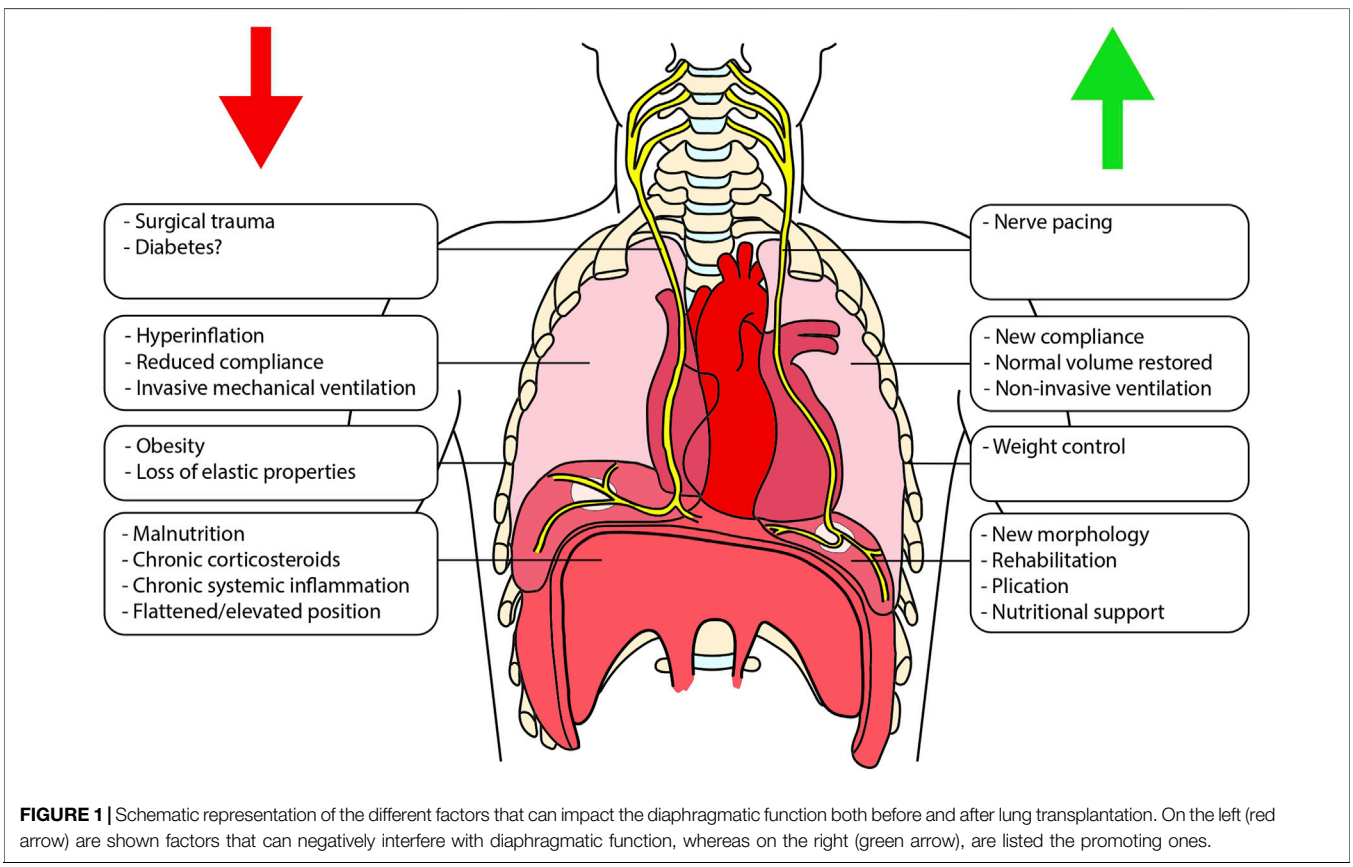

FIGURE 1 | Schematic representation of the different factors that can impact the diaphragmatic function both before and after lung transplantation. On the left (red arrow) are shown factors that can negatively interfere with diaphragmatic function, whereas on the right (green arrow), are listed the promoting ones.

restrictive disorder, exerting a higher body mass load both on the chest wall and on the abdomen, leading to an increased respiratory workload [10]. A correlation between malnutrition and diminished diaphragmatic strength in patients with cystic fibrosis was found [46], and some post-transplantation studies have reported reduced survival in malnourished individuals [47, 48]. Nevertheless, the relationship between nutrition and diaphragm function and structure remains a complex and incompletely understood area of study [47].

Both acute and chronic inflammation may affect the respiratory muscle function [49]. Some Authors speculate that in respiratory diseases with chronic systemic inflammation, such as

idiopathic lung fibrosis, this mechanism may participate in muscle weakness [50]. Inflammation may also result from infections, especially in patients affected by cystic fibrosis [51]. Patients affected by severe respiratory diseases may also experience acute septic states. Although sepsis has been associated with temporary and reversible diaphragm dysfunction [52], more evidence is needed to fully grasp the correlation between acute inflammation and abnormal diaphragmatic function.

Chronic corticosteroid use-related myopathy is a known adverse effect and can affect function and volume of skeletal muscles [1, 53, 54]. Even if there are no studies specifically addressing the diaphragm in humans, particularly those waiting for LTx, a loss of respiratory strength has been described in chronic corticosteroid treatment. However, it is reasonable to postulate the presence of multiple confounding factors that warrant consideration (e.g., patients with worse respiratory function and overall health possibly receive higher corticosteroid doses compared to healthier counterparts).

Finally, another pre-LTx factor that may influence the diaphragm is the respiratory support. The impact of chronic non-invasive ventilation (NIV) on diaphragm function remains underexplored. While some Authors suggest potential improvements in diaphragmatic contraction with NIV, other studies report no discernible effects [55]. The impact of NIV on patient survival remains controversial. Nonetheless, NIV is a widely employed treatment for chronic respiratory failure and is accepted as a bridge to LTx [56]. Conversely, invasive mechanical ventilation has been associated with diaphragm atrophy, with links to prolonged mechanical ventilation, ICU admission, and complications during acute respiratory failure [57]. Currently, there is a lack of data to clarify the impact of extracorporeal membrane oxygenation (ECMO) on diaphragmatic function, in the setting of bridging to LTx [58, 59].

The Interplay Between the Diaphragm and Lung Transplantation

LTx potentially triggers significant alterations in the diaphragm. Concurrently, the diaphragm influences the course of LTx (**Figure 1**). Herein, we provide an overview of the key aspects governing the reciprocal relationship between the two.

Diaphragm Activity, Outcomes, and Respiratory Function

Diaphragm contraction depends on various factors (e.g., neural function, diaphragmatic morphology and structure), although isolating their individual significance can be challenging. The incidence of a diaphragmatic dysfunction can vary across studies due to different identification methods and definitions [3, 27]. When referring to multiple parameters, such as respiratory function test, chest X-ray, ultrasound, opto-electronic plethysmography, and electromyography, in the early postoperative phase of LTx a diaphragm dysfunction may be systematically detected, although it might not necessarily be clinically significant [3]. This was shown in a cohort of 30 BTLx patients and the abnormalities persisted for 6 months, with full recovery at one-year post-transplantation [3]. Diaphragmatic dysfunction has been noted in terms of force, weakness, electrical activity, and kinematics. Despite that, an improvement in global spirometry and the six-minute walking test (6MWT) was reported. An incidence of 62% for diaphragmatic dysfunction was observed using ultrasound assessment, which decreased till 22% at 3 months from LTx, without impacting outcomes [60]. When concerning only chest X-ray findings, a unilateral elevated hemidiaphragm was detected in 23% of a cohort of 1,100 LTx patients in the early postoperative period (with a median of 21 days post-surgery) [27]. This abnormality reverted in 38% of cases, but in the remaining 62% a permanent elevation over time was seen, though no significant impact on outcomes (e.g., survival, chronic lung allograft dysfunction) was noted despite worse lung function tests. Additionally, a diaphragmatic elevation was present before LTx in nearly 3% of the study cohort, and this was a significant risk factor for presenting postoperative diaphragmatic elevation ($p < 0.001$), predominantly permanent ($p < 0.001$). More than half (58%) of these patients had pulmonary fibrosis as indication to transplantation. Interestingly, diaphragm elevation reverted after LTx in 45% of cases.

Huh et al. analysed the clinical relevance of a pre-LTx diaphragmatic dysfunction and its possible evolution after transplantation. Of 102 BLTx patients, 32% presented preoperative diaphragmatic dysfunction during ultrasound assessments [61]. After surgery, 12% and 3% of them showed a persistent (same side) and new (contralateral side) dysfunction, respectively. Moreover, nearly 55% recovered at 3 months from surgery, and an additional 30% within one-year. The presence of preoperative diaphragmatic dysfunction was found to be a negative prognostic factor. These patients experienced prolonged mechanical ventilation, extended ICU and hospital stays, and showed a significantly lower FVC. The difference in in-hospital mortality between patient with and without pre-LTx dysfunction was not statistically significant. However, the subgroup with the highest mortality was the one with preoperative diaphragmatic dysfunction that did not recover at 3 months.

Diaphragm dysfunction following BLTx, identified by ultrasound, has been associated with difficult weaning in the ICU setting [4]. In patients experiencing challenging weaning, nearly 78% exhibited diaphragmatic dysfunction. Neuro-ventilatory efficiency (NVE), defined as the ratio of tidal volume to peak electrical activity of the diaphragm, was also linked to difficult weaning in these patients. Longer durations of ventilation inversely correlated with both TFdi and NVE.

The improvement of the diaphragmatic function goes in parallel to the respiratory function gain following BLTx, as demonstrated in a cohort of patients with cystic fibrosis and bronchiectasis [62]. The study monitored pulmonary function tests, MIP, and surface diaphragmatic electromyogram, since the pre-LTx phase. It was shown that maximal contraction strength and diaphragmatic resistance increased, and this positive effect tended to stabilize after 6 months post-surgery. In the early phase, at one-month post-LTx, MIP was not significantly improved, but the time limit (i.e., the duration between contraction onset and exhaustion) value was already substantially increased compared

to the preoperative period. This could be attributed to increased Vital Capacity (VC) along with reduced hypoxic drive, allowing for longer breath-holding periods. However, diminished MIP values at this time may also be linked to pain or other inhibitory pathways. Noticeable MIP improvements tend to be perceived at 6 months post-LTx. In these cases, the negative correlation between MIP and RV/TLC (total lung capacity) observed pre-transplantation subsequently disappears. Another study showed that 2 years post-LTx, diaphragmatic and abdominal muscles' thickness and strength were comparable to healthy controls [63]. However, quadriceps strength and cross-section were decreased by nearly 30% in LTx patients, with cumulative corticosteroid dosage emerging as an independent predictor of quadriceps atrophy.

When concerning long follow-up, in a cohort of 15 patients after 5 years post-surgery, lower diaphragm thickening ratios (DTR) but normal TFdi at FRC were found, and the DTR was unrelated to 6MWT distance but strongly correlated with forced expiratory volume in one second (FEV1) [64]. Nonetheless, when compared to normative data, most LTx patients exhibited nearly normal DTR values, indicating preserved or regained diaphragm contractility post-LTx. The presence of diaphragmatic muscular abnormalities was hypothesized after excluding a neural dysfunction, achieved through further testing of the phrenic nerve response to stimulation. This revealed normal electrical activity in both groups, but lower TwPdi in LTx patients. Whether these abnormalities existed pre-LTx or developed post-LTx remains unclear [64].

Neural Function

Neural activity, encompassing both the phrenic nerve and the central neural drive, plays a pivotal role in diaphragm function. During LTx surgery, the phrenic nerve is exposed to potential damage and subsequent dysfunction, which can range from complete (paralysis) to partial (neurapraxia or moderate axonotmesis). The duration of such dysfunction varies, spanning from permanent to temporary. The definition of phrenic nerve injury and, consequently, diaphragmatic dysfunction significantly varies across studies [3, 65, 66]. The incidence of phrenic nerve injury after LTx can range between 3%–43%, with complete permanent paralysis being relatively rare. Partial dysfunction potentially results from surgical manipulation (primarily of the pericardium and mediastinum) or the use of cold solutions/ice during surgery [3, 66]. When measuring diaphragmatic compound muscle action potential area and phrenic nerve latency, this dysfunction might be constantly present after LTx, with a return to normality over several months, and may be sub-clinical (i.e., asymptomatic, or not visible at chest X-ray) [3]. When the phrenic nerve injury is defined as the presence of both ultrasound and neurophysiological abnormalities, this incidence can reach almost 43% in LTx patients [66]. Around 29% of phrenic nerves exposed to injury during surgery on the same side possibly sustain damage. Identified risk factors include right lung grafts and mediastinal adhesiolysis. LTx patients with phrenic nerve injury often experience longer ICU stays, increased reintubation rates, and more frequent use of NIV [66, 67]. Nevertheless, without a standardized definition and evaluation of phrenic nerve injury, determining its true impact on diaphragm function after LTx remains challenging. Moreover, the optimal management of this condition has yet to be defined. Even in the absence of detected diaphragmatic electrical activity, in the first 48 h post-LTx no respiratory impairment may be observed so far [68]. This finding emerged from a study involving the use of Neurally Adjusted Ventilatory Assist, which provides ventilatory assistance proportional to the diaphragm's electrical activity. This activity was detectable in 63% of patients. Additionally, in two patients with long-term diaphragmatic dysfunction, normal electrical activity was recorded in the early postoperative period.

Another significant effect of LTx may relate to neural drive. In COPD patients undergoing LTx, a reduction in inspiratory effort sensation during ventilatory stress can lead to improved quality of life [69]. Compared to COPD patients, a decrease of the neural drive to the diaphragm with a normal endurance of inspiratory muscles was found after LTx. In single lung transplantation (SLTx) patients even the native side had a lower diaphragmatic neural activation. This suggests an involvement of the diaphragm at transplanted side to support the work of the other one.

Studying LTx patients, Kinnear et al. postulated the hypothesis that ventilatory compensation does not depend on vagal information from intrapulmonary or tracheal airway stretch receptors, but on diaphragmatic Golgi tendon organs [70]. In fact, with postural changes, the respiratory function tests revealed no differences between LTx patients and healthy controls. A tilt table test after the blockade of tracheal stretch receptors with aerosolized lidocaine have shown an immediate and unchanged ventilatory response. This hypothesis might be supported by the finding of unchanged respiratory pattern adaption to variations in ventilatory assistance and positive end-expiratory pressure (PEEP) in the early postoperative setting of LTx [68]. Notably, surgery determines interruption of vagal continuity at the bronchial anastomoses level, theoretically disabling the volume-feedback response.

Diaphragmatic Morphology

An interesting area of study concerns the modifications of diaphragmatic morphology following LTx and their consequent functional implications. In patient suffering from chronic lung hyperinflation, such as those with COPD, LTx reduces diaphragmatic flattening. This mitigation permits positional adjustments that bring mechanical advantages to ventilation, thereby achieving a respiratory gain [71]. One-month post-surgery, chest X-rays of LTx recipients can reveal extended diaphragm length compared to COPD patients. Additional findings may include higher sniff-Pdi values but similar TwPdi. Notably, the restoration of diaphragmatic morphology in emphysematous patients may need up to 2 years post-surgery [72]. Even in the presence of a positional recovery, the diaphragmatic surface may remain smaller on the graft side of patients receiving SLTx, when compared to the native side and to the ipsilateral side of healthy controls [72]. This

phenomenon appears to be determined by a mediastinal displacement toward the transplanted lung. A similar finding was previously described by Groote et al. [73]. In addition, they demonstrated a bidirectional lateral movement of the mediastinum during respiration. Dynamic CT-scans of a single thoracic slice in both healthy controls and SLTx patients showcased mediastinal movement towards the native lung during inspiration and towards the graft during expiration. These movements seemed to be unaffected by changes in respiratory rate or position (supine or standing). However, it remains unclear whether these movements coincide with asymmetrical diaphragmatic motion.

Conversely, patients affected by restrictive disorders may tend to show a higher diaphragm. In a cohort of 37 SLTx patients, the pre- and post-LTx CT scans were compared to assess diaphragmatic changes. In restrictive disorders (i.e., fibrosis), while the native side had no modifications, diaphragmatic height significantly reduced on the graft side [74]. This may reflect an increased lung volume as well as an efficient diaphragmatic contraction with a more compliant lung, due to a previous chronic overload. In addition, the diaphragmatic thickness of the graft side also significantly increased in all patients. A negative association was found between diaphragmatic height and FVC and TLC, confirming the benefit of a diaphragmatic remodelling. However, diaphragmatic elevation in restrictive disorders may not always revert after LTx [27].

The Role of the Chest Wall

Following bilateral lung transplantation (BLTx) and heart-lung transplantation (HLTx), TLC tends to align with predicted values for recipients and does not correlate with pre-LTx values [75]. Nonetheless, despite TLC returning to normal values post-LTx, VC may decrease while FRC and residual volume (RV) increase. Normal FEV1/FVC ratio values might not be indicative of airway obstruction or muscle weakness but could signify irreversible alterations in the static elastic properties of the chest wall, possibly related to chronic lung hyperinflation. There is evidence concerning the role of the different surgical incisions (i.e., clamshell, thoracotomy, median sternotomy) on respiratory function and chest wall elasticity [76–78], however similar findings were not shown for the diaphragmatic function [3].

Diaphragm and Sleep-Disordered Breathing

Both LTx and diaphragmatic dysfunction are linked to sleep-disordered breathing [5, 6, 14, 19, 21, 22, 79]. In the setting of LTx, this sleep-related disorder can occur both before (18%–45% prevalence) and after (30%–64%) surgery, and patients without NIV or oxygen supplementation are at higher risk [5, 6, 22]. Interestingly, approximately 50% of pre-LTx sleep-disordered breathing cases may resolve after transplantation. The presence of this disorders was shown to not affect survival in LTx patients [5]. In non-LTx patients, diaphragmatic dysfunction was related to worse sleep-disordered breathing scenarios (e.g., increased respiratory disturbance index, lower oxygen saturation) and a different NIV management [21, 79]. However, literature lacks studies addressing the association between diaphragmatic dysfunction and sleep-disordered breathing in the LTx context. At present, only one case report exists, which showed that despite treatment and resolution of the diaphragmatic dysfunction in a LTx patient, the sleep disorder persisted [80]. Patients affected by sleep-disordered breathing usually require therapy (i.e., nocturnal NIV support) to alleviate symptoms, however the ideal management of a post-LTx sleep-related breathing disorder is yet to be defined [5, 6, 22]. Indeed, early diagnosis has been emphasized [6].

Postoperative Complications

Complications of LTx surgery may also directly involve the diaphragm. A spontaneous rupture of the diaphragm is a rare but possible complication of LTx [81, 82]. Literature cases have been described to appear in the early postoperative phase. Clinical manifestations include chest pain, subcutaneous emphysema, and dyspnoea. At chest X-ray abdominal visceral herniation in the thorax may be identified. The surgical repair usually consists of both a direct suture of the laceration or the positioning of a prosthesis (e.g., Goretex). A thinner diaphragm can be found intraoperatively. Some Authors suggested an association with lung emphysema.

Potential Treatments

There is still no univocal evidence that a specific management of diaphragm function is effective in improving the outcomes after LTx. Many studies on this topic have been published and the interest continues to grow.

Diaphragm Plication

Lawrence et al. distinguished two indications for diaphragm plication: anatomical (i.e., size matching between recipient chest cavity and donor graft) and functional (i.e., clinically evident diaphragm dysfunction) [83]. They showed that most (78%) of the 38 diaphragmatic plication procedures were performed for anatomical reasons during LTx surgery, to increase recipient space and avoiding graft volume reduction. Almost 11% of patients receiving LTx during the study period underwent diaphragm plication, confirming the low frequency of this procedure. In the functional indication setting (22%), diaphragm plication was performed subsequently to LTx. Most were unilateral. Patients reported varying degrees of dyspnoea, orthopnoea, and persistent supplemental oxygen needs. Adhesions were almost constantly present, requiring an open surgical approach through a thoracotomy. Overall, unilateral right plication was the most common (57%), followed by bilateral plication (32%). A pre-LTx severe diaphragmatic dysfunction was detected on fluoroscopy only in 3% of patients. Interrupted sutures (62%) were more common than running sutures. Postoperative outcomes were satisfying. Only two (5%) asymptomatic patients had an incidental finding of liver laceration as a complication. In patients that received plication, the 6MWT distance at 1 year was not altered. When compared to patients without plication, FEV1 and FVC were consistently lower. Moreover, three-year survival

and chronic lung allograft dysfunction-free survival were similar.

Other experiences of diaphragm plication in patients receiving LTx are limited mainly to case reports [84, 85].

This evidence confirms the extremely low frequence of diaphragmatic plication during LTx surgery for functional reasons. Future research could address whether it could be possible to identify ideal candidates that may benefit diaphragmatic plication at LTx time.

Non-Invasive Ventilation

Domiciliary NIV for post-LTx patients is uncommon; however, diaphragm palsy is one of the two main indications, as found in a retrospective study on 488 LTx over a 6.5-year period [86]. Five out of 20 (25%) patients requiring NIV had diaphragm dysfunction as an indication. Three LTx patients required NIV immediately after extubation while other two recipients started NIV within 1 month of being transplanted. All these patients had diaphragm palsies confirmed by ultrasound screenings. The incidence of diaphragm palsies in LTx patients ranged from 6.9% to 20%; however, only 1% of the patients required NIV for this indication. Further research is needed to further clarify the potential benefits of NIV in diaphragmatic dysfunction following LTx, along with its optimal introduction and discontinuation timing.

Physiotherapy and Nutritional Support

Respiratory physiotherapy is part of routine management in the early postoperative period after LTx and it is highly recommended [87, 88]. In the preoperative setting, respiratory rehabilitation has been shown to reduce dyspnoea and increase respiratory function parameters (e.g., 6MWT distance and DLCO) along with quality of life [89]. A trial showed that the addition of specific inspiratory muscle training (namely, the diaphragm) may empower the increase of walking distance, MIP and DLCO [90]. In the late post-LTx setting, respiratory rehabilitation is also linked to better respiratory performances [89]. It could be interesting to further investigate the optimal timing and methodology to rehabilitate the diaphragmatic activity in patients both waiting and that received LTx, especially when a clinically evident diaphragmatic dysfunction is present. In the late postoperative period, a comparison of the respiratory rehabilitation results between patients with and without dysfunction may also help determine the ideal candidates for this treatment.

Currently, there are no studies directly addressing the nutrition status effect on the diaphragm in the LTx setting. However, given the existence of a relationship between the nutritional status and the diaphragmatic performance [91], it could be useful to examinate the effect of nutrition correction in pre- and post-LTx patients.

Phrenic Nerve Pacing

Phrenic nerve pacing consists in direct electrical stimulation of the phrenic nerve through surgically implanted electrodes, to support a diaphragmatic dysfunction. In a feasibility trial from the Leuven group on three LTx patients, it was shown that intrathoracic intermittent pacing may help wean from mechanical ventilation and reduce the incidence of diaphragm dysfunction [92]. The electrodes were implanted at the time of LTx surgery and removed after up to 7 days. Electric stimulation managed to trigger ventilation and offer monitoring of changes of the diaphragm activity. In another study with a larger cohort, 11 patients received a temporary pacing system positioned at LTx surgery, whereas five patients underwent a laparoscopic positioning of a chronic pacing system, remotely after LTx [93]. In these patients, it was demonstrated that diaphragm stimulation helped both weaning from mechanical ventilation and recovery from phrenic nerve injury. In selected high risk LTx patients, this technology may effectively manage both temporary and chronic diaphragmatic dysfunction.

CONCLUSION

A renewed interest has recently emerged in understanding the role of the diaphragm in the LTx setting. Nonetheless, in this context, the identification and definition of diaphragmatic dysfunction remain highly heterogeneous across the literature. Furthermore, the clinical significance and optimal management of these diaphragmatic abnormalities are still unclear. Future research should focus not only on gathering more evidence but also on enhancing standardization of methods for assessment and treatment.

AUTHOR CONTRIBUTIONS

AP and GM contributed to the study conception and design. Material preparation, data collection and analysis were performed by GM, AL, and VM. The first draft of the manuscript was written by GM, AP, AL, and VM, and all Authors commented on previous versions of 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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Systemic Inflammation Differences in Brain-vs. Circulatory-Dead Donors: Impact on Lung Transplant Recipients

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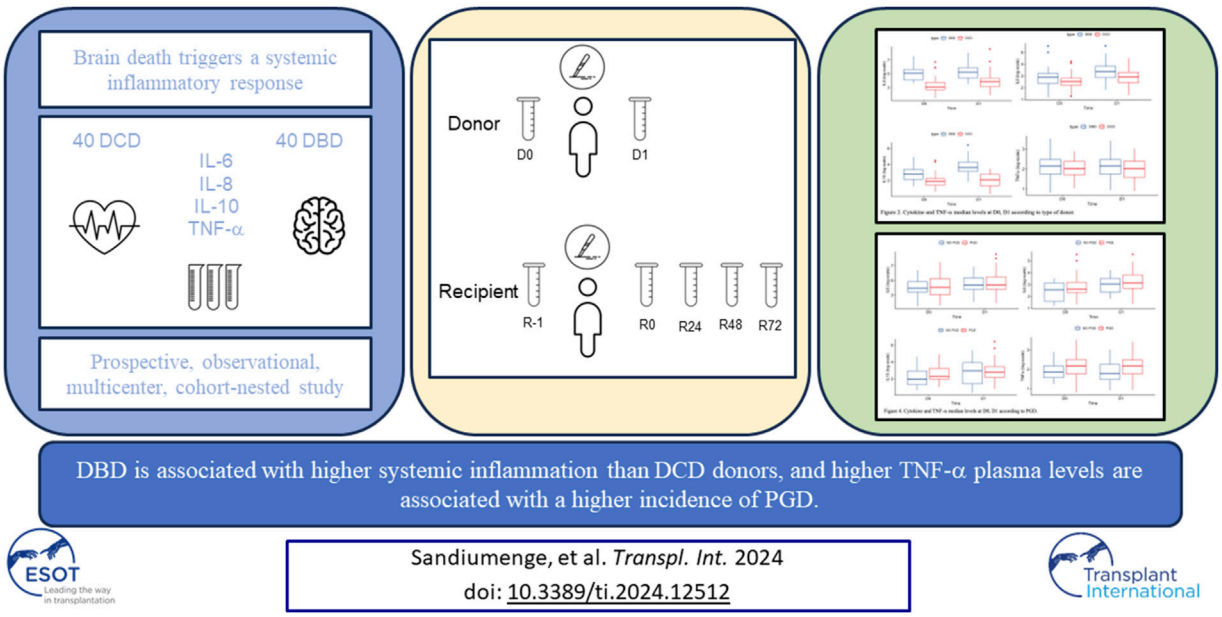
Brain death triggers a systemic inflammatory response. Whether systemic inflammation is different in lung donors after brain- (DBD) or circulatory-death (DCD) is unknown, but this may potentially increase the incidence of primary graft dysfunction (PGD) after lung transplantation. We compared the plasma levels of interleukin (IL)-6, IL-8, IL-10 and TNF-α in BDB and DCD and their respective recipients, as well as their relationship with PGD and mortality after LT. A prospective, observational, multicenter, comparative, cohort-nested study that included 40 DBD and 40 DCD lung donors matched and their respective recipients. Relevant clinical information and blood samples were collected before/during lung retrieval in donors and before/during/after (24, 48 and 72 h) LT in recipients. Incidence of PGD and short-term mortality after LT was recorded. Plasma levels of all determined cytokines were numerically higher in DBD than in DCD donors and reached statistical significance for IL-6, IL-10 and IL-8. In recipients with PGD the donor's plasma levels of TNF-α were higher. The post-operative mortality rate was very low and similar in both groups. DBD is associated with higher systemic inflammation than DCD donors, and higher TNF-α plasma levels in donors are associated with a higher incidence of PGD.

Keywords: lung transplantation, brain-death donation, circulatory-death donation, interleukin, cytokine storm



GRAPHICAL ABSTRACT |

INTRODUCTION

Donation after brain death (DBD) is the main source of organ donation for transplantation worldwide. Brain death (BD) usually induces a systemic inflammatory response. This "cytokine storm" may damage different body organs in donors, which may in turn have a deleterious impact on their function and survival in recipients after transplantation [1–3] since this can further aggravate the insults that occur during warm and cold ischemia and the subsequent reperfusion of the transplanted organ, by amplifying an inflammatory response in the recipient [4]. Lungs are especially sensitive to the BD-induced cytokine storm, which enhances the likelihood of ischemia-reperfusion-induced primary graft dysfunction (PDG) [5]. PDG is one of the main complication during the early post-operative period of lung transplantation and is the first cause of mortality during the first month and second one during the first year after transplantation [6] significant morbidity, as well as longer hospital length of stay and duration of mechanical ventilation. Experimental evidence has strongly suggested that DBD increases the incidence and severity of PGD(3,7).

Given the shortage of DBD donors, in recent years, donation after circulatory death (DCD) has been increasingly used as a source of organs for transplantation. The cytokine storm that follows BD and the potential deleterious impact on the lungs could theoretically be prevented or minimized during a DCD process [7]. Based on previous studies, we hypothesized that the plasma level of several inflammatory cytokines would be higher in DBD vs. DCD. To test this hypothesis, we conducted a prospective study that sought to compare: (1) the plasma levels of the pro-inflammatory (IL-6, IL-8, TNF-α) and anti-inflammatory cytokines (IL-10) in BDD vs. DCD; (2) the plasma levels of pro-inflammatory (IL-6, IL-8, TNF-α) and anti-inflammatory cytokines (IL-10) in recipients with PGD; and (3) the incidence of PGD and short-term mortality in LT recipients from DBD or DCD.

MATERIAL AN METHODS

Study Design and Ethics

This was a prospective, observational cohort-nested study conducted in four transplant centers in Spain that included adult patients undergoing uni- or bilateral LT between July 2018 and July 2019 and their respective BD or CD donors. The type of death was certified in accordance with Spanish legislation[25].

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the Human Research Committees of all participating hospitals (PR [AG]202/2017). All recipients provided written informed consent before being included in the study. Informed consent to participate in the study from donors was included in the donation consent. No deviation from the standard management of lung recipients took place except for serial blood sampling.

Patients

LT recipients from DBD and DCD were matched individually for sex, age (±5 years), and indication for LT (**Figure 1**). We excluded

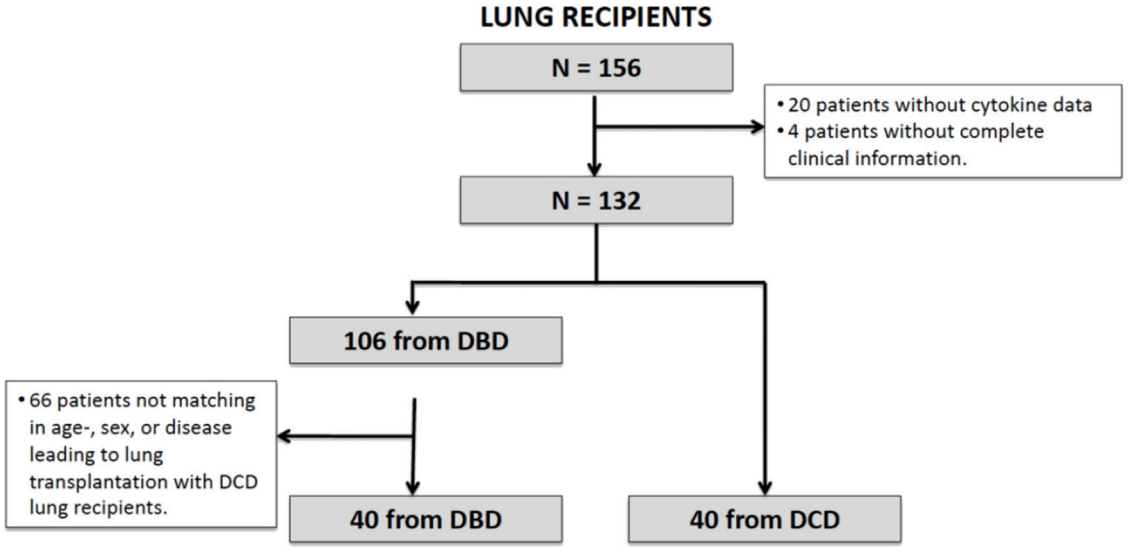


FIGURE 1 | Consort diagram of the study.

from this analysis patients undergoing lung re-transplantation, cardiopulmonary transplantation or those who had previously undergone or were to receive more than one simultaneous solid organ transplantation, as well as those for whom the graft ischemic time was >420 min. Following previous reports by Koukoulis et al.[27] and considering a confidence interval of 95%, statistical power of 80% and patient loss of 15%, a sample size of 36 LT recipients for each group (DCD and DBD) was estimated to detect a difference of at least one SD (15 pg/mL) in IL-6 plasma levels.

Study Variables

The following donor and recipient variables were prospectively collected: demographic (sex, age) and anthropometric (body mass index) measurements; clinical data (cause of death, corticosteroid pretreatment of donors and indication for transplantation of recipients); and surgical data (transfusion of blood products, vasoactive support or application of veno-venous [VV-] or veno-arterial [VA-] extracorporeal membrane oxygenation [ECMO], ischemic times for the first and second lungs and use of cardiopulmonary bypass). In all recipients, we registered the development of PGD (any grade and grade III) within 72 h post-transplantation according to the International Society for Heart and Lung Transplantation (ISHLT) Working Group criteria [8]. Mortality during the first 3 months after the transplant was also recorded.

Measurements

Blood samples (10 mL) were obtained from donors before skin incision (D0) and before organ perfusion (D1). In recipients, blood samples were obtained in the operating room before implantation surgery (R-1), just after graft reperfusion (R0) and 24 (R24), 48 (R48) and 72 (R72) hours after LT. All blood samples were collected in EDTA tubes and centrifuged at 1,000 g for 10 min at room temperature (22oC–23°C). Plasma was separated, divided into 2 aliquots of 2 mL each, and immediately stored at −80°C until analysis. At the end of the inclusion period, samples were shipped together in dry ice containers to the central laboratory located at the coordinating site (Hospital Universitari Vall d'Hebron, Barcelona) for cytokine analysis by immunofluorescence assays based on microfluidics using ELLA Simple Plex (Protein Simple, Biotechne, CA, United States) to simultaneously detect IL-6, IL-10, IL-8 and TNF-α. Triplicates of each cytokine result were obtained (the maximum allowed variation among triplicates was 5%).

Data Analysis

Data were collected and stored in an *ad hoc* database on the website of the *Organización Nacional de Trasplantes* (ONT, Spanish Transplant Organization) and made accessible only to the principal investigator of each participating site. A member of the ONT was commissioned to monitor the study.

The Shapiro-Wilk test was performed as a test of normality for IL level distribution. Categorical variables are expressed as n and percentages. Quantitative data is presented as mean ± SD if normally distributed or as median (Q1, Q3) if not. Chi-square test, or Fisher's exact test were used to compare categorical variables. Change over time was analyzed with non-parametric 2-way mixed repeated-measures ANOVA. The basic model included a group factor (DBD or DCD), a time factor, and an interaction between group and time. The main effects of group and time were explored for nonsignificant interactions. Bonferroni correction was applied to adjust for multiple comparisons in posthoc tests to determine specific pairwise differences between groups. All statistical analyses were performed using R version 4.3.1 (R Core Team, 2023). A p-value < 0.05 was considered statistically significant.

TABLE 1 | Donor and recipient characteristics.

		DBD (n = 40)	DCD (n = 40)	*p* -value
Donor	Sex, male, n (%)	22 (55)	18 (45)	0.799
	Age, years, mean ± SD	54 ± 16	56 ± 14	0.435
	BMI, kg/m^2, mean ± SD	26 ± 5	26 ± 5	0.754
	Corticosteroids treatment, n (%)	35 (87.5)	22 (55)	0.002
	Transfusion with blood products, n (%)	2 (5)	8 (20)	0.043
	Vasoactive drugs treatment, n (%)	34 (85)	12 (30)	0.001
	VV- or VA-ECMO, n (%)	0 (0)	4 (10)	0.116
	Cause of death, n (%)			0.014
	Stroke	31 (77.5)	19 (47.5)	
	Anoxia	3 (7.5)	13 (32.5)	
	Traffic head injury	2 (5)	1 (2.5)	
	Traffic non head injury	4 (10)	4 (10)	
	Other	0 (0)	3 (7.5)	
Recipient	Sex, male, n (%)	23 (57.5)	24 (60)	0.820
	Age, years, mean ± SD	56 ± 10	54 ± 10	0.500
	BMI kg/m^2± SD	25 ± 4	24 ± 4	0.189
	Ischemic time 1st graft, min, mean ± SD	238 ± 61	256 ± 48	0.213
	Ischemic time 2nd graft, min, mean ± SD	341 ± 82	353 ± 56	0.213
	Cardiopulmonary bypass, n (%)	20 (20)	7 (17.5)	0.775
	Transfusion with blood products, n (%)	27 (67.5)	19 (47.5)	0.070
	Vasoactive drugs intake, n (%)	33 (82.5)	35 (87.5)	0.630
	VV- or VA-ECMO after transplantation, n (%)	2 (5)	4 (10)	0.675
	Indication for lung transplantation (%)			-
	Bronchiectasis	3 (7.5)	3 (7.5)	
	Diffuse interstitial lung disease	17 (42.5)	18 (45)	
	Occupational lung disease	1 (2.5)	1 (2.5)	
	COPD/Emphysema	12 (30)	11 (27.5)	
	Cystic fibrosis	3 (7.5)	3 (7.5)	
	Pulmonary hypertension	3 (7.5)	3 (7.5)	
	Other	1 (2.5)	1 (2.5)	

BMI: body mass index; COPD, chronic obstructive pulmonary disease; ECMO: extracorporeal membrane oxygenation; VA: veno-arterial; VV: veno-venous.

RESULTS

Patient Characteristics

Figure 1 presents the consort diagram of the study. A total of 156 LT recipients were initially included, but 24 were later excluded because of incomplete data. Of the remaining 132 patients, 106 were DBD recipients and 40 were DCD recipients. Forty of the 106 DBD recipients were matched individually to the 40 DCD ones by sex, age (±5 years) and indication for lung transplantation.

Table 1 contrasts the main characteristics of both donors and recipients. More DBD received corticosteroids and vasoactive support before lung retrieval than DCD (p = 0.02 and p = 0.043, respectively), while more DCD than DBD received transfusions of blood products (p = 0.001). No other significant differences were observed between groups in donor or recipient characteristics.

Systemic Inflammation

At D0 and D1 the levels of IL-6, IL-10 and IL-8 were higher in the DBD group than in DCD, and a significant main effect of time with a higher concentrations at D1 compared to D0 with statistical significant increase the IL-10 levels at D1 in DBD group. There was no statistical differences of type or time on TNFα (**Table 2**; **Figure 2**).

In recipients, there was no statistical significance of type of donor on IL-6, IL-10, IL-8 and TNF. Before LT (R-1), the plasma level of these cytokines was similar in both groups (DBD and DCD) and immediately after LT (R0), the plasma levels of IL-6, IL-8 and IL-10, increased in both groups of recipients and decreased thereafter during the next 72 h without differeces. There was a statistically significant effect of time on TNFα only for the DBD group (**Table 3**; **Figure 3**).

Primary Graft Dysfunction (PGD)

The incidence of PGD of any grade was similar in DCD and DBD recipients (**Table 4**). Therefore, to further investigate the evolution of systemic inflammation in PGD we merged both groups for analysis. We found that the donor plasma levels of all cytokines were comparable between patients who developed any grade of PGD or not, except for TNF-α, which was higher at D0 and D1 in those recipients with PGD (**Table 5**; **Figure 4**). Specific analysis of PGD grade 3 did not show differences between groups (**Table 6**).

On the other hand, *recipients* experiencing PGD demonstrated elevated levels of IL-6 at R0 and a trend towards R48 and IL-8 at R0, R48, without differences between groups (**Table 7**; **Figure 5**).

TABLE 2 | Levels (pg/mL) of cytokines according to the type of donor at D0 and D1. Data expressed as median (Q1, Q3). *p*-values corresponding to non-parametric two-way mixed ANOVA.

	D0		D1		*p*-values		
	DBD	DCD	DBD	DCD	p:time	p:type	p:interaction
IL10	16.4 (8.6, 29.0)	6.8 (4.2, 9.4)	37.9 (22.8, 73.7)	8.1 (4.0, 15.9)	<0.001	<0.001	0.004
IL 6	164.3 (61.9, 278.9)	21.1 (15.1, 43.2)	189.4 (81.5, 363.3)	46.5 (23.0, 76.3)	0.040	0.003	0.381
IL 8	18.1 (11.3, 25.3)	12.5 (9.1, 16.5)	28.6 (17.5, 45.7)	18.5 (11.3, 27.4)	<0.001	0.010	0.210
TNF α	8.6 (5.7, 11.9)	7.5 (5.6, 10.7)	8.6 (5.7, 11.8)	7.5 (4.9, 10.8)	0.883	0.479	0.467

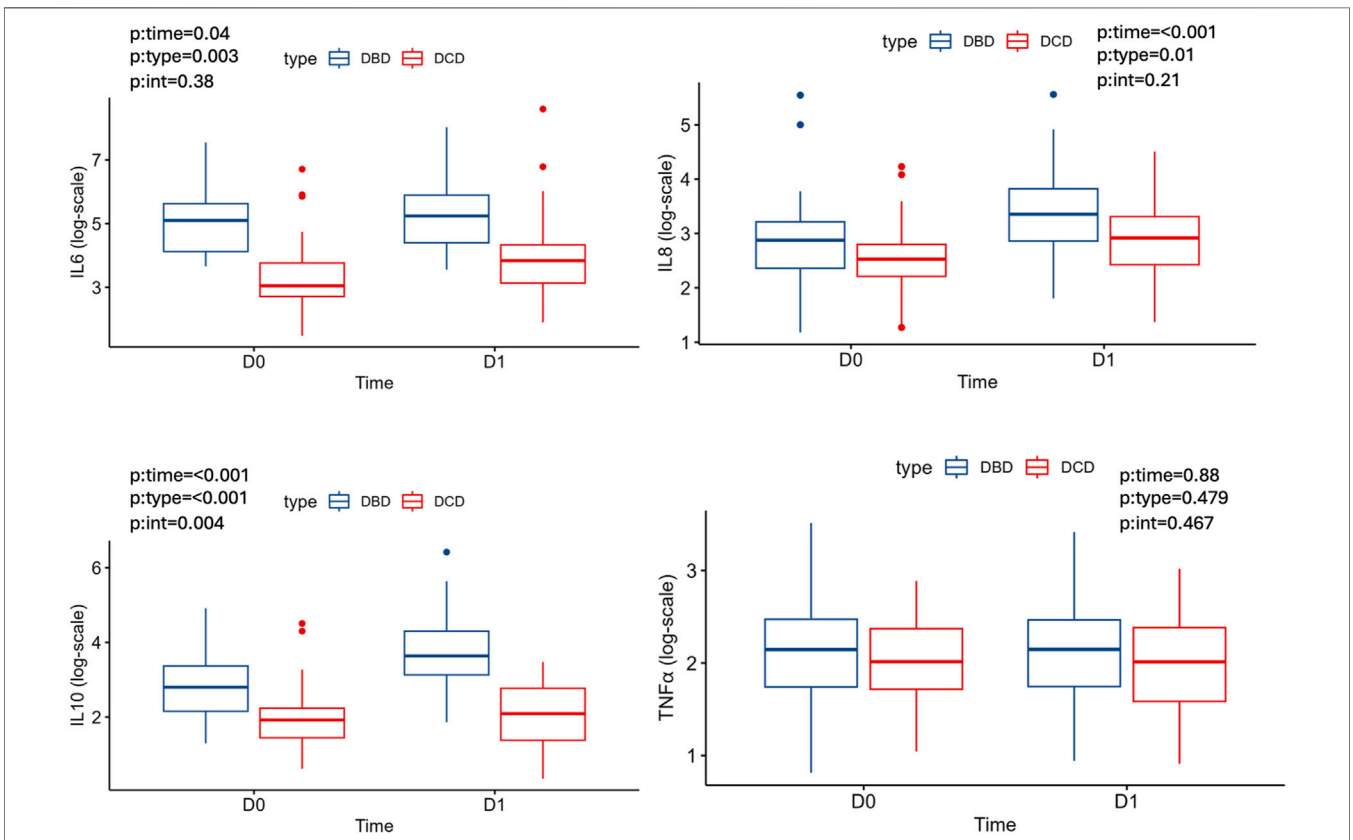


FIGURE 2 | Cytokine and TNF-α median levels at D0, D1 according to type of donor.

Furthermore, in the PGD grade 3 group, recipient plasma levels of IL-6 and IL-8 were significantly elevated at R0, R24 and R48 (**Table 8**).

Mortality

Post-operative mortality rate was very low and similar in both groups without any additional deaths within 3 months after surgery (**Table 4**).

DISCUSSION

The main results of this prospective, controlled, multicenter, cohort-nested study are that: (1) DCD presents lower systemic inflammation than DBD in donors; (2) after LT, the time course of systemic, the incidence of PGD and mortality after LT is similar in BDB and DCD recipients; and (3)recipients from donors with elevated levels of tumor necrosis factor-alpha (TNF-α) have a higher incidence of PGD and PGD grade 3 with elevated levels of IL-6 and IL-8. These observations support the DCD as a viable LT option.

To our knowledge, this is the first prospective multicenter study comparing systemic inflammation, PGD and mortality in LT recipients according to DCD vs. DBD donors. Yet, previous experimental studies have shown the development of systemic inflammation following BD [4, 5, 9] Also, it is known that increased systemic inflammation may worsen ischemia-reperfusion-induced lung injury [9, 10], and has been

TABLE 3 | Levels (pg/mL) of cytokines according to the type of donor at R-1 to R72. Data expressed as median (Q1, Q3). *p*-values corresponding to non-parametric two-way mixed ANOVA.

	R-1		R0		R24		R48		R72		*p*-values		
	DBD	DCD	DBD	DCD	DBD	DCD	DBD	DCD	DBD	DCD	p:time	p: type	p: interaction
IL10	2.9 (2.3, 4.3)	2.4 (1.9, 3.2)	151.6 (63.7, 624.0)	233.3 (69.2, 397.4)	17.4 (11.4, 24.1)	14.9 (9.8, 25.4)	7.9 (5.3, 10.5)	7.8 (6.3, 11.5)	7.9 (5.4, 10.8)	6.6 (4.6, 10.1)	<0.001	0.947	0.614
IL 6	8.1 (3.1, 23.7)	4.0 (2.1, 8.1)	329.2 (244.3, 848.8)	352.5 (107.4, 565.9)	56.7 (35.7, 93.4)	58.0 (41.6, 89.1)	25.3 (17.1, 39.9)	29.2 (18.9, 59.5)	19.9 (10.1, 30.7)	15.7 (8.9, 21.6)	<0.001	0.293	0.146
IL 8	14.8 (8.8, 29.0)	12.3 (8.2, 17.6)	56.2 (35.2, 119.0)	46.7 (19.1, 114.7)	12.7 (8.2, 21.8)	13.3 (9.0, 33.5)	10.6 (7.9, 16.2)	11.0 (8.0, 19.8)	12.3 (8.5, 16.7)	11.5 (8.0, 14.4)	<0.001	0.706	0.361
TNF α	5.9 (5.2, 7.3)	5.0 (4.1, 6.5)	7.1 (5.1, 10.1)	5.2 (4.1, 6.8)	4.8 (3.5, 6.7)	5.4 (3.7, 6.6)	4.9 (4.1, 7.9)	5.3 (4.0, 7.2)	6.2 (4.7, 9.8)	4.9 (3.7, 6.5)	0.062	0.069	0.005

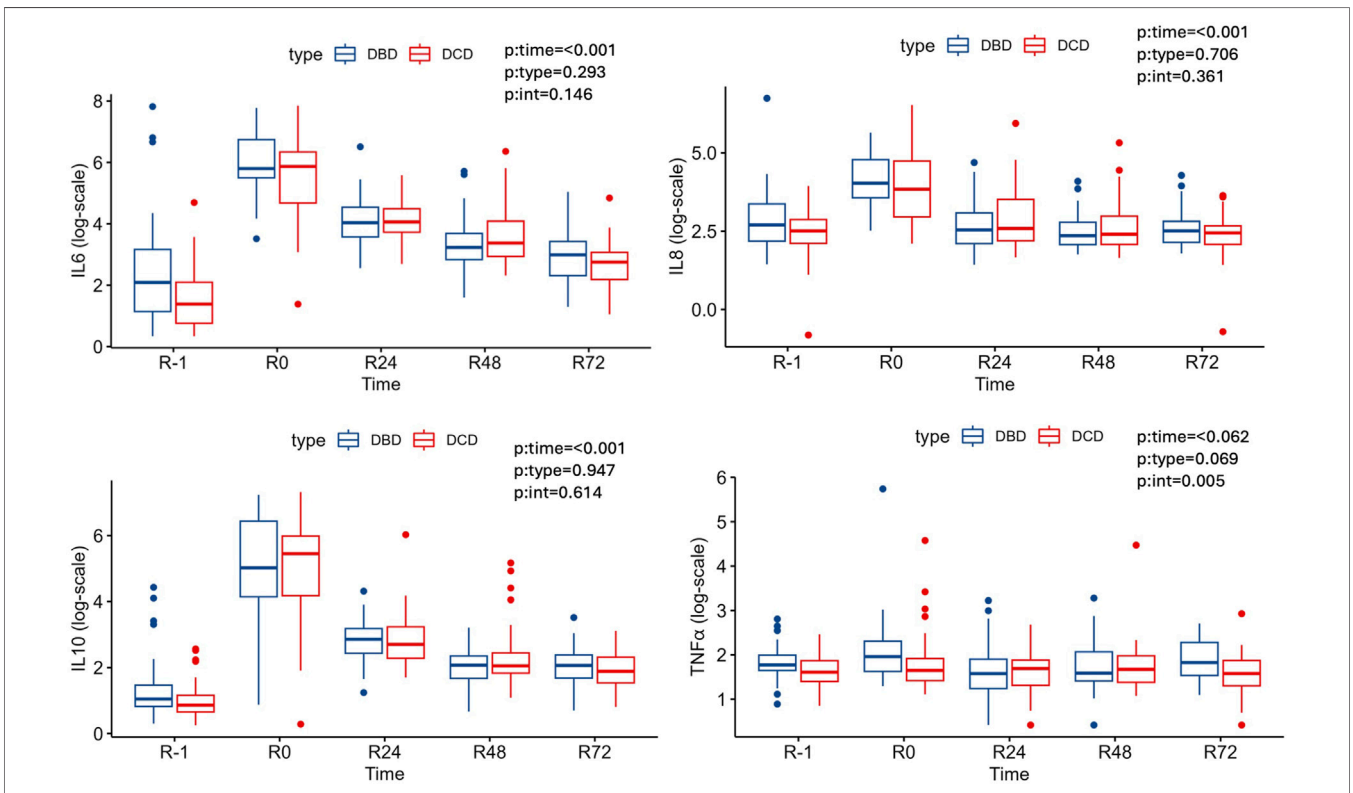


FIGURE 3 | Cytokine and TNF-α median levels at R-1, R0, R24, R48, R72 according to type of donor.

associated with a higher incidence of PGD [11, 12]. Our results confirm that cytokine levels are increased after BD.

The first goal of this study was to investigate if the systemic inflammatory response elicited in DBD or DCD was different. We found that the plasma levels of IL-6, IL-8, IL-10, all well-established inflammatory markers [13] were higher in donors (D1) in DBD than in DCD. Of note, this occurred despite DBD had been treated with systemic corticosteroids more often. The role of treatment with corticosteroids in the management of DCD is a matter of debate [14] although it is widely used in practice. So far, only two experimental studies have indicated that it significantly reduces the plasma levels of several pro-inflammatory cytokines, warm ischemic injury [15] or myocardial edema [16]. However, these studies were

TABLE 4 | Outcomes after lung transplantation from DBD and DCD.

	DBD (n = 40)	DCD (n = 40)	*p* -value
Primary graft dysfunction, n (%)			
Of any grade	27 (67.5)	26 (65)	0.100
Grade III	14 (35)	11 (27.5)	0.469
Post-operative mortality	0 (0)	2 (5)	0.494
Three-month mortality	0 (0)	2 (5)	0.494

conducted during *ex vivo* lung perfusion. Our results in real clinical practice suggest a small role for corticosteroid treatment in preventing systemic inflammation in lung DCD.

The second goal of our study was to compare the relationship of systemic inflammation with the incidence of PGD and short-term mortality in LT recipients from DBD and DCD donors. The relationship of PGD and systemic inflammation is a controversial issue since some previous studies have reported such a relationship [13, 17–20] whereas others did not [21]. We observed that recipients who presented PGD were transplanted from donors with elevated levels of TNF-α.

Finally, we found that lung reperfusion was followed by a rapid and similar increase in IL-6, IL-8 and IL-10 (not TNF-α) plasma levels in DBD and DCD recipients, followed by a reduction to normal levels in the next few hours, with a similar pattern in the two groups. These similarities likely explain why the incidence of PGD and mortality rate was not different in our study between DBD or DCD.

Our results may have clinical implications because they clearly show that DCD is not associated with increased

TABLE 5 | Levels (pg/mL) of cytokines according to PGD at D0 and D1. Data expressed as median (Q1, Q3). *p*-values corresponding to non-parametric two-way mixed ANOVA.

	D0		D1		*p*-values		
	No PGD	PGD	No PGD	PGD	p:time	p:PGD	p:interaction
IL10	7.0 (3.8, 18.9)	9.7 (7.2, 25.4)	19.2 (4.0, 50.0)	15.9 (8.8, 32.1)	0.018	0.903	0.291
IL 6	51.8 (30.4, 124.7)	58.2 (21.3, 188.6)	78.5 (43.5, 209.0)	81.8 (44.8, 237.5)	0.003	0.773	0.314
IL 8	12.9 (4.9, 23.1)	14.1 (10.3, 24.2)	21.1 (10.6, 33.5)	23.2 (14.6, 45.1)	<0.001	0.226	0.555
TNF α	6.4 (4.8, 8.8)	8.8 (5.8, 12.3)	6.0 (4.4, 9.5)	8.7 (5.8, 12.0)	0.769	0.022	0.648

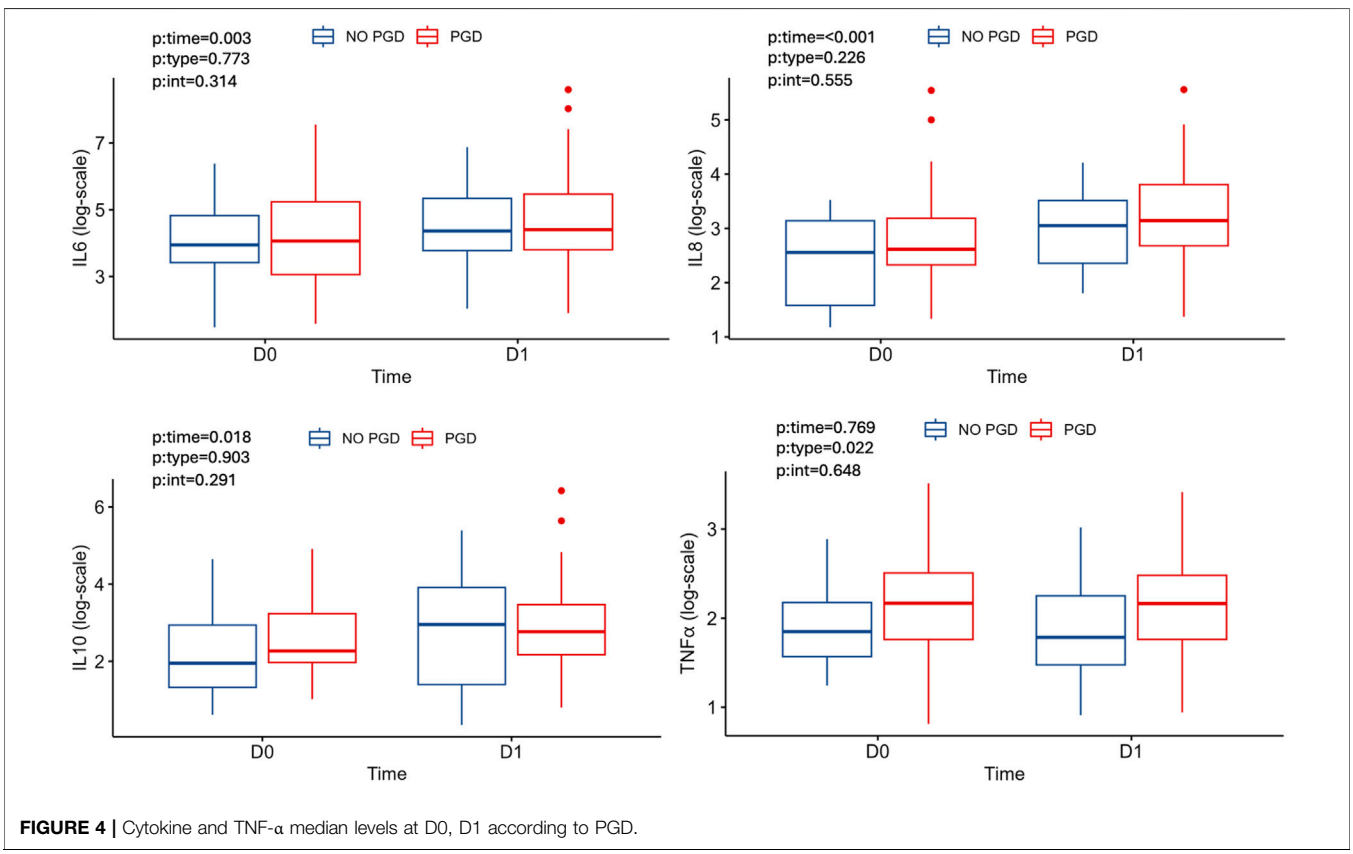


FIGURE 4 | Cytokine and TNF-α median levels at D0, D1 according to PGD.

TABLE 6 | Levels (pg/mL) of cytokines according to PGD0-2 vs. PGD3 at D0 and D1. Data expressed as median (Q1, Q3). *p*-values corresponding to non-parametric two-way mixed ANOVA.

	D0		D1		*p*-values		
	No PGD/PGD12	PGD3	No PGD/PGD12	PGD3	p:time	p:PGD3	p:interaction
IL10	8.8 (5.8, 23.2)	9.2 (6.8, 23.8)	20.6 (7.9, 40.7)	14.8 (6.4, 28.2)	0.001	0.419	0.040
IL 6	48.4 (20.9, 112.0)	164.3 (38.3, 302.2)	68.2 (35.7, 194.9)	125.8 (60.5, 443.6)	0.165	0.091	0.595
IL 8	13.0 (9.0, 22.7)	16.4 (10.2, 26.0)	19.9 (11.6, 34.3)	27.1 (17.1, 50.2)	<0.001	0.099	0.090
TNF α	7.0 (5.4, 10.5)	9.3 (6.0, 11.6)	7.4 (4.9, 11.8)	9.6 (6.4, 11.3)	0.182	0.618	0.615

TABLE 7 | Levels (pg/mL) of cytokines according to PGD at R-1 to R72. Data expressed as median (Q1, Q3). *p*-values corresponding to non-parametric two-way mixed ANOVA.

	R-1		R0		R24		R48		R72		*p*-values		
	No PGD	PGD	No PGD	PGD	No PGD	PGD	No PGD	PGD	No PGD	PGD	p:time	p: PGD	p: interaction
IL10	2.6 (1.9, 3.1)	2.9 (2.2, 4.4)	146.5 (44.1, 402.0)	184.9 (95.7, 409.6)	13.3 (10.0, 20.5)	19.0 (12.0, 28.1)	7.0 (5.3, 9.6)	8.4 (6.2, 11.7)	7.6 (4.9, 12.1)	7.3 (5.2, 9.6)	<0.001	0.654	0.212
IL 6	3.9 (2.4, 15.3)	4.7 (2.9, 12.6)	205.2 (79.1, 407.5)	462.9 (269.4, 792.3)	51.0 (33.6, 71.9)	63.6 (43.5, 99.2)	20.7 (14.8, 38.1)	28.5 (20.7, 52.7)	17.1 (7.2, 28.2)	17.7 (10.8, 27.0)	<0.001	0.009	0.114
IL 8	13.1 (9.0, 19.6)	12.8 (8.2, 24.1)	30.2 (16.2, 52.1)	67.1 (35.9, 120.4)	11.4 (8.5, 14.5)	13.5 (9.9, 27.5)	9.4 (7.0, 12.7)	11.6 (8.7, 21.2)	11.0 (7.9, 14.1)	12.4 (9.0, 15.8)	<0.001	0.003	0.068
TNF α	5.0 (4.2, 5.8)	5.7 (4.6, 7.0)	5.8 (4.0, 10.3)	5.6 (4.7, 8.8)	4.4 (3.1, 6.8)	5.3 (3.9, 6.3)	4.9 (4.0, 8.3)	5.2 (4.2, 6.8)	5.4 (4.5, 8.3)	5.3 (4.2, 7.1)	0.132	0.927	0.617

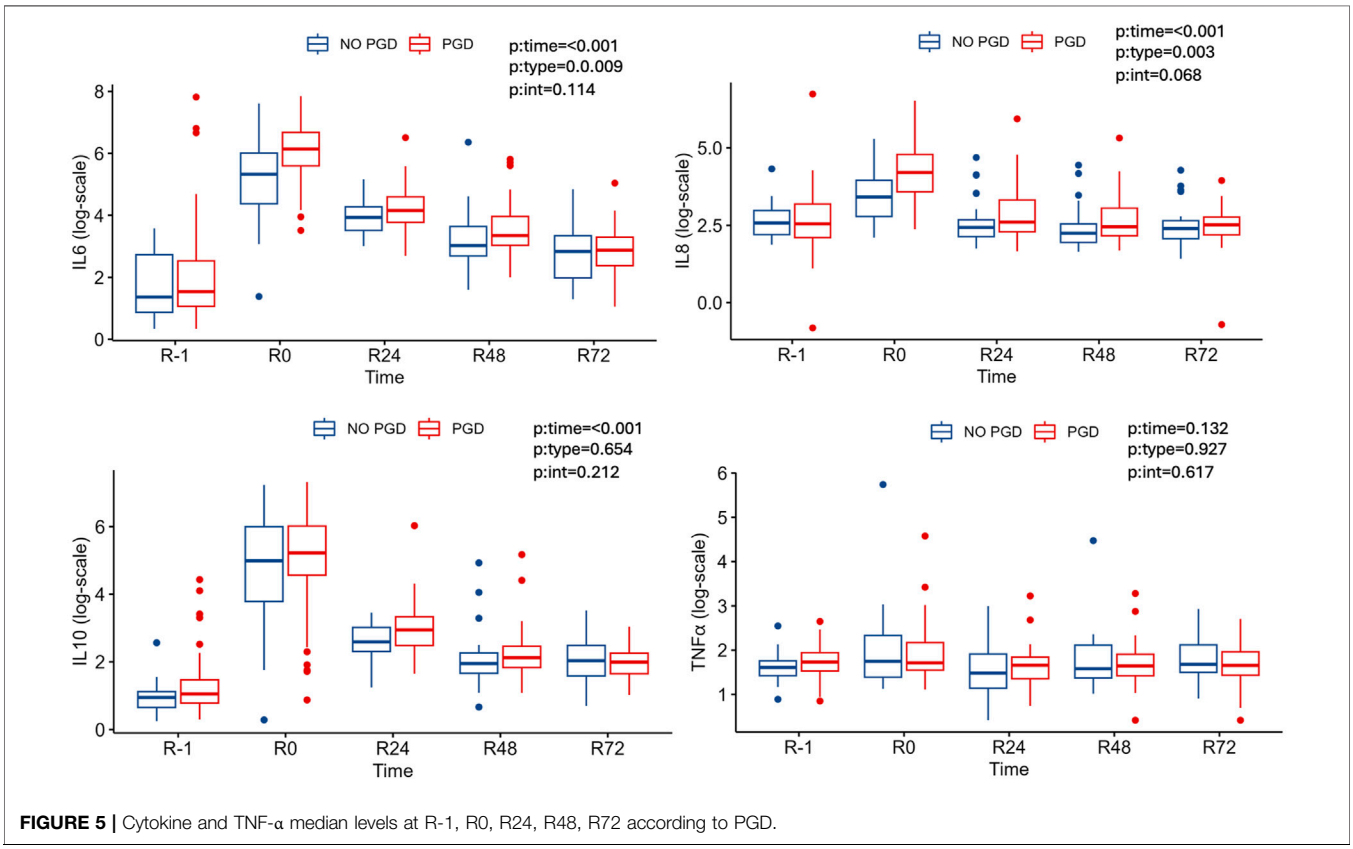


FIGURE 5 | Cytokine and TNF-α median levels at R-1, R0, R24, R48, R72 according to PGD.

TABLE 8 | Levels (pg/mL) of cytokines according to PGD0-2 vs. PGD3 at R-1 to R72. Data expressed as median (Q1, Q3). *p*-values corresponding to non-parametric two-way mixed ANOVA.

	R-1		R0		R24		R48		R72		*p*-values		
	No PGD/ PGD12	PGD3	No PGD/ PGD12	PGD3	No PGD/ PGD12	PGD3	No PGD/ PGD12	PGD3	No PGD/ PGD12	PGD3	p:time	p: PGD3	p: interaction
IL10	2.6 (2.0, 3.2)	2.7 (1.9, 4.3)	200.8 (44.9, 477.2)	160.6 (96.6, 290.4)	15.4 (10.0, 22.5)	22.0 (14.4, 33.2)	7.7 (5.7, 9.7)	9.2 (6.8, 18.8)	7.1 (4.9, 10.3)	7.7 (6.3, 10.4)	<0.001	0.576	0.321
IL 6	4.1 (2.7, 12.8)	5.2 (3.0, 13.3)	262.3 (101.1, 581.8)	441.0 (324.0, 1153.8)	53.3 (35.6, 78.0)	79.3 (44.7, 104.3)	25.3 (16.8, 55.2)	27.7 (23.6, 44.5)	17.3 (9.9, 27.8)	18.5 (9.6, 25.7)	<0.001	0.063	0.097
IL 8	12.2 (8.6, 19.2)	17.2 (8.3, 30.0)	42.3 (20.1, 88.9)	74.1 (48.1, 128.5)	11.8 (8.4, 16.1)	20.4 (11.8, 37.8)	10.1 (7.6, 14.4)	15.0 (10.6, 27.3)	11.2 (8.4, 13.8)	13.7 (8.5, 19.6)	<0.001	0.010	0.315
TNF α	5.4 (4.5, 6.7)	5.9 (5.1, 7.4)	5.5 (4.3, 9.2)	5.9 (4.7, 8.9)	4.6 (3.2, 6.5)	5.6 (4.3, 7.3)	5.0 (4.0, 7.1)	5.4 (4.2, 7.5)	5.2 (4.3, 7.6)	5.8 (4.1, 7.0)	0.270	0.374	0.834

systemic inflammation (as compared to DBD) and that the incidence of PGD and post-operative mortality seemed to be similar in LT recipients from DBD or DCD donors. This provides further support for the feasibility and safety of LT from DCD donors which, in turn, can stimulate DCD donation and contribute to alleviate the shortage of DBD donors and waiting lists.

The prospective, cohort-nested design of our study and the provision of the inflammatory status of donors before lung retrieval are strengths of our study. Among potential limitations we acknowledge that, although we estimated the needed sample size based on one of the most prominent cytokines investigated here (IL6), our results need to be replicated in other larger cohorts.

Brain death in humans is associated with higher levels of IL-6, IL-8 and IL-10, but this does not alter the biologic or clinical response of LT recipients. Yet, recipients transplanted from donors with higher TNF-α plasma levels (irrespective of DBD or DCD) have an increased incidence of PGD. These observations support the use of DCD in clinical practice.

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 studies involving humans were approved by the Human Research Committees of all participating hospitals (PR[AG]202/2017). 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, EC, and TP participated in the design, interpretation of the studies and analysis of the data; all authors conducted the experiments and review of the manuscript, IB and AS 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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Risk Factors, Incidence, and Outcomes Associated With Clinically Significant Airway Ischemia

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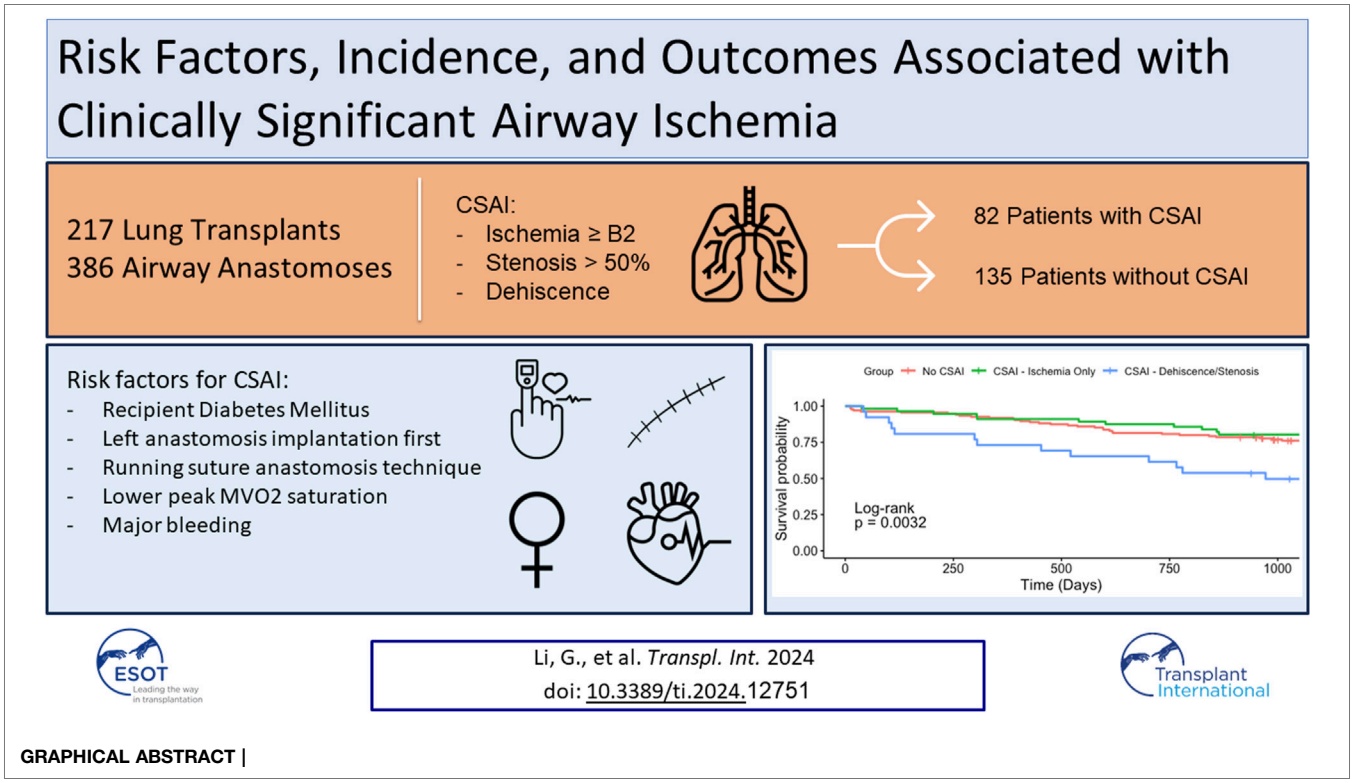
Airway complications following lung transplantation remain an important cause of morbidity and mortality. We aimed to identify the incidence, risk factors and outcomes associated with clinically significant airway ischemia (CSAI) in our center. We reviewed 217 lung transplants (386 airway anastomoses) performed at our institution between February 2016 and December 2020. Airway images were graded using the 2018 ISHLT grading guidelines modified slightly for retrospective analysis. Airways were considered to have CSAI if they developed ischemia severity >B2, stenosis >50%, and/or any degree of dehiscence within 6-months of transplant. Regression analyses were used to evaluate outcomes and risk factors for CSAI. Eighty-two patients (37.8%) met criteria for CSAI. Of these, twenty-six (32%) developed stenosis and/or dehiscence, and 17 (21%) required interventions. Patients with CSAI had lower one-year (80.5% vs. 91.9%, $p = 0.05$) and three-year (67.1% vs. 77.8%, $p = 0.08$) survival than patients without CSAI. Factors associated with CSAI included younger recipient age, recipient diabetes, single running suture technique, performance of the left anastomosis first, lower venous oxygen saturation within 48-h, and takeback for major bleeding. Our single-center analysis suggests that airway ischemia remains a major obstacle in contemporary lung transplantation. Improving the local healing milieu of the airway anastomosis could potentially mitigate this risk.

Keywords: lung transplant, airway stenosis, airway ischemia, airway anastomosis, airway dehiscence

Abbreviations: AIC, Akaike information criterion; CSAI, Clinically significant airway ischemia; ECD, Extended criteria donors; ECMO, Extracorporeal membrane oxygenation; EVLP, *Ex-vivo* lung perfusion; ICU, Intensive care unit; ISHLT, International Society of Heart and Lung Transplantation; LAS, Lung allocation score; LOS, Length of stay; MDS, Macroscopic aspect, diameter of anastomosis, sutures of anastomosis; MVO2, Mixed Venous Oxygen Saturation; OCS, Organ care system; PGD, Primary graft dysfunction; SRTR, Scientific Registry for Transplant Research; TEGLA, Thickness, extent of injury, granulation tissue, loose sutures, anastomotic complications; UNOS, United Network for Organ Sharing.



GRAPHICAL ABSTRACT |

INTRODUCTION

Lung transplant is an effective treatment for patients with irreversible lung disease, but impaired airway healing remains a constant threat affecting patient outcomes. Perfusion to the airway anastomosis relies on collateral vessels, fed from the pulmonary artery circulation in a retrograde fashion; therefore, ischemia is inevitable. While many ischemic lesions are not clinically significant, some are severe enough to warrant intervention. Severe airway ischemia leading to dehiscence and/or stenosis may require balloon dilation, stents, or operative interventions and have been associated with reduced long-term survival [1–4].

Accurate assessment of the risk of airway complications is important for clarifying the clinical sequelae and identifying preventive strategies. In the early days of lung transplant, airway complication rates were reported to exceed 50% and were associated with substantial morbidity [5, 6]. Risk factors included rejection, limitations in organ preservation, and tracheal anastomosis. Despite improvements, there has been considerable variability in the reported incidence of this complication in contemporary series. The past 15 years have seen reports of airway complications ranging from as low as 1.4% to as high as 38% [1, 3, 7–10]. This variability stems mostly from a lack of consensus around the classification of anastomotic lesions [11].

Several grading systems have been proposed to report airway complications including the TEGLA classification by Chajed et al. [12], the six category airway complications system by Santacruz and Mehta [13], and the MDS grading system by the French Language Pulmonary Society [14]. However, there are pitfalls to each method, and none has been universally adopted. To address this, a working group of the ISHLT convened in 2018 to create a consensus document to standardize airway assessments [15]. Reports demonstrating the utility and clinical integration of the updated guidelines are lacking. Such reports are needed to revisit and validate previously reported donor, technical, and postoperative risk factors while identifying potentially novel risk factors [1, 2, 7, 8, 16–20]. Studies integrating the updated guidelines could provide new benchmarks for the incidence of airway complications and their clinical sequelae [1–3].

We adapted the 2018 ISHLT guidelines to grade individual airway anastomoses in a single-center cohort of lung transplant recipients to establish the incidence of clinically significant airway ischemia and identify the clinical and physiologic risk factors associated with this complication.

PATIENTS AND METHODS

Patient Population

This was a retrospective, single-center study of all lung transplants performed between February 2016 and December 2020 at our institution. Patients were included if they had 6 months of bronchoscopic airway pictures available for

TABLE 1 | Grading systems.

ISHLT's proposed grading system		Our Study's adapted grading system	
Ischemia and Necrosis (I)		**Ischemia and Necrosis (I)**	
Location	A. Perianastomotic - Within 1 cm of anastomosis B. Extending >1 cm from anastomosis to major airways (bronchus intermedium and distal left main-stem) C. Extending >1 cm from anastomosis into lobar or segmental airways	*Location*	A Perianastomotic - Within 1 cm of anastomosis B. Extending >1 cm from anastomosis to major airways (bronchus intermedium and distal left main-stem) C. Extending >1 cm from anastomosis into lobar and segmental airways
Extent	a. < 50% circumferential ischemia b. > 50%–100% circumferential ischemia c. < 50% circumferential necrosis d. > 50%–100% circumferential necrosis	*Extent*	1. < 50% circumferential ischemia or necrosis 2. > 50%–100% circumferential ischemia or necrosis
		Dehiscence (D)	Presence of any
		Stenosis (S)	
Dehiscence (D)		*Extent*	<50% stenosis
Location	a. Cartilaginous c. Membranous c. Both		>50% stenosis
Extent	a. 0%–25% of circumference b. > 25%–50% of circumference c. > 50%–75% of circumference d. > 75% of circumference		
Stenosis (S)			
Location	a. Anastomotic b. Anastomotic plus lobar/segmental c. Lobar/segmental only		
Extent	a. 0%–25% reduction in cross-sectional area b. > 25%–50% reduction in cross-sectional area c. > 50% but <100% reduction in the cross-sectional area d. 100% obstruction		
Malacia (M)			
Location	a. Perianastomotic - within 1 cm of anastomosis b. Diffuse - involving anastomosis and extending beyond 1 cm		

grading and three-year clinical follow-up. Patients who died within this timeframe were included if postoperative airway images were available for review. Single, double, dual-organ and re-do lung transplants were included. This study was approved by the Baylor College of Medicine Institutional Review Board with waiver of consent.

Surgical Technique

There were four surgeons who performed lung transplants during the study interval. Each surgeon utilized their preferred surgical technique for the airway anastomosis, including either an interrupted or running suture technique. Either of the following techniques were characterized as "interrupted suture technique": 1) interrupted figure of 8 poly-p-diaxanone (PDS) for the cartilaginous portion and running PDS for the membranous portion or 2) interrupted 4-O prolene for the cartilaginous portion and running 4-O prolene for the membranous portion (our current center preference). The running suture technique was defined as the use of a single running circumferential suture line in a continuous fashion using 4-O prolene. All anastomoses were routinely reinforced with an onlay patch of donor pericardium. Patient medication and donor allograft preservation protocols are detailed in **Supplementary Methods S1**.

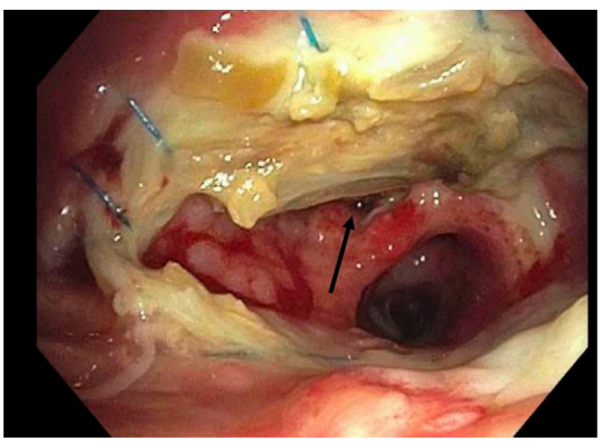


FIGURE 1 | Example of grading airway ischemia. Right Anastomosis. Ischemia: C2. Stenosis: No. Dehiscence: Yes (arrow).

Airway Grading

At our program, it is standard for the transplant pulmonologist to digitally archive two-dimensional color images of the anastomosis and distal airways. We reviewed both the images

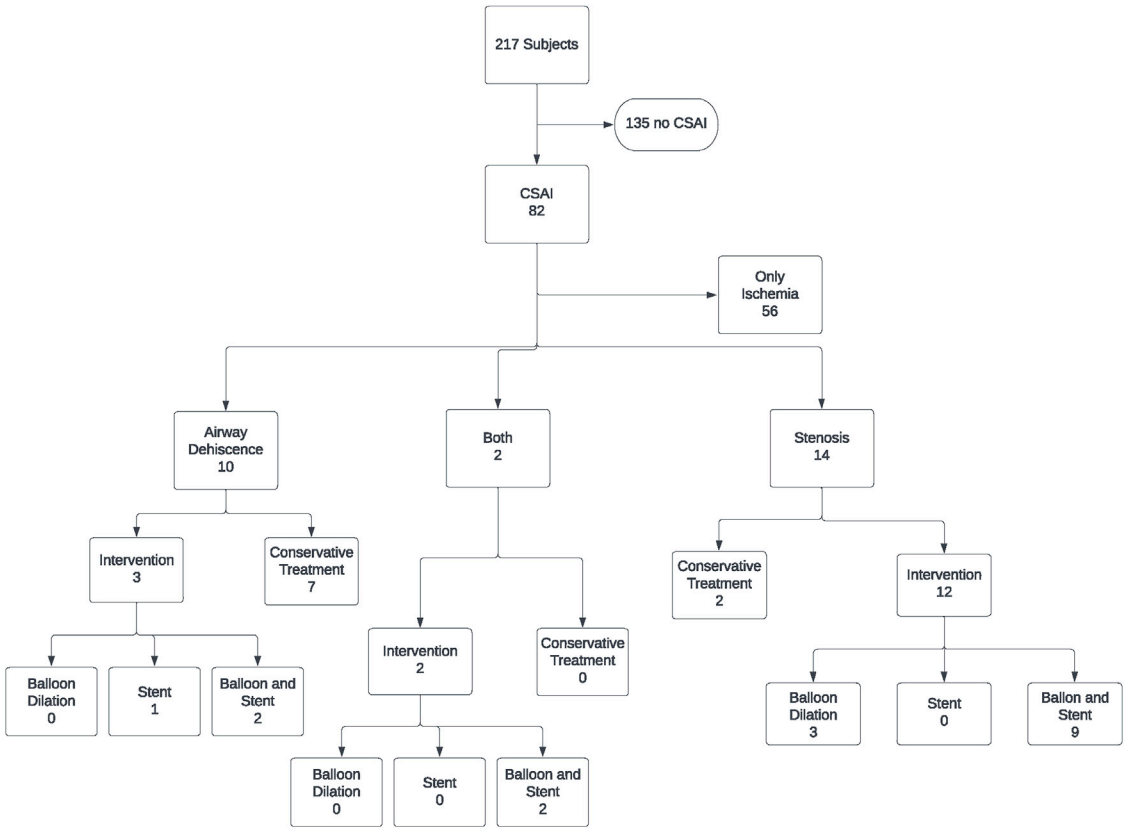


FIGURE 2 | Breakdown of the patients who developed CSAI and associated interventions.

and the bronchoscopy reports for all patients who underwent lung transplant within the study interval. One of three transplant pulmonologists reviewed the images and reports obtained at time points closest to 15, 30, 60, 90, and 180 days after transplant. Airways were graded only if the pictures were available for review.

In 2018 the ISHLT convened a workgroup which proposed a detailed grading system for airway complications after transplant [15]. We modified this grading system to allow retrospective grading of archived images (**Table 1**). For example, ischemia and necrosis were combined into one category ("ischemia") because they could not be easily distinguished on two-dimensional bronchoscopic digital images. We also simplified the reporting of dehiscence and stenosis for easier statistical analysis. Malacia was not evaluated as this diagnosis can only be confirmed by assessing the airway in motion. **Figure 1** provides an example of how an anastomosis was graded in this study.

Clinically Significant Airway Ischemia

To facilitate reporting and analysis of bronchoscopic images, we simplified the reporting scheme to focus on a clinically significant and sensitive composite endpoint. We termed our composite endpoint "clinically significant airway ischemia" (CSAI), which was defined as the presence of airway ischemia severity >B2 (extending beyond 1 cm of the airway anastomosis and involving >50% of the anastomotic circumference), >50% stenosis, and/or presence of any dehiscence occurring at any timepoint within 6 months of the transplant. These findings were deemed clinically significant because they warranted either bronchoscopic interventions, changes in patient management or at least frequent bronchoscopies beyond routine surveillance procedures. Only balloon dilation and/or stent placement were considered interventions in this study.

Study Outcomes

The primary outcome of this study was overall survival. The secondary outcomes included primary graft dysfunction (PGD), post-operative use of extracorporeal membrane oxygenation (ECMO), ventilation time, atrial fibrillation, major bleeding requiring take back to the operating room, acute cellular rejection, hospital and intensive care unit (ICU) length of stay (LOS), hospital readmission within 1 year, tracheostomy, acute kidney injury requiring dialysis, or pneumonia. PGD was defined as the presence of PGD grade 3 at 48 and/or 72 h post-reperfusion. Pneumonia was defined by positive bronchial cultures requiring antibiotic treatment. Finally, we sought to determine clinical and physiologic risk factors associated with CSAI using individual airways.

TABLE 2 | Patient outcomes.

Postoperative and survival outcomes with and without clinically significant airway ischemia			
	CSAI (N = 82)	**Non-CSAI (N = 135)**	***p*-values**
Postoperative Outcomes			
PGD grade 3 at 48–72 h	24 (29.3%)	40 (29.6%)	0.95
Post-Op ECMO	11 (13.4%)	15 (11.1%)	0.61
Ventilator Support >5 days	24 (29.3%)	37 (27.4%)	0.77
Atrial Fibrillation	36 (43.9%)	60 (44.4%)	0.94
Major Bleeding	11 (13.4%)	10 (7.4%)	0.15
Acute Cellular Rejection	9 (11%)	8 (5.9%)	0.19
Hospital Length of Stay	32.68 (30.13)	25.90 (29.64)	0.12
ICU Length of Stay	20.77 (26.38)	15.43 (21.48)	0.11
Hospital Readmission within 1 year	66 (52.4%)	103 (39.6%)	0.09
Tracheostomy	21 (25.6%)	30 (22.2%)	0.57
Dialysis	12 (14.6%)	13 (9.6%)	0.27
Pneumonia	20 (24.4%)	23 (17.0%)	0.19
Survival			
90-day	78 (95.1%)	131 (97%)	0.47
1 year	66 (80.5%)	124 (91.9%)	0.05
3 years	55 (67.1%)	105 (77.8%)	0.08

Continuous variables expressed as Mean (SD); Categorical variables expressed as frequency (%).
CSAI: clinically significant airway ischemia, PGD*: primary graft dysfunction.*

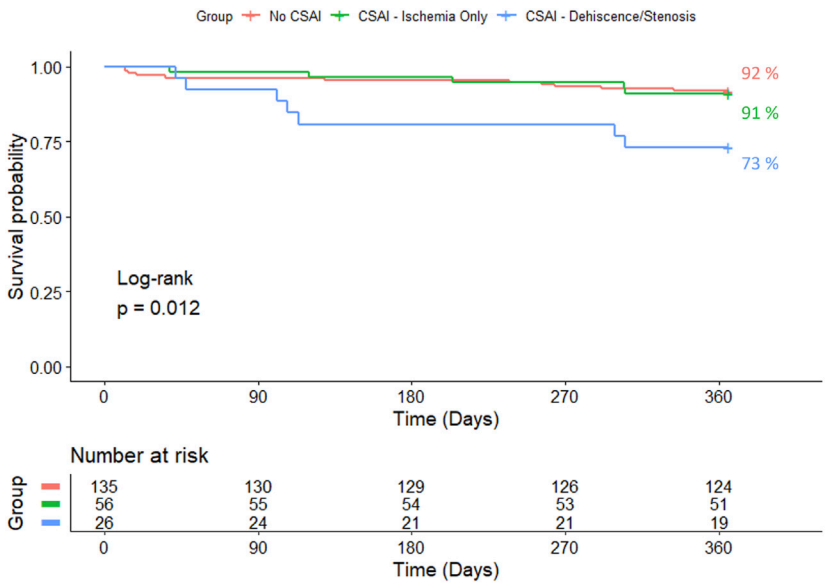


FIGURE 3 | Kaplan-Meier 1-year survival estimates for patients without CSAI, with CSAI - Ischemia Only, and with CSAI—Dehiscence/Stenosis.

Statistical Analysis

Continuous data are expressed as mean +/- standard deviation. Nominal variables are expressed as percentages. Statistical significance of continuous data was calculated using the unpaired two-tailed *t*-test for normally distributed variables and the Mann-Whitney *U*-test for variables showing a skewed distribution. Contingency analysis of nominal data was performed using a two-sided Fisher's exact test. Both unadjusted and adjusted analysis were performed. For unadjusted analysis, a univariate logistic regression of CSAI of each factor was conducted, and the *p*-value of Student's t-test, odds ratio, and 95% confidence interval were reported. A *p*-value of <0.05 was considered statistically significant. Contingency tables were made between CSAI and each categorical factor and computed for marginal percentage. For the adjusted analysis, a multivariate logistic regression model was employed, starting with a list of clinically significant variables identified *a priori* based on the published literature (**Supplementary Table S1**). This was followed by a forward stepwise variable selection process, guided by the Akaike information criterion (AIC), to selectively add factors from the unadjusted analysis that had a *p*-value <0.1. This approach was

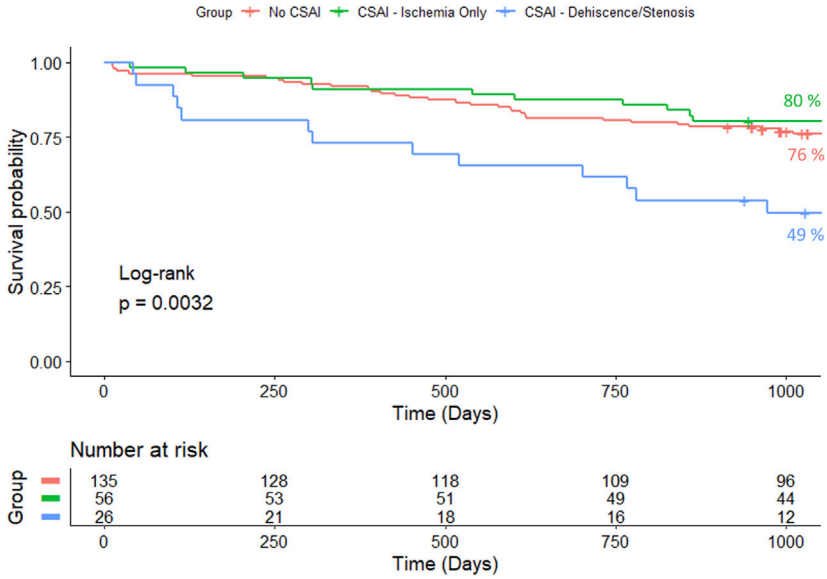


FIGURE 4 | Kaplan-Meier 3-year survival estimates for patients without CSAI, with CSAI - Ischemia Only, and with CSAI—Dehiscence/Stenosis.

used to assess the strength of the association between CSAI and all potential risk factors and to determine the significant factors in the full model.

Actuarial survival rates were estimated with the Kaplan-Meier method and compared with the log-rank test. An adjusted analysis including recipient age, pulmonary artery pressures, PGD, and LAS was used to establish the association between CSAI and survival.

RESULTS

Incidence and Outcomes Associated With Clinically Significant Airway Ischemia

217 patients underwent lung transplantation between February 2016 and December 2020. Of these, 169 patients underwent double lung transplant, and 48 patients underwent single lung transplant. Bronchoscopic images were available for all 386 airway anastomoses. Eighty-two patients out of the 217 in the study cohort (37.8%) met the definition of CSAI in at least one of their airway anastomoses. Of these patients, 56 (68.3%) had ischemic lesions only and 26 (31.7%) had dehiscence and/or stenosis, with 17 (21%) requiring intervention (balloon dilation and/or stent placement) (**Figure 2**).

Supplementary Table S2 outlines the patient, donor, and operative characteristics between patients that did and did not develop CSAI. No statistically significant differences were identified between groups. **Table 2** summarizes the postoperative outcomes associated with CSAI. One-year survival was lower in the CSAI group compared to the non-CSAI group (80.5% vs. 91.9%, $p = 0.05$). This reduction in one-year survival persisted after adjusting for recipient age, pulmonary artery pressure, PGD, and LAS. Three-year survival was non-significantly lower in the CSAI-group compared to the non-CSAI group (67.1% vs. 77.8%, $p = 0.08$). There was no difference in 90-day survival between the two groups. Additionally, we did not identify a difference in secondary outcome (**Table 2**).

Figure 3 and **Figure 4** show one and three-year survivals, respectively, for patients with no CSAI, CSAI patients with ischemia only, and CSAI patients with dehiscence and/or stenosis. The one-year survival rates were 92% for no CSAI, 91% for CSAI with ischemia only, and 73% for CSAI with dehiscence and/or stenosis ($p = 0.012$) (**Figure 3**). Three-year survival rates were 76% for no CSAI, 80% for CSAI with ischemia only, and 49% for CSAI with dehiscence and/or stenosis ($p = 0.0032$) (**Figure 4**). Thus, reduction in survival associated with CSAI appeared to be driven by the effect of dehiscence and/or stenosis. This was confirmed in a refined analysis using a Cox regression model, which showed there is a significant reduction in one-year survival in the CSAI with dehiscence and/or stenosis group compared with the non-CSAI group ($p = 0.005$) but no difference in one-year survival between the non-CSAI and CSAI with ischemia only groups ($p = 0.46$).

Overall, the average time between lung transplant and detection of CSAI was 23.5 days (SD + 14.2). In the subgroup of patients who only had CSAI with ischemia, the average time between lung transplant and detection of CSAI was 20.1 days (SD + 10). In the subgroup of 26 patients who had CSAI with dehiscence and/or stenosis, the average time between lung transplant to detection of airway ischemia was 32.3 days (SD + 19.1) and the average time from airway ischemia to detection of dehiscence and/or stenosis was 32.6 days (SD + 30.9). Every case of dehiscence and/or stenosis was preceded by bronchoscopic evidence of ischemia.

TABLE 3 | Unadjusted analysis.

Unadjusted analysis for risk factors associated with clinically significant airway ischemia					
	CSAI (N = 126)	**Non-CSAI (N = 260)**	***p*-value**	**OR**	**CI**
Patient Demographics					
Age (years)	51.30 (15.86)	53.29 (15.31)	0.24	0.99	0.98, 1.01
Gender					
Female	52 (41.3%)	105 (40.4%)			
Male	74 (58.7%)	155 (59.6%)	0.87	0.96	0.63, 1.49
Body-Mass Index	25.36 (5.71)	25.39 (5.41)	0.96	1	0.96, 1.04
Type of Transplant					
Single	15 (11.9%)	33 (12.7%)			
Double	111 (88.1%)	227 (87.3%)	0.83	1.08	0.57, 2.11
First Anastomosis					
Left	61 (48.4%)	103 (39.6%)			
Right	65 (51.6%)	157 (60.4%)	0.1	0.7	0.45, 1.07
Primary Diagnosis					
ILD/Restrictive Lung Disease	68 (54%)	152 (58.5%)			
COPD	23 (18.3%)	42 (16.2%)	0.5	0.82	0.46, 1.48
Cystic Fibrosis	25 (19.8%)	47 (18.1%)	0.38	1.83	0.46, 7.21
PAH/PVD	5 (4%)	5 (1.9%)	0.94	0.97	0.48, 1.97
Other	5 (4%)	14 (5.4%)	0.46	0.65	0.19, 1.95
Multiorgan Transplant	4 (3.2%)	7 (2.7%)	0.79	1.19	0.31, 4.00
LAS score	43.10 (11.53)	45.79 (15.65)	0.09	0.99	0.97, 1.00
ABO Type					
A	46 (36.5%)	97 (37.3%)			
B	12 (9.5%)	26 (10%)	0.95	0.97	0.44, 2.07
O	61 (48.4%)	122 (46.9%)	0.82	1.05	0.66, 1.69
AB	7 (5.6%)	15 (5.8%)	0.97	0.98	0.36, 2.51
Condition at Transplant					
Hospitalized	4 (3.2%)	8 (3.1%)			
ICU	8 (6.3%)	31 (11.9%)	0.37	0.52	0.13, 2.32
Not hospitalized	114 (90.5%)	221 (85%)	0.96	1.03	0.32, 3.93
Life support prior to transplant	5 (4%)	28 (10.8%)	0.03	0.34	0.11, 0.84
Preoperative Ventilator Use	0 (0%)	4 (1.5%)	0.98	0	NA, 5.68E+29
Preoperative ECMO	2 (1.6%)	10 (3.8%)	0.22	0.38	0.06, 1.48
Preoperative Noninvasive Ventilation	3 (2.4%)	14 (5.4%)	0.17	0.41	0.09, 1.29
Mean PAP (mmHg)	26 (9.44)	26.80 (9.87)	0.71	1	0.97, 1.02
Creatinine (mg/dL)	0.86 (0.20)	0.86 (0.45)	0.93	0.97	0.50, 1.67
Prior cardiac surgery	0 (0%)	9 (3.5%)	0.98	0	NA, 1.23E+21
Prior lung surgery	23 (18.3%)	39 (15%)	0.42	1.27	0.71, 2.21
Type 2 diabetes mellitus	39 (31%)	55 (21.2%)	0.04	1.67	1.03, 2.70
History of Smoking	64 (50.8%)	134 (51.5%)	0.89	0.97	0.63, 1.49
Re-Transplant	1 (0.8%)	9 (3.5%)	0.16	0.22	0.01, 1.21
Chronic steroid use	46 (36.5%)	111 (42.7%)	0.25	0.77	0.50, 1.19
Donor Characteristics					
Donor Type					
DBD	120 (95.2%)	241 (92.7%)			
DCD	6 (4.8%)	19 (7.3%)	0.34	0.63	0.23, 1.54
Age (years)	36.16 (12.69)	35.07 (12.75)	0.43	1.01	0.99, 1.02
Gender					
Female	54 (42.9%)	86 (33.1%)			
Male	72 (57.1%)	174 (66.9%)	0.06	0.66	0.43, 1.02
Diabetes	16 (12.7%)	24 (9.2%)	0.3	1.42	0.72, 2.77
>20 py smoking history	78 (61.9%)	139 (53.5%)	0.13	1.4	0.91, 2.18
Extended Criteria Donor[a]	55 (43.7%)	102 (39.2%)	0.41	1.2	0.78, 1.85
Donor Cultures					
Candida species	41 (32.5%)	66 (25.4%)	0.10	1.49	0.93, 2.38
Any positive donor cultures	101 (80.2%)	202 (77.7%)	0.24	1.40	0.81, 2.51
Perioperative Characteristics					
ECLS					
Off-Pump	20 (15.9%)	63 (24.2%)			
ECMO	35 (27.8%)	57 (21.9%)	0.05	1.93	1.01, 3.77
CPB	71 (56.3%)	140 (53.8%)	0.11	1.6	0.91, 2.90
EVLP	28 (22.2%)	42 (16.2%)	0.15	1.48	0.86, 2.52

(Continued on following page)

TABLE 3 | (*Continued*) Unadjusted analysis.

Unadjusted analysis for risk factors associated with clinically significant airway ischemia					
	CSAI (N = 126)	**Non-CSAI (N = 260)**	***p*-value**	**OR**	**CI**
Total Ischemic Time (min)	330.00 (153.93)	320.95 (132.66)	0.55	1	1.00, 1.00
Warm Ischemic Time (min)	42.72 (14.84)	45.20 (13.00)	0.19	0.99	0.96, 1.01
Suture Technique					
Running	100 (79.4%)	171 (65.8%)			
Interrupted	26 (20.6%)	89 (34.2%)	0.01	0.5	0.30, 0.82
PGD 3 at 48–72 h	28 (22.2%)	57 (21.9%)	0.95	1.02	0.60, 1.69
Post-Op ECMO	18 (14.3%)	33 (12.7%)	0.67	1.15	0.61, 2.11
Ventilator Support >5 days	40 (31.7%)	76 (29.2%)	0.61	1.13	0.71, 1.78
Peak mixed venous O2 within 48 h	72.46 (11.55)	76.00 (10.02)	0.01	0.97	0.95, 0.99
Peak Creatinine within 48 h	1.02 (0.34)	1.04 (0.50)	0.61	0.88	0.53, 1.41
Peak Lactate within 72 h	7.10 (3.62)	6.96 (3.72)	0.73	1.01	0.95, 1.07
Atrial Fibrillation	56 (44.4%)	110 (42.3%)	0.69	1.09	0.71, 1.67
Major Bleeding[b]	21 (16.7%)	19 (7.3%)	0.01	2.54	1.31, 4.95
Acute Cellular Rejection	14 (11.1%)	16 (6.2%)	0.09	1.9	0.88, 4.03

Continuous variables expressed as Mean (SD); Categorical variables expressed as frequency (%).

[a]*Extended Criteria Donor: Age >55, DCD, PF < 300, Anticipated ischemia >6 h, abnormal chest X-ray, >20 py smoking history.*

[b]*Major bleeding within the early postoperative period requiring surgical intervention.*

CSAI: clinically significant airway ischemia, ILD: interstitial lung disease, PAH: pulmonary arterial hypertension, PVD: pulmonary vascular disease, LAS: lung allocation score, ICU: intensive care unit, ECMO: Extra-Corporeal Membrane Oxygenation, PAP: pulmonary arterial pressure, DBD: donor after brain death, DCD: donor after circulatory death, CPB: Cardio-Pulmonary Bypass, EVLP: Ex-Vivo *Lung Perfusion.*

PGD: primary graft dysfunction.

TABLE 4 | Adjusted analysis.

Adjusted analysis for risk factors associated with clinically significant airway ischemia			
	***p*-value**	**OR**	**CI**
Recipient age (years)	0.09	0.98	0.95, 1.00
Recipient gender: Male (vs. Female)	0.16	1.51	0.86, 2.72
Type of Transplant: Single (vs. Double)	0.11	0.51	0.22, 1.17
First Anastomosis: Right (vs. Left)	**0.01**	0.54	0.32, 0.88
Primary Diagnosis			
ILD/Restrictive Lung Disease			
COPD	0.19	1.57	0.79, 3.08
Cystic Fibrosis	0.32	0.57	0.19, 1.72
PAH/PVD	0.68	1.37	0.29, 6.27
Other	0.27	0.37	0.05, 1.76
Condition at Transplant			
Not hospitalized			
ICU	0.99	1.01	0.29, 3.29
Hospitalized	0.39	0.46	0.06, 2.28
Life support prior to transplant	0.06	0.24	0.05, 1.00
Type 2 diabetes mellitus	0.06	1.79	0.97, 3.29
Candida albicans	0.12	1.61	0.88, 2.92
Donor gender: Male (vs. Female)	0.08	1.69	0.94, 3.08
Total Ischemic Time (min)	0.71	1.00	1.00, 1.00
Suture Technique: Interrupted (vs. Running)	**0.01**	0.47	0.25, 0.84
PGD 3 at 48–72 h	0.76	0.90	0.44, 1.78
Ventilator Support >5 days	0.77	1.10	0.58, 2.05
Major Bleeding[a]	0.10	1.89	0.88, 4.08
Acute Cellular Rejection	0.20	1.80	0.73, 4.43
Pneumonia	0.18	1.49	0.83, 2.64

[a]*Major bleeding within the early postoperative period, requiring surgical intervention. Bold values represent clinically significant* p*-values.*

Risk Factors for Clinically Significant Airway Ischemia

Of the 386 airway anastomoses reviewed, 222 (57.5%) were right-sided and 164 (42.5%) were left-sided. A total of 126 out of 386 (32.6%) anastomoses developed CSAI; 65 (51.6%) were right-sided and 61 (48.4%) were left-sided anastomoses.

Univariate analysis was performed to determine clinical and physiologic risk factors associated with airways that developed CSAI. Significant clinical risk factors included the following: lack of life support prior to lung transplant, recipient diabetes, intraoperative ECMO, use of a single running suture technique versus an interrupted suture technique, and major bleeding associated with takeback to the operating room (**Table 3**). The only significant physiologic risk factor associated with airways that developed CSAI in the unadjusted analysis was a reduced peak mixed venous oxygen saturation (MVO2) within 48 h (mean MVO2 of 72% in CSAI vs. 76% in no CSAI, p = .01) (**Supplementary Table S3**). Notably, lactate, vasopressor requirements, albumin, and hemoglobin values were not significantly associated with CSAI. Despite a trend towards greater donor culture positivity in the CSAI group, it did not reach significance.

The adjusted analysis identified the following risk factors for CSAI: younger recipient age, diabetes in the recipient, performance of the left anastomosis first, single running suture technique versus an interrupted suture technique, and major bleeding associated with takeback to the operating room (**Table 4**). Of note, not all patients had a MVO2 drawn after transplant. Thus, a similar adjusted analysis using stepwise variable selection with AIC was performed with a smaller cohort of airways (n = 306) from patients that had complete MVO2 data. This analysis showed that a higher peak MVO2 within 48 h after transplant was associated with a reduced risk of CSAI (**Supplementary Table S4**).

DISCUSSION

Our study incorporated the 2018 ISHLT consensus-based guidelines to retrospectively grade airway anastomoses in our center and to identify the incidence, risk factors, and outcomes associated with clinically significant airway complications. We found the grading system to be practical, reproducible, and efficient with only minor modifications needed for retrospective analysis of bronchoscopic images. We focused on a clinically significant composite outcome of the grading system which was the presence of any of the following: >B2 severity ischemia, >50% stenosis, and/or any evidence of dehiscence occurring at any time within 6 months of transplant.

The incidence of CSAI in our cohort was 37.8%. This was at the higher end of the 1.4%–38% range reported in recent series on post-transplant airway complications [1–3, 8, 11, 16]. Our higher incidence of airway complications was likely due to the sensitivity of our composite outcome which included ischemic lesions (>B2) with or without bronchoscopic interventions, many of which may not have qualified as an airway complication in other studies. However, we believe that these precursor lesions are important as evidenced by the high rate of dehiscence and stenosis (32%) seen in patients that developed > B2 ischemia. The incidence of dehiscence and/or stenosis in our study cohort was 11.9%, and the incidence of airway complications requiring interventions was 7.8%. These rates are similar to those reported in the literature [11, 13, 17, 18]. Also, like prior reports, our study showed that patients that developed CSAI with dehiscence and/or stenosis were at significantly greater risk of having reduced survival than patients that developed CSAI with ischemia only [1, 2].

We identified several risk factors associated with CSAI on multivariate analysis. Airway anastomosis with interrupted sutures along the anterior cartilaginous portion of the airway and a running posterior membranous suture line was superior to a single running Prolene suture. Of note, we routinely trim back the airway as close as possible to the secondary carina as suggested by several authors [17, 18, 20–22]. This modification to reduce the length of the bronchus has been key for reducing airway ischemia over the past two decades. The finding of an association between interrupted suture technique and reduction in airway ischemic complications has been observed by others [18, 20].

However, this finding is not ubiquitous. Schweiger et al. reported a low rate of severe airway complications requiring interventions in their series of lung transplants using exclusively a single running technique [23]. In contrast, their study did not have a comparison group, did not focus on early ischemic lesions, and did not have all bronchoscopic images available for review. Olland et al. also showed that a single running suture technique was not associated with increased airway complications if the donor airway was trimmed back substantially to include a wedge of the bronchus intermedius [24]. This modification was first described by Weder et al. who showed that extensive donor bronchial trimming on the left and the right was associated with a near absence of airway stenosis [25]. Unlike the study by Olland, Weder utilized an interrupted suture technique in their series. We hypothesize that interrupted sutures provide two advantages: 1) greater opportunity for microvascular connections and oxygen delivery, and 2) better alignment of the airway anastomosis.

Our multivariate analysis suggested that diabetes in a recipient was associated with greater odds of CSAI. This is consistent with the study by Olland et al., which found that recipient diabetes was independently associated with airway complications after transplant [24]. Diabetes affects the microvascular beds increasing the risk of tissue ischemia. Whether postoperative control of hyperglycemia is associated with reduced CSAI is intriguing and requires further investigation.

Major bleeding requiring takeback to the operating room was also a risk factor that, to our knowledge, has not been previously reported. We hypothesize that acute bleeding results in hypotension and prioritization of blood distribution to vascular beds in critical need leaving the airway anastomosis more vulnerable to ischemia. The association between major bleeding and CSAI underscores the potential vulnerability of the anastomosis to systemic changes that affect oxygen delivery

and the healing milieu. However, it is important to note that neither nadir hemoglobin levels, nor vasopressor requirements were associated with CSAI. Perhaps the airway anastomosis is only vulnerable to major changes in these values associated with a takeback for bleed. Transient fluctuations in hemodynamics and blood requirements during the takeback for bleeding were not captured in this study.

Our multivariate analysis suggests that a right lung-first approach is associated with less risk of CSAI than a left lung-first approach. This finding requires further analysis, and we would not advocate for one approach over the other based on this finding alone. It is possible, however, that the airway anastomosis is subject to different perfusion patterns depending on which lung is implanted first. For example, when the right lung is implanted first, there is more space for it to ventilate and perfuse while working on the left lung. Conversely, when the left lung is implanted first, there may be less space to ventilate and perfuse because of external compression from the heart.

Importantly, we found that the peak MVO2 level was inversely associated with CSAI. Therefore, patients with a greater oxygen content in the pulmonary artery circulation had a lower rate of severe ischemia. This is also intuitive because, in the absence of bronchial artery reconstruction, the pulmonary artery is the sole blood supply to the transplanted lung. Maneuvers to increase the amount of oxygen in the venous return may be advantageous for reducing the risk of airway ischemia although this requires further study.

There were a few findings that were counterintuitive. The association between older age and reduced risk of airway ischemia was difficult to explain. The ages between patients in the CSAI and non-CSAI groups were similar. It was only after incorporating age as a previously reported risk factor, that we obtained a significant odds ratio suggesting an inverse relationship between age and airway risk. This finding requires further study. One possible explanation is that older patients free of comorbid conditions such as diabetes are more likely to receive lung transplant than those with multiple comorbid conditions. At our program, older recipients are more likely to receive single lung transplants to reduce surgical stress. In addition, postoperative albumin levels were not associated with CSAI. This is counterintuitive because one would assume that a lower albumin level would suggest worse nourishment and diminished wound healing. Perhaps this is explained by the low number of recipients in our cohort that were malnourished during the preoperative period. Our program makes every effort to optimize patient nutrition and weight prior to transplant.

In our series, severe airway ischemia was first detected approximately 4 weeks after transplant. Ischemic airways that went on to develop dehiscence and/or stenosis did so, on average, 4 weeks after the detection of ischemia. All airways that developed dehiscence/stenosis had evidence of severe ischemia first (i.e., > B2 by 2018 ISHLT guidelines - defined as ischemia >1 cm from anastomosis and >50% of the circumference). Thus, ischemia of this severity is an important precursor for greater complications. Frequent monitoring for progression or resolution of ischemia may improve outcomes through prompt recognition and treatment of advanced lesions [2, 3, 13, 26].

Prior studies have utilized novel grading systems for airway complications after transplantation. Yserbyt et al. utilized the MDS classification system in 2016 and performed a similar analysis looking at severe and less severe airway grades [21]. Contrary to our results, they showed that advanced recipient age was associated with airway complications and that right-sided anastomoses were at greater risk of complications than left sided anastomosis. We found that older age recipients were at lower risk of airway complications, and we did not find a difference in laterality although we noticed a trend towards greater complications in the right-sided anastomoses. The difference in results could certainly be due to differences in patient cohorts as well as the differences in airway grading schemes. Yserbyt et al also determined that recipient microbiological colonization and postoperative infections were associated with airway complications. Olland et al. also identified postoperative infections as being important for the development of airway complications [24]. In our study patients with CSAI did not have higher rates of post-operative pneumonia compared to those without CSAI. Additionally, while we noted a trend towards greater donor culture positivity and incidence of *candida* species in patients with CSAI compared to no CSAI, these differences were not statistically significant in our cohort. It is known that fungal infections are a significant risk factor for airway complications [27]; however, our use of fungal prophylaxis with voriconazole or itraconazole has likely reduced this risk. It is conceivable that these trends could have been significant if we had analyzed a greater number of patients.

Moreover, previous literature has shown associations between various additional risk factors and airway complications. A retrospective study of the United Network for Organ Sharing (UNOS) database evaluated risk factors associated with airway complications [1]. They showed an incidence of 1.4% and found the following risk factors: ICU hospitalization before transplant, advanced recipient age, male recipient, bilateral lung transplantation, and diagnosis other than emphysema, cystic fibrosis, or idiopathic pulmonary fibrosis. We did not identify these risk factors, although this could be due to differences in the airway grading, transplant eras, patient population, and inclusion of covariates.

Other series have reported unique risk factors such as: donor and recipient ventilation times, early rejection, donor recipient size mismatch, cold ischemic interval, and PGD [1, 2, 8, 9, 16–19, 24, 28]. We did not study donor ventilation times in the current analysis. However, we were surprised that total organ ischemic time, PGD, and recipient ventilation times were not associated with airway complications in our series. It is conceivable that with a larger sample size, these factors may emerge as significantly associated with CSAI and at present we would not dismiss them as being potentially important factors affecting airway healing.

Our study has several limitations. Its retrospective nature relies on accurate chart review and assessment of airways. Grading airways remains somewhat subjective and biased, and grading retrospectively from 2-D bronchoscopic images is less reliable than grading them in real time. To mitigate this, prior to the study, pulmonologists graded a sample of airways to ensure consistency. This study modified the ISHLT 2018 grading system by combining ischemia and necrosis into one category as it is

difficult to distinguish between the two from retrospective review of images. Our team recognizes that combining ischemia and necrosis could have led to an overestimation of the incidence of airway complications in our study group. On the other hand, our study suggests that without dehiscence/stenosis, isolated ischemic lesions had little impact on survival. Our study did not look at malacia because this diagnosis requires bronchoscopic visualization on forced exhalation, and it is a complication that may not be seen within 180 days [4]. As mentioned previously, we included presence of any dehiscence or stenosis rather than specifying the exact location of these lesions as suggested by the ISHLT 2018 airway guidelines. We agree that real time imaging and reporting of exact locations is ideal, however this was not possible in our current analysis. We also recognize that modifying the ISHLT grading system undermines its purpose of standardization and that the scoring guidelines were not intended to prognosticate patient outcomes. Despite the study's limitations, it provides one of the largest series with 386 graded airways across multiple time-points. This is an important contribution to our existing knowledge of airway complications after lung transplant.

In conclusion, CSAI was a common complication after lung transplantation in our large single center experience. This complication was associated with reduced patient survival. However, this reduction in patient survival was driven by dehiscence/stenosis rather than by severe ischemia alone. While ischemia alone was not associated with reduced survival, it was an important precursor to severe complications. The proposed 2018 ISHLT guidelines for grading airway complications are functional in clinical practice and useful for standardizing the reporting of important post-transplant airway complications. Our findings establish the utility of the updated guidelines while highlighting potential methods to mitigate the risk of airway ischemia: achievement of euglycemia in diabetic recipients, establishment of hemostasis and avoidance of take back for bleeds, optimization of MVO2 levels, and use of interrupted suture technique for the airway anastomosis. Prospective research should evaluate these findings using real time bronchoscopic images across multiple centers.

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 studies involving humans were approved by the Baylor College of Medicine Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because of the observational and retrospective nature of the study.

AUTHOR CONTRIBUTIONS

Study Concept and Design: GlL, GaL, ZL, BM, and ML. Acquisition of Data: ED'S, EK, JC, AM, AE, BM, GlL, BR, MS-G, BR, ML, and GaL. Analysis and Interpretation of Data: GlL, GaL, ZL, BM, MS-G, ML, and AK. Drafting of Manuscript: GlL, GaL, EK, ED'S, AK, MS, and ML. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

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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.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.12751/full#supplementary-material

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Impact of Transient and Persistent Donor-Specific Antibodies in Lung Transplantation

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Lung transplantation (LuTx) is an established treatment for patients with end-stage lung diseases, however, outcomes are limited by acute and chronic rejection. One aspect that has received increasing attention is the role of the host's humoral alloresponse, particularly the formation of *de novo* donor-specific antibodies (dnDSAs). The aim of this study was to investigate the clinical significance of transient and persistent dnDSAs and to understand their impact on outcomes after LuTx. A retrospective analysis was conducted using DSA screening data from LuTx recipients obtained at the Medical University of Vienna between February 2016 and March 2021. Of the 405 LuTx recipients analyzed, 205 patients developed dnDSA during the follow-up period. Among these, 167 (81%) had transient dnDSA and 38 (19%) persistent dnDSA. Persistent but not transient dnDSAs were associated with chronic lung allograft dysfunction (CLAD) and antibody-mediated rejection (AMR) ($p < 0.001$ and $p = 0.006$, respectively). CLAD-free survival rates for persistent dnDSAs at 1-, 3-, and 5-year post-transplantation were significantly lower than for transient dnDSAs (89%, 59%, 56% vs. 91%, 79%, 77%; $p = 0.004$). Temporal dynamics of dnDSAs after LuTx have a substantial effect on patient outcomes. This study underlines that the persistence of dnDSAs poses a significant risk to graft and patient survival.

Keywords: donor specific antibody (DSA), humoral rejection, lung transplantation, AMR, antibody-mediated rejection

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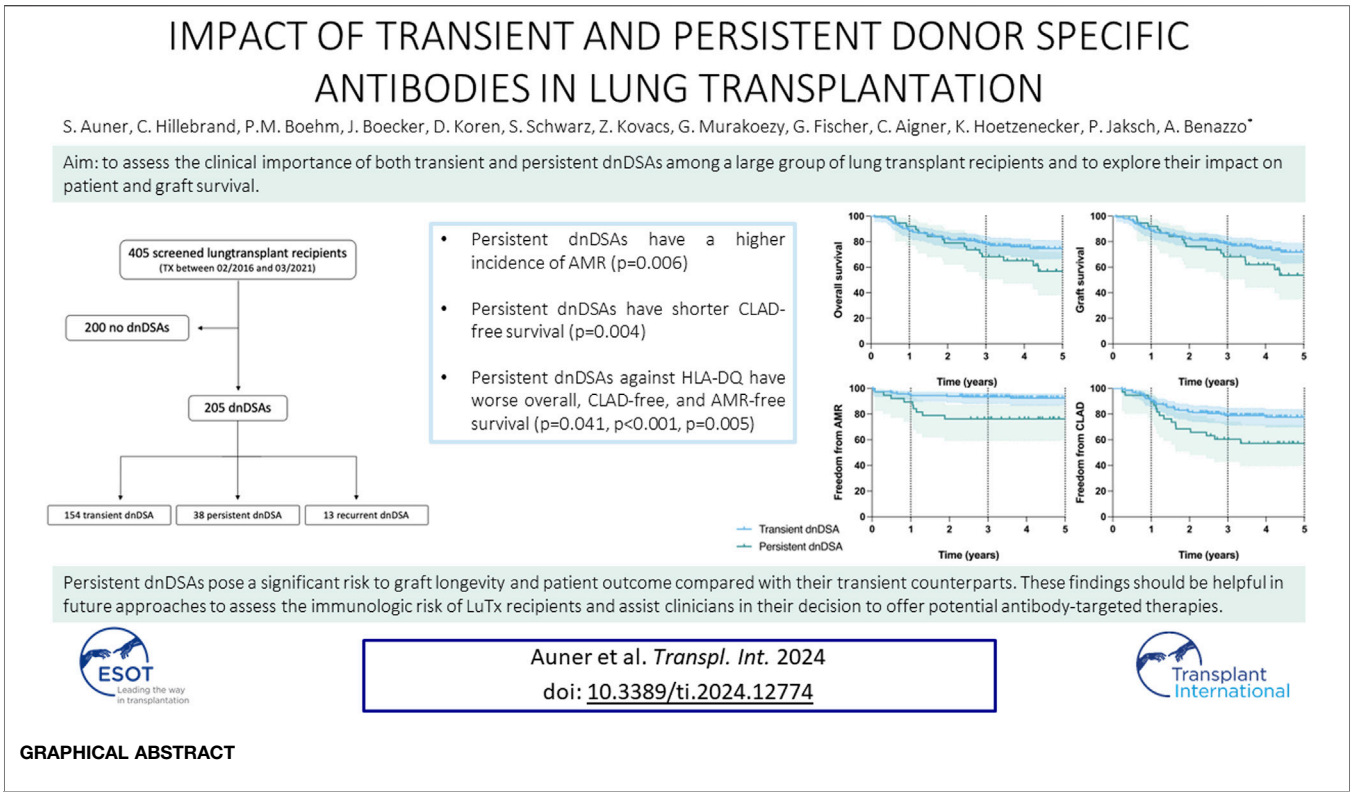
INTRODUCTION

Lung transplantation represents a life-saving therapeutic option for patients with end-stage lung diseases, however, outcomes remain impaired by acute and chronic rejection. Over the last decade, there has been growing recognition of the pathogenic significance of the host's humoral response against the pulmonary allograft in addition to cellular immunity. It has been observed that patients with antibody-mediated rejection who survive the acute phase often develop long-term structural derangements of the allograft, leading to CLAD [1, 2]. However, to date, dnDSAs without clinical signs of graft dysfunction are not considered a stringent indication for treatment, primarily because currently available therapeutic interventions carry significant associated risks.

Few studies have already shown the pathogenic role of persistent DSAs. Schmitzer et al. drew attention to the contrasting outcomes linked with transient versus persistent DSAs, and



GRAPHICAL ABSTRACT

showed a significantly reduced survival in patients with persistent DSAs [3]. Lobo et al. highlighted the significant correlation between DSA presence, particularly with anti-HLA DQ specificity, and increased AMR and CLAD [4].

We hypothesized that transient dnDSAs, which may appear briefly after transplantation, lack clinical significance. Persistent dnDSAs, on the other hand, reflect an ongoing subclinical humoral response against the graft. The primary aim of this study was to assess the clinical importance of both transient and persistent dnDSAs among a large group of lung transplant recipients and to explore their impact on patient and graft survival.

MATERIALS AND METHODS

Study Design

This study was a retrospective single-center analysis of data obtained at the Medical University of Vienna between February 2016 and March 2021. We reviewed DSA screening data from 405 lung transplant recipients. The analysis included adult patients with *de novo* DSAs after primary transplantation. Patients who underwent retransplantation or multi-organ transplantation were excluded from the study (as shown in **Figure 1**).

This study has been approved by the Institutional Ethical Committee of the Medical University of Vienna [EK-Nr. 1899/2023] and was performed according to the Declaration of Helsinki. Due to the retrospective nature of the study patient consent was waived.

Pretransplant Screening

Prior to transplantation, all patients underwent screening for potential pre-sensitization using the complement-dependent lymphoma cytotoxicity assay and the Luminex multiplex assay. If positive, a Single Antigen bead assay (LABScreen® Single Antigen; OneLambda) was performed. Based on the results of the Single Antigen bead assay, unacceptable antigens (UAGs) were defined based on MFI > 10,000 and on clinical plausibility, i.e., previous sensitization events (e.g., pregnancies). When UAGs were present, the virtual panel reactive antibody score (vPRA) was calculated using the "Eurotransplant Reference Laboratory virtual PRA Calculator."[1] On the day of transplantation, a single antigen bead assay was performed. Based on this assay, antibodies present with an MFI > 1,000 were classified as "preformed" DSAs. Antibodies post-transplant with an MFI > 1,000, which were not detected or were below this threshold before transplant, were classified as *de novo* DSAs.

Flow cytometric crossmatch (FCXM) was conducted for every patient immediately after transplantation. Donor lymphocytes were incubated with the patient's serum, along with both negative and positive controls. Differently from other centers that might employ the median channel shift of median MFI for analysis, our center utilizes a linear acquisition approach. Accordingly, an FCXM test is deemed positive when the fluorescence intensity measurement exceeds 6,000 units above the mean of the negative controls.

[1]https://www.etrl.org/InformationVPRA.aspx

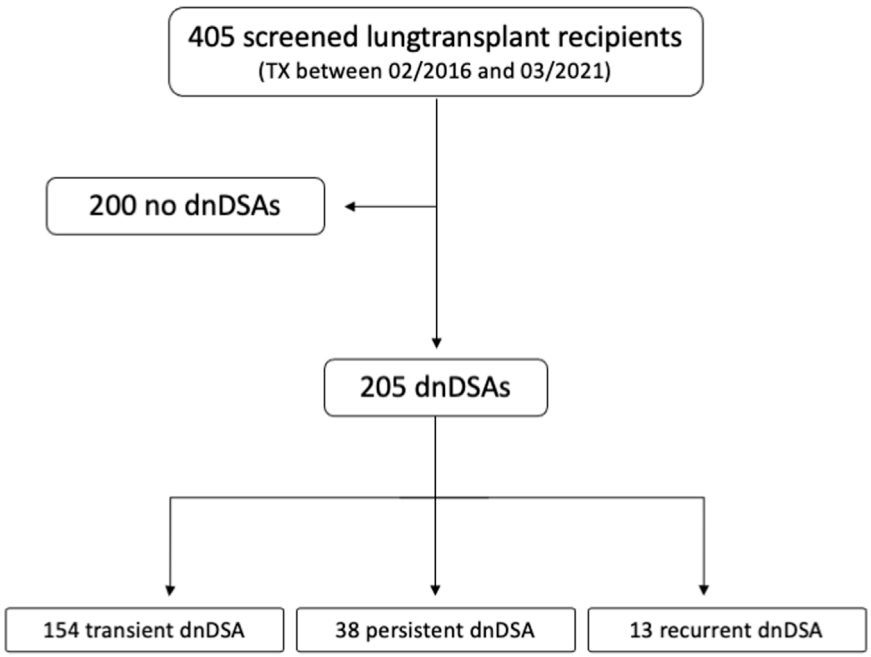


FIGURE 1 | Flowchart showing patient selection.

Clinical Protocol

Upon arrival at the intensive care unit, patients either received either a single 30 mg dose of alemtuzumab (Genzyme/Sanofi, Cambridge, United States), anti-thymocyte globulin (ATG, Neovii, Rapperswil-Jona, Switzerland) or no induction therapy after transplantation. If alemtuzumab therapy was given, a low-dose maintenance immunosuppression protocol based on tacrolimus and steroids was followed for the first year, with the addition of mycophenolate mofetil from the second year onward [5]. Otherwise, patients received a standard triple-drug maintenance immunosuppression. Since 2009 our center routinely used alemtuzumab as induction therapy agent. Since 2009, however, the induction policy changed over time. At the beginning of our experience, alemtuzumab was not administered in patients with multi-resistant bacteria, in sensitized patients or in patients with connective-tissue diseases (CTD). Patients with multi-resistant bacteria did not receive any induction, while sensitized or CTD patients received ATG. However, due to the excellent results with alemtuzumab, sensitized and CTD patients have been treated with alemtuzumab for approximately 5 years. More details regarding our induction and immunosuppression strategies have been published elsewhere [6]. All LuTx recipients received lifelong pneumocystis prophylaxis with trimethoprim-sulfamethoxazole or atovaquone. Inhalation therapy with Amphotericin B was administered for a minimum of 3 months. For cytomegalovirus (CMV) prevention, patients received CMV hyperimmunoglobulins and valganciclovir for at least 3 months, while patients identified as high-risk (donor CMV positive, recipient CMV negative) receiving this prophylaxis for an extended period of up to 1 year.

Follow-Up

Surveillance bronchoscopy with transbronchial biopsy (TBB) and bronchoalveolar lavage (BAL) were scheduled at 2 weeks, 1-, 2-, 3-, 6-, and 12-month post-transplant. TBBAs were graded according to the latest ISHLT criteria [7]. Annual lung CT scans were part of the follow-up protocol. The diagnosis of CLAD was attributed by two independent transplant pulmonologists following the ISHLT consensus report guidelines [8]. If a patient's lung function deteriorated without reversible reasons, patients received 250 mg azithromycin three times weekly for at least 3 months. If the lung function continued to decline, extracorporeal photopheresis (ECP) was initiated.

According to the ISHLT, clinical antibody-mediated rejection (AMR) was identified by the presence of donor-specific antibodies, pathological signs of tissue injury, complement activation (evidenced by c4d deposition), or detectable graft dysfunction [2]. Acute cellular rejection (ACR) was histologically graded based on the intensity of cellular infiltrates and fibrosis found in transbronchial biopsies and ranged from grade A0 (no acute rejection) to A4 (severe acute rejection) [9].

Measurement of Post-Transplant Donor Specific Antibodies

HLA class I and class II specific antibodies in patients' sera were detected using single antigen beads (SAB) (LABScreen Single Antigen HLA Class I and Class II, One Lambda, Canoga Park, CA, United States) on the Luminex Flexmap 3D platform (Luminex Corporation, Austin, TX, United States). The assay was performed according to the manufacturer's protocol with

TABLE 1 | Patient characteristics for patients with transient and persistent dnDSA.

Patients' characteristics					
		Overall (*n* = 205)	**Transient (*n* = 167)**	**Persistent (*n* = 38)**	***p*-value**
Female (*n*, %)		73 (36%)	67 (40%)	6 (16%)	0.005
Age in years (median, IQR)		56 (42–62)	57 (44–62)	53 (35–60)	0.11
Type of TX (*n*, %)	DLuTx	202 (99%)	165 (99%)	37 (97%)	0.5
	SLuTx right	2 (1.0%)	1 (0.6%)	1 (2.6%)	
	SLuTx left	1 (0.5%)	1 (0.6%)	0 (0%)	
Underlying diagnosis (n, %)	Obstructive	84 (41%)	72 (44%)	12 (31%)	0.13
	Restrictive	65 (32%)	52 (31%)	13 (34%)	
	Vascular	9 (4.4%)	9 (5.4%)	0 (0%)	
	Suppurative	38 (19%)	27 (16%)	11 (29%)	
	Others	9 (4.4%)	7 (4.2%)	2 (5.3%)	
UAGs (*n*, %)		11 (5.9%)	10 (6.4%)	1 (3.1%)	0.7
Crossmatch positive (*n*, %)		1 (0.5%)	1 (0.6%)	0 (0%)	>0.9
High grade ACR (*n*, %)		8 (3.9%)	4 (2.4%)	4 (11%)	0.041
High grade LB (*n*, %)		9 (4.4%)	5 (3.0%)	4 (11%)	0.063
Immuno-suppression (*n*, %)	Ciclosporin	4 (2.0%)	4 (2.4%)	0 (0%)	>0.9
	Tacrolimus	198 (98%)	161 (98%)	37 (100%)	
CMV risk (*n*, %)	D+/R–	58 (28%)	42 (25%)	16 (42%)	0.079
	D+/R+	68 (33%)	61 (37%)	7 (18%)	
	D–/R+	58 (28%)	45 (27%)	13 (34%)	
	D–/R–	20 (9.8%)	18 (11%)	2 (5.3%)	
AMR (*n*, %)		23 (11%)	14 (8.4%)	9 (24%)	0.018
HLA class I (*n*, %)		133 (65%)	106 (63%)	27 (71%)	0.4
HLA class II (*n*, %)		143 (70%)	108 (65%)	35 (92%)	<0.001
DSA against HLA—A (*n*, %)		69 (34%)	51 (31%)	18 (47%)	0.048
DSA against HLA—B (*n*, %)		75 (37%)	55 (33%)	20 (53%)	0.023
DSA against HLA—C (*n*, %)		43 (21%)	33 (20%)	10 (26%)	0.4
DSA against HLA—DQ (*n*, %)		116 (57%)	84 (50%)	32 (84%)	<0.001
DSA against HLA—DP (*n*, %)		18 (8.8%)	13 (7.8%)	5 (13%)	0.3
DSA against HLA—DR (*n*, %)		56 (27%)	40 (24%)	16 (42%)	0.023
mean MFI intensity score (median, IQR)		2.00 (1.25–2.40)	2.00 (1.00–2.00)	2.53 (2.00–2.96)	<0.001
CLAD (*n*, %)		52 (25%)	35 (21%)	17 (45%)	0.002

Abbreviations: TX, transplantation; DLuTx, double lung transplantation; SLuTx, single lung transplantation; HLA, human leukocyte antigen; UAG, unacceptable antigen; ACR, acute cellular rejection; LB, lymphocytic bronchiolitis; D, donor; R, recipient; AMR, antibody mediated rejection; DSA, donor specific antibody; MFI, mean fluorescence intensity; CLAD, chronic lung allograft dysfunction.

TABLE 2 | Patients with unacceptable antigens.

Patients with UAGs										
Patients	**UAGs**	**vPRA (%)**	**Matched organ**	**preTX therapy**	**postTX therapy**	**Crossmatch**	**Induction**	**dnDSAs**	**AMR**	**CLAD**
patnr001	B17	8	Yes	No	No	Negative	None	Transient	No	No
patnr002	B12, Dr13, DQ1	78	Yes	ECP	No	Negative	Alemtuzumab	Transient	No	No
patnr003	A2	51	Yes	ECP	No	Negative	Alemtuzumab	Transient	No	No
patnr004	A2, B17, DR4, DR53	72	Yes	ECP+IAS	No	Negative	ATG	Transient	No	No
patnr005	B46, B73, Cw7, Cw8, Cw3, C*12, C*16	81	Yes	ECP	No	Negative	ATG	Transient	Yes	No
patnr006	B18, DR4, DR6, DR2, DR3, DR52, DQ1	98	Yes	ECP	No	Negative	ATG	Transient	No	No
patnr007	B12	23	Yes	No	No	Negative	Alemtuzumab	Transient	No	Yes
patnr008	A25, B22, Cw3	5	Yes	No	No	Negative	Alemtuzumab	Persistent	No	No
patnr009	B12	23	Yes	No	No	Negative	Alemtuzumab	Transient	No	Yes
patnr010	DR7	23	Yes	No	No	Negative	Alemtuzumab	Transient	No	No
patnr011	A80	1	Yes	No	No	Negative	ATG	Transient	No	No

Abbreviations: vPRA, virtual panel reactive antibodies; preTX, pretransplantation; postTX, post-transplantation; ECP, extracorporeal photopheresis; IAS, immunoadsorption; ATG, anti-thymocyte globulin.

minor modifications. Sera were subjected to pretreatment with ethylenediaminetetraacetic acid (EDTA) at a final concentration of 50 mM to avoid a prozone effect, followed by incubation with the beads. After three wash phases, a fluorescence-labelled IgG antibody was added and further incubated. After three additional wash steps, fluorescence intensity of the beads was measured. The

observed mean fluorescence intensity (MFI) of the beads was reported after subtracting the value of the negative control bead (normalized values). An MFI value > 1,000 was considered positive. Initially, the sera were evaluated in their undiluted form. However, if MFI values of the beads exceeded 20,000, implying antibody saturation of the beads, the serum was diluted with PBS prior to reassessment. In this case, the MFI values were reported as the initial observed values multiplied by the dilution factor. Measurements were analyzed using the HLA Fusion software (Thermo Fisher Scientific Inc.). DSAs were screened at every follow-up visit at 2 weeks, 1-, 2-, 3-, 6-, and 12-month post-transplantation and in case of clinical deterioration. DnDSAs were classified as "transient" when they were detected for a period of less than 6 months following transplantation. Conversely, dnDSAs were classified as "persistent" when their presence extended for 6 months or longer. Recurrent DSAs were defined as circulating dnDSAs, which disappeared without treatment and later reemerged. Based on their initial detection post-transplantation, dnDSAs were further categorized into "early" dnDSAs (emerging within the first 6 months) and "late" dnDSAs (manifesting after 6 months). Subsequently, dnDSAs were classified into subgroups according to their MFI value: MFI class I: 1,000–2,000, MFI class II: 2,000–5,000, MFI class III: 5,000–10,000, MFI class IV: >10,000. For further comparative analysis, a "*mean MFI intensity score*" was computed for each individual, calculated as the average MFI class of all dnDSAs identified in that particular patient.

Statistical Analysis

Categorical variables are expressed as absolute and relative frequencies and were compared using a chi-square test. Continuous variables were expressed as median and interquartile range (IQR) or mean and standard deviations. Chi-square tests, Fisher exact tests, Mann-Whitney U-tests, or ANOVA were used to compare variables as applicable. Patient and graft survival as well as freedom from AMR and CLAD were displayed with Kaplan-Meier-curves and compared using a log-rank test. Univariate and multivariable Cox regression were performed to find risk factors for mortality and CLAD. Variables that reached significance in the univariate analyses, they were included in a multivariable Cox regression. Data was analyzed using SPSS version 27.0 software, graphics were designed with GraphPad Prism 6.

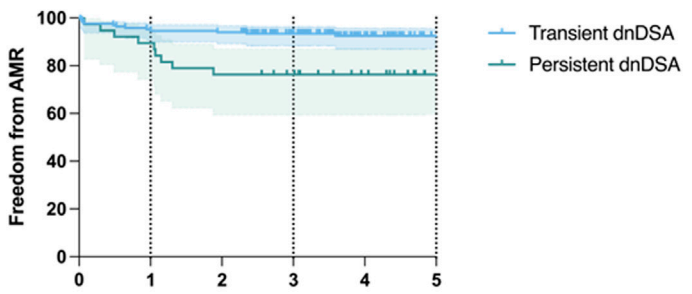
RESULTS

Within the study period, we analyzed data from 405 LuTx recipients. Of these patients, 205 developed dnDSAs during the median follow-up period of 3.44 years (IQR 2.45–4.81) and were included in the present study. 167 (81%) patients accounted for the transient dnDSA group and 38 (19%) for the persistent dnDSA group. The median age at the time of transplantation was 56 years (IQR: 42–62) and 36% of the cohort were female. 202 (99%) patients underwent a double lung transplantation (DLuTx), while only three received a single lung transplantation (SLuTx). Chronic obstructive pulmonary disease (COPD) was the most common underlying diagnosis, accounting for 41% of the cohort. Detailed patient characteristics, including patients with recurrent dnDSAs, are summarized in **Table 1**. Patient characteristics for transient, persistent, and recurrent dnDSAs are summarized in **Supplementary Table S2**.

Pretransplant Immunization

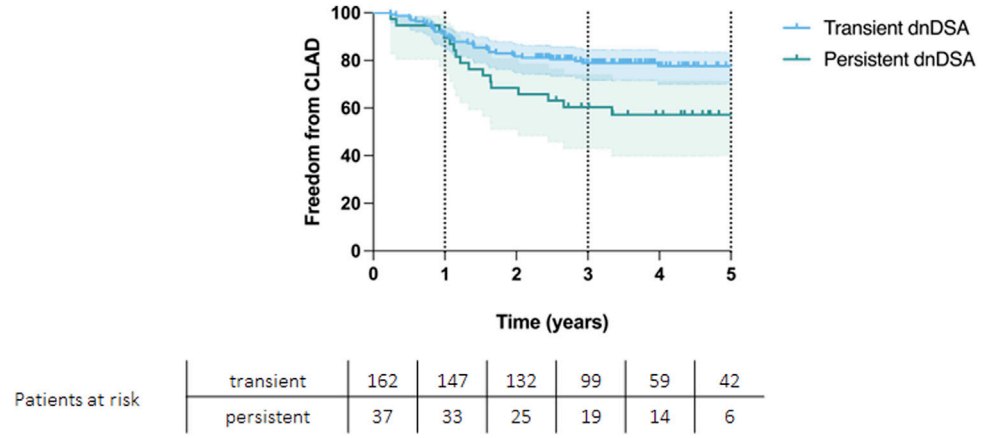
Eleven patients (5.9%) had UAGs with a mean vPRA of 42% (range 1%–98%). Characteristics of those 11 patients are displayed in **Table 2**. All presensitized patients received an organ matched on all 6 HLA donor-recipient loci.

Only one patient in the cohort had a positive FCXM. This patient, diagnosed with usual interstitial pneumonia (UIP) and rheumatoid arthritis had previously been treated with rituximab. The patient was bridged to transplantation on veno-venous ECMO. Prior to transplantation, the patient was negative for both HLA class I and II in the single antigen bead assay. The patient developed dnDSAs and clinical AMR 190 days after



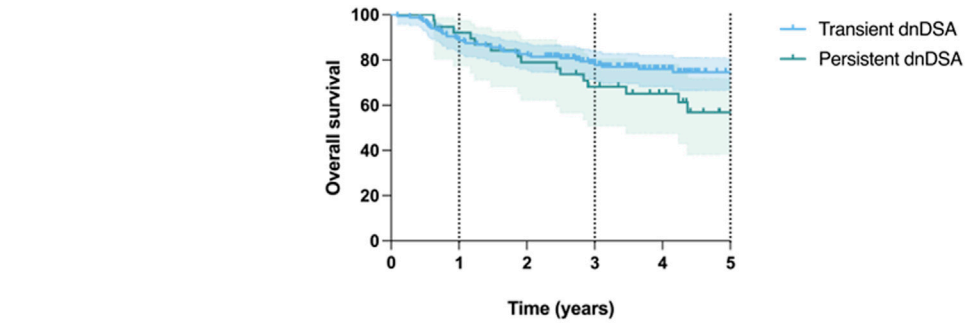
Patients at risk							
	transient	166	158	156	124	84	59
	persistent	38	34	29	26	19	10

FIGURE 2 | Kaplan-Meier curve showing AMR-free survival for patients with transient and persistent DSAs (p = 0.004).



Patients at risk							
	transient	162	147	132	99	59	42
	persistent	37	33	25	19	14	6

FIGURE 3 | Kaplan-Meier curve showing CLAD-free survival for patients with transient and persistent DSAs (p = 0.004).



Patients at risk							
	transient	167	149	138	102	61	39
	persistent	38	35	30	24	18	9

FIGURE 4 | Kaplan-Meier curve showing overall survival for patients with persistent and transient *de novo* DSAs (p = 0.074).

transplantation, which was treated with ECP, IAS and ATG. The patient died 26 days after AMR diagnosis due to respiratory failure.

DSA Profile

A complete list and median MFI class of all timepoints for each dnDSA based on the Luminex Single Antigen bead assay is shown in **Supplementary Table S1**. After transplantation, 65% of the overall cohort (n = 133) tested positive for HLA class I antibodies with a slightly higher proportion in the persistent group (71%) than in the transient group (63%). This difference was not statistically significant (p = 0.4). A more notable discrepancy was observed for DSA class II, for which 70% of the total cohort (n = 143) tested positive. Of these patients, 92% accounted for the persistent group and 65% for the transient group (p < 0.001).

When assessing the frequency of specific transient or persistent dnDSAs, significant variations were evident for dnDSAs against HLA-A, -B, -DQ and -DR (p = 0.048, p = 0.023, p < 0.001, and p = 0.023, respectively) (**Table 1**).

The median of the *mean MFI intensity score* was significantly higher in the persistent DSA group with a score of 2.53 (IQR: 2.00–2.96), than in the transient group with a score of 2.00 (IQR: 1.00–2.00) (p < 0.001).

Next, we analyzed the impact transient and persistent dnDSAs against a specific HLA subclass on AMR and CLAD development. Patients with persistent dnDSA against HLA-DQ had a higher incidence of AMR (p = 0.004) and CLAD (p = 0.002). Furthermore, persistent dnDSA against HLA-A showed a significantly higher rates of AMR, but not CLAD (p = 0.046). Patients with persistent dnDSA against HLA-DQ showed a significantly worse overall, CLAD-free, and AMR-free survival (p = 0.041, p < 0.001, and p = 0.005, respectively), whereas persistent dnDSAs against HLA-A had a significantly worse overall and AMR free survival (p = 0.020 and p = 0.012). Specifically, persistent dnDSA against HLA-DQB had a significantly worse overall, CLAD-free, and AMR-free survival (p = 0.026, p = 0.007, and p = 0.016, respectively).

Rejections and Long-Term Survival

Eleven percent (n = 22) of the entire cohort developed an AMR. Within the persistent dnDSA group, the incidence was 24%

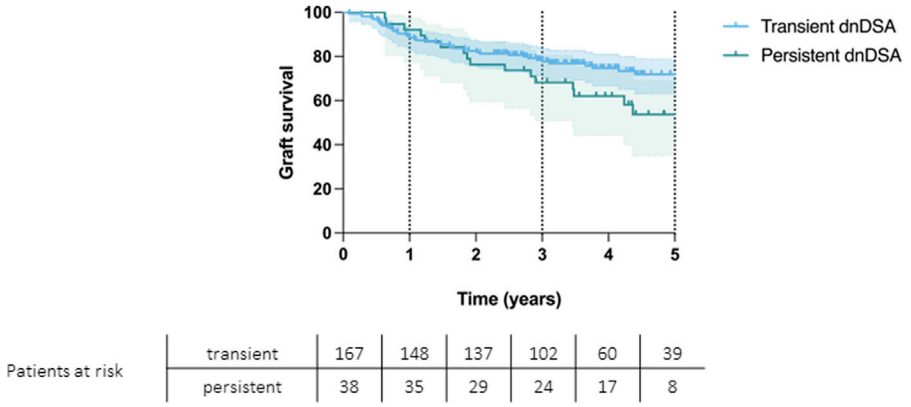


FIGURE 5 | Kaplan-Meier curve showing graft survival for patients with persistent and transient *de novo* DSAs ($p = 0.069$).

($n = 9$) as compared to 7.8% ($n = 13$) in the transient group ($p = 0.008$). Patients with AMR had significantly worse survival than those without AMR episodes ($p < 0.001$). AMR free survival rates at 1, 3 and 5 years of 89%, 76%, and 76% for persistent DSA group and 95%, 93%, and 92% for transient DSA group ($p = 0.004$, **Figure 2**). Patients with AMR, that received any form of treatment are displayed in **Supplementary Table S3**.

ACR free survival rates at 1, 3 and 5 years were 99%, 97% and 97% for the transient group and 95%, 89% and 89% for the persistent group ($p = 0.021$).

CLAD-free survival significantly differed between the groups, with CLAD free survival rates at 1, 3 and 5 years of 89%, 59%, and 56% for persistent DSA group and 91%, 79%, and 77% for transient DSA group ($p = 0.004$, **Figure 3**).

Patients' overall survival rates at 1, 3 and 5 years were 92%, 68%, and 57% for persistent DSAs and 89%, 79%, and 75% for transient DSAs ($p = 0.074$) (**Figure 4**). Graft survival rates at 1, 3, and 5 years were 92% 68% and 54% for persistent DSAs and 89%, 78%, and 71% for transient DSAs ($p = 0.069$) (**Figure 5**).

Risk Factor Analysis

Univariate and multivariable cox regression for overall survival and CLAD occurrence were performed to identify risk factors. AMR was identified as the only independent risk factors for impaired survival ($p < 0.001$) and for CLAD occurrence ($p = 0.030$) in the multivariable cox regression model (**Table 3**). Interestingly, dnDSAs against HLA-DQ, were not identified as risk factors in both adjusted models. To elucidate the potential impact of MFI intensity on outcomes, we analyzed MFI classes one to four in our risk factor analysis. While MFI class 1 exhibited a protective trend against mortality in the univariate analysis, it did not reach statistical significance in the multivariate model. Similarly, MFI class 4 appeared to be a risk factor for CLAD occurrence in the univariate analysis, yet this did not remain significant in the multivariate analysis.

DISCUSSION

The current study provides valuable insights into the temporal dynamics of *de novo* donor-specific antibodies after lung transplantation and their potential role in allograft dysfunction. The primary aim of this study was to assess the clinical importance of both transient and persistent dnDSAs and to explore their impact on patient and graft survival. We could demonstrate that CLAD-free and AMR-free survival was significantly higher in patients with transient DSAs, signaling the potential negative impact of persistent dnDSAs on outcomes after LuTx.

DSAs have been associated with glomerulopathy in renal transplant recipients and cardiac allograft vasculopathy in cardiac transplant recipients [10, 11]. Similarly, observational studies in LuTx suggested that dnDSAs could have a deleterious effect on survival and CLAD [12–16]. In addition, some publications report a beneficial effect of treating dnDSAs in the absence of graft dysfunction. For example, a single-center retrospective study analyzed the effects of preemptive treatment of early DSAs with IVIG and showed comparable graft survival in patients receiving preemptive treatment compared to patients without DSAs [17]. Hachem et al performed a prospective cohort study showing that patients who developed DSAs and received antibody-directed therapy had similar rates of CLAD and acute rejection as patients without DSAs [18]. Finally, in a recent multicenter retrospective analysis, Keller et al provided evidence that asymptomatic patients with dnDSAs who received preemptive treatment of any kind had a lower risk of CLAD or death than untreated patients with dnDSAs [19]. Based on these findings, it is meaningful to speculate that an active approach towards patients with dnDSAs could result in improved outcomes. Nevertheless, there is only limited evidence on the efficacy of available therapies and most of them are associated with a high-risk side effect profile. As a consequence, treating asymptomatic patients with dnDSAs remains clinically and ethically questionable and it is of paramount importance to

TABLE 3 | Risk factor analysis for mortality and CLAD occurrence.

Univariate cox regression for mortality					Multivariable cox regression for mortality			
Variable	**HR**	**CI**		***p*-value**	**HR**	**CI**		***p*-value**
		Lower limit	**Upper limit**			**Lower limit**	**Upper limit**	
Age	**1.02**	**1.002**	**1.04**	**0.040**	**1.03**	**1.01**	**1.05**	**0.008**
Female	1.01	0.98	1.04	0.421				
Preformed DSA	0.83	0.37	1.82	0.636				
DSAclass I	1.16	0.67	2.02	0.586				
DSAclass II	0.98	0.55	1.73	0.946				
persistent dnDSA	1.69	0.94	3.01	0.078				
MFI class 4	1.69	0.94	3.05	0.082				
MFI class 3	0.98	0.51	1.89	0.943				
MFI class 2	1.39	0.82	2.34	0.223				
MFI class 1	**0.32**	**0.14**	**0.75**	**0.008**	0.38	0.14	1.03	0.061
dnDSA against HLA—A	1.05	0.61	1.80	0.871				
dnDSA against HLA—B	1.48	0.87	2.51	0.151				
dnDSA against HLA—C	1.15	0.62	2.14	0.653				
dnDSA against HLA—DP	0.96	0.38	2.40	0.925				
dnDSA against HLA—DQ	1.10	0.65	1.86	0.793				
dnDSA against HLA—DQA	1.14	0.62	2.09	0.668				
dnDSA against HLA—DQB	1.00	0.60	1.69	0.996				
dnDSA against HLA—DR	1.48	0.85	2.57	0.167				
Persistent dnDSA against HLA—A	7.99	1.00	34.88	0.059				
Persistent dnDSA against HLA—B	0.05	0.01	18.27	0.551				
Persistent dnDSA against HLA—C	1.21	0.16	9.34	0.853				
Persistent dnDSA against HLA—DQ	**2.00**	**1.01**	**3.93**	**0.046**	0.834	0.17	4.05	0.822
Persistent dnDSA against HLA—DQA	1.73	0.54	5.53	0.359				
Persistent dnDSA against HLA—DQB	**2.34**	**1.08**	**5.04**	**0.031**	2.03	0.36	11.56	0.424
Persistent dnDSA against HLA—DR	1.96	0.77	4.97	0.156				
AMR	**4.33**	**2.42**	**7.74**	**<0.001**	**4.14**	**2.11**	**8.12**	**<0.001**
Mean MFI intensity score	**1.64**	**1.17**	**2.22**	**0.003**	0.93	0.58	1.48	0.742

Univariate cox regression for CLAD occurrence					Multivariable cox regression for CLAD occurrence			
Variable	**HR**	**CI**		***p*-value**	**HR**	**CI**		***p*-value**
		Lower limit	**Upper limit**			**Lower limit**	**Upper limit**	
Age	0.99	0.98	1.02	0.831				
Female	0.81	0.45	1.46	0.487				
DSAclass I	1.06	0.60	1.88	0.586				
DSAclass II	0.89	0.50	1.61	0.741				
Persistent dnDSA	**2.30**	**1.29**	**4.11**	**0.005**	0.60	0.08	4.64	0.621
MFI class 4	**2.57**	**1.44**	**4.59**	**0.001**	1.43	0.65	3.14	0.372
MFI class 3	0.80	0.39	1.65	0.549				
MFI class 2	0.78	0.44	1.39	0.395				
MFI class 1	0.63	0.31	1.29	0.206				
dnDSA against HLA—A	0.84	0.46	1.51	0.561				
dnDSA against HLA—B	1.29	0.69	2.41	0.429				
dnDSA against HLA—C	1.13	0.61	2.19	0.690				
dnDSA against HLA—DP	1.06	0.61	1.85	0.837				
dnDSA against HLA—DQ	0.57	0.18	1.82	0.325				
dnDSA against HLA—DQA	0.89	0.46	1.74	0.737				
dnDSA against HLA—DQB	1.06	0.62	1.83	0.829				
dnDSA against HLA—DR	1.34	0.75	2.39	0.327				
Persistent dnDSA against HLA—A	0.05	0.01	20.23	0.738				
Persistent dnDSA against HLA—B	0.05	0.01	34.62	0.593				
Persistent dnDSA against HLA—C	0.97	0.13	7.43	0.973				
Persistent dnDSA against HLA—DQ	**3.25**	**1.61**	**6.58**	**0.001**	6.23	0.53	73.64	0.147
Persistent dnDSA against HLA—DQA	3.07	0.89	10.53	0.075				
Persistent dnDSA against HLA—DQB	**2.82**	**1.29**	**6.19**	**0.010**	0.58	0.13	2.55	0.470
Persistent dnDSA against HLA—DR	2.48	0.81	7.63	0.113				
AMR	**3.10**	**1.63**	**5.92**	**<0.001**	**2.20**	**1.08**	**4.48**	**0.030**
Mean MFI class score	**1.64**	**1.15**	**2.35**	**0.006**	1.24	0.78	1.97	0.368

Abbreviations: CLAD, chronic lung allograft dysfunction; DSA, donor specific antibodies; dnDSA, de-novo *donor specific antibodies; HLA, human leukocyte antigen; AMR, antibody mediated rejection; MFI, mean fluorescence intensity.*

Bold values are statistically significant.

identify which patients could profit from such an aggressive approach.

In particular, patients with persistent antibodies have worse freedom from AMR and CLAD compared to patients with transient dnDSAs. Our findings confirm previous observations. Schmitzer et al. investigated the relevance of DSA prospectively in 72 patients and showed that persistent DSAs had a significantly reduced survival compared to transient or no DSAs [3]. Especially patients developing AMR had dramatic outcomes in our cohort. These patients could benefit from antibody-directed therapies and further prospective studies should aim to assess possible strategies in this high-risk cohort. Furthermore, our analysis of MFI intensity classes suggests its role is not straightforward and may be moderated by other factors. The potential collinearity between higher MFI classes and persistent dnDSA groups was considered, however, our analysis indicates that the relationship between MFI intensity, dnDSA persistence, and clinical outcomes is complex and warrants further investigation.

Since 2016, our center has started to routinely screen patients for dnDSAs, before 2016, patients were only tested in case of functional decline or clinical suspicion of rejection. This practice is not common in every transplant program yet, partly explaining the high variability in the reported incidence of dnDSAs [15, 20, 21]. Starting a screening program had significantly affected our current practice. Indeed, dnDSA were present in more than half of the study population and one-third of them were persistent. Moreover, based on the current findings, it is meaningful to argue that a large proportion of these patients would require some antibody-directed therapies, which can on one hand improve long-term outcomes and on the other hand, reduce healthcare costs by decreasing future hospitalization, intensive supportive care or more expensive treatments.

In the follow-up period, the majority of dnDSA were directed against antibodies of HLA class II antigens. Especially antibodies against HLA-DQ antigens were significantly higher in the persistent group than in the transient group. Remarkably, the development of persistent dn-DSA-DQ was significantly associated with a higher incidence of CLAD and AMR. This association has also been found in other retrospective single-center studies [4, 22, 23]. For example, Tikkanen et al. showed that recipients with *de novo* DSAs against HLA-DQ had an increased risk for developing CLAD [22]. Also, Roux et al. analyzed data from 206 LuTx recipients with and without AMR. They showed that the DSA-DQ was associated with AMR and CLAD [23].

We acknowledge that our study is not free of limitations. Given the retrospective design of our study and the limited number of observations, there is a potential for miscoded data and an increased risk of Type I error. Then, different induction therapies have been used in the study cohort, which might complicate the interpretation of the results. Furthermore, different desensitization strategies as well as multiple AMR treatments have been implied in our center overtime. This reflects the lack of efficient treatment but can represent possible confounders. We furthermore must acknowledge the lack of sequential screening pretransplant after potentially sensitizing exposures as a limitation. Another limitation of our study is the lack of additional confirmatory testing for atypical DQ bead reactions, despite the known potential for artifacts in bead array assays. Finally, the definition used to distinguish transient from persistent dnDSAs is based on our clinical experience and past literature, instead of being based on robust mechanistic data.

In summary, our study demonstrated that persistent dnDSAs pose a significant risk to graft longevity and patient outcome compared with their transient counterparts. These findings should be helpful in future approaches to assess the immunologic risk of LuTx recipients and assist clinicians in their decision to offer potential antibody-targeted therapies. Finally, our results may provide a rationale starting point on defining a high-risk population that ought to be included in future randomized controlled intervention trials.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: The dataset are property of the Medical University of Vienna. Access to the dataset can be provided after formal approval of the legal departments of the three involved centers and the of the first and last authors. Requests to access these datasets should be directed to rechtsabteilung@meduniwien.ac.at.

AUTHOR CONTRIBUTIONS

Study design: SA and AB; Contributed to data collection: SA, CH, PB, AB, and PJ; Wrote the manuscript: SA and AB; Statistical analysis and interpretation of results: SA, AB, and KH. 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.2024.12774/full#supplementary-material

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REVIEW
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Lung Transplantation in Controlled Donation after Circulatory-Determination-of-Death Using Normothermic Abdominal Perfusion

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The main limitation to increased rates of lung transplantation (LT) continues to be the availability of suitable donors. At present, the largest source of lung allografts is still donation after the neurologic determination of death (brain-death donors, DBD). However, only 20% of these donors provide acceptable lung allografts for transplantation. One of the proposed strategies to increase the lung donor pool is the use of donors after circulatory-determination-of-death (DCD), which has the potential to significantly alleviate the shortage of transplantable lungs. According to the Maastricht classification, there are five types of DCD donors. The first two categories are uncontrolled DCD donors (uDCD); the other three are controlled DCD donors (cDCD). Clinical experience with uncontrolled DCD donors is scarce and remains limited to small case series. Controlled DCD donation, meanwhile, is the most accepted type of DCD donation for lungs. Although the DCD donor pool has significantly increased, it is still underutilized worldwide. To achieve a high retrieval rate, experience with DCD donation, adequate management of the potential DCD donor at the intensive care unit (ICU), and expertise in combined organ procurement are critical. This review presents a concise update of lung donation after circulatory-determination-of-death and includes a step-by-step protocol of lung procurement using abdominal normothermic regional perfusion.

Keywords: lung transplantation, normothermic regional perfusion, DCD donors, lung procurement, donation after circulatory determination-of-death

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INTRODUCTION

Lung transplantation (LT) has become a viable life-saving therapy for patients with a variety of end-stage lung diseases. Donation after neurologic determination of death (brain death donors, DBD) remains the main source of lungs for transplantation. However, the persistent scarcity of suitable lung donors remains a major limiting factor to the number of transplants performed [1]. Among the available multiorgan donors, only 20%–25% are typically acceptable for lung donation, as the lung is particularly vulnerable to injury after brain death. To overcome organ shortage, different strategies have been applied over time, including the liberalization of standard criteria for lung donation [2], lobar lung

transplantation [3], the use of donors after circulatory death (DCD donors) [4] and *ex-vivo* lung perfusion [5].

This article focuses on the expansion of DCD donors in lung transplantation, including the latest findings on the topic. We also describe our technique of combined lungs and abdominal organs procurement using normothermic abdominal perfusion step-by-step.

BACKGROUND FOR DONATION AFTER CIRCULATORY DEATH IN LUNG TRANSPLANTATION

In 1963, James Hardy performed the world's first human lung transplant procedure. The patient had a squamous cell carcinoma on his left lung and recurrent episodes of pneumonia. He was a prisoner sentenced to death due to murder, which was commuted to life in prison in exchange for undergoing a lung transplant. No details on ischemic time or lung preservation were provided. The recipient survived for 18 days but finally succumbed to renal failure and "malnutrition" [6]. Just a few days later, George Magovern and Adolph Yates reported the second human lung transplant at the University Hospital in Pittsburgh [7]. The patient survived for only 1 week. It was not until 1971 that the first medium-term successful human lung transplantation was performed by Fritz Derom in Belgium [8]. Both the first world's lung transplant and the first medium-term successful human lung transplants utilized DCD donors. Over the next two decades, approximately 38 lung, lobe, or heart-lung transplant procedures were attempted, with no long-term success [9, 10]. All used DCD donors, as formal criteria for brain death had not yet been established. When the Harvard criteria for brain death were accepted [11], DBD became the standard method for organ donation, with DCD donation being abandoned.

DCD donation was then reintroduced by Thomas M. Egan in 1991, following a series of canine experiments demonstrating its feasibility [12]. In 1995, Robert Love reported the first controlled DCD lung transplant with success. Loves's group performed a left single lung re-transplantation in a patient on ECMO for severe primary graft dysfunction (PGD) [13]. An international workshop organized in 1995 led to the Maastricht classification of DCD donors [14], to categorize DCD donors based on the duration of warm ischemia. This classification has since been updated [15–17].

Steen and others reported a successful right single LT from a uDCD donor after failed resuscitation in 2001 [18]. The authors preserved the lungs by topical cooling inside the body of the potential donor, while consent for donation was obtained from the next of kin, and *ex-vivo* evaluation of lung function (EVLP) was done. EVLP is crucial when considering uDCD for LT, as premortem functional evaluation is not possible. Worldwide experience with uDCD donors is still limited to small case series, as logistics are complex.

DEFINITIONS AND CATEGORIES OF DONATION AFTER CIRCULATORY DEATH

Maastricht Classification

The Maastricht classification organizes DCD into five categories. Categories I (dead on arrival) and II (unsuccessful resuscitation) are considered "uncontrolled" donors (uDCD), whereas categories III (awaiting cardiac arrest), IV (unexpected cardiac arrest in a brain-dead donor), and V (euthanasia) comprise "controlled" DCD (cDCD) (**Table 1**) [17]. In the uncontrolled DCD scenario, a patient suffers from sudden death, so cardiopulmonary resuscitation maneuvers are initiated and continued during transport to hospital. In controlled DCD, a patient with catastrophic brain injury for whom supportive care is thought to be futile is subjected to withdrawal of life-sustaining therapies (WLST) in a planned way. Nowadays, controlled DCD donation is the most used DCD type used for transplantation.

TABLE 1 | Modified Maastricht classification of donation after circulatory death [15].

Category		Definition	Subclassification	
Uncontrolled	I	Found dead	Ia	Out-of-hospital
			Ib	In hospital
	II	Witnessed cardiac arrest	IIa	Out-of-hospital
			IIb	In hospital
Controlled	III	Planned WLST	IIIa	In ICU
			IIIb	In OR
	IV	Cardiac arrest while brain death prior to organ recovery	IVa	Unexpected in ICU
			IVb	Expected in OR/ICU
	V	Medically assisted death/ euthanasia	Va	Out-of-OR
			Vb	In OR

ICU, Intensive Care Unit; OR, Operating Room; WLST, Withdrawal of life-sustaining therapies.

Warm Ischemia

Organs retrieved from DCD donors are vulnerable to warm ischemic injury, especially the heart and the liver. However, the lung is unique among solid organs that are transplanted, as the alveoli remains filled with oxygen despite not being perfused. Nevertheless, this is an important difference to DBD, as in DCD an additional warm ischemia interval exists. Worldwide, there is no consensus regarding the definition of warm ischemic time in DCD donors. Whereas WIT can be generally described as the time between the withdrawal of life-sustaining therapies (WLST) and the initiation of organ preservation (*total* WIT), the critical period starts when significant hypoperfusion occurs. This defines the *functional WIT* and corresponds to a drop in systolic blood pressure below 50 mmHg. In Spain, the cut-off of systolic arterial pressure is slightly higher, at 60 mmHg. Some additional terms and definitions have been also proposed:

-**Relative WIT:** From WLST to significant organ hypoperfusion (mean arterial pressure <50 mmHg). **Absolute or functional WIT** is the time between significant organ hypoperfusion (mean arterial pressure <50 mmHg) and initiation of organ preservation. **Acirculatory WIT:** From cardiac arrest to cold flush. **Agonal phase**: from WLST to circulatory arrest. **No-touch period**: from circulatory arrest to death declaration. **Warm to cold interval**: from death declaration to cold preservation.

TABLE 2 | Major time-points suggested by ISHLT DCD Working Group [19].

Time Point	Description
T0	Withdrawal of life-sustaining therapies or euthanasia
T1	Oxygen saturation <80%
T2	Systolic blood pressure <50 mmHg
T3	Cessation of cardiac output/asystole
T4	Resumed lung inflation/ventilation
T5	Start of pulmonary flush

ISHLT, International Society for Heart and Lung Transplantation; DCD, donation after cardiac death.

The International Society for Heart and Lung transplantation (ISHLT) DCD Working Group recommended different time points and intervals for lung donation, as depicted in **Table 2**; **Figure 1** [19]:

-T0: withdrawal of life-sustaining therapies or euthanasia
-T1: oxygen saturation <80%
-T2: systolic blood pressure <50 mmHg
-T3: cessation of cardiac output/asystole
-T4: resumed lung inflation/ventilation
-T5: start of pulmonary flush

Interval 1 is the time from the withdrawal of life-sustaining therapy (WLST) to hypotension with systolic blood pressure <50 mmHg (T0 to T2); Interval 2 is the time from WLST to cessation of cardiac output/asystole (T0 to T3); Interval 3 is the time from WLST to start of pulmonary flush; and Interval 4 is the time from systolic blood pressure <50 mmHg to start of pulmonary flush (T2 to T5).

According to the ISHLT DCD Registry Report, no differences in one-year survival after LT were observed irrespective of the duration of Intervals 1, 2, or 3 [19].

The acceptable upper limit of WIT for DCD LT is still debatable [20]. During the agonal phase, progressive hypoxemia and hypoperfusion occur before cardiac arrest. This interval varies among donors and may result in organ injury. It has been suggested that the most important parameters associated with organ damage are oxygen saturation below 85% and systemic arterial pressure below 50 mmHg [21]. On the contrary, in a multicenter ISHLT DCD Registry analysis, the authors did not find an association between the duration of the agonal phase or functional WIT up to 60 min and early survival after LT [22]. In this report, 84.5% of 465 DCD donors reached asystole in 30 min and 96.5% reached it in >60 min after WLST. Furthermore, the Toronto group has reported good outcomes using DCD donors taking more than 120 min from WLST to cardiac arrest [23]. Nevertheless, most centers agree on 60–90 min of WIT [22].

An important difference between DCD and DBD is the possibility of autoresuscitation in DCD donors when spontaneous resumption of cardiopulmonary activity after circulatory arrest occurs. Thus, a "no-touch" or observation period has been established. This timeframe is debatable and depends on legal and ethical criteria. Many countries accept no-touch periods between 2 to 5 min, although this is variable and may extend up to 20 min, such as in Italy [24].

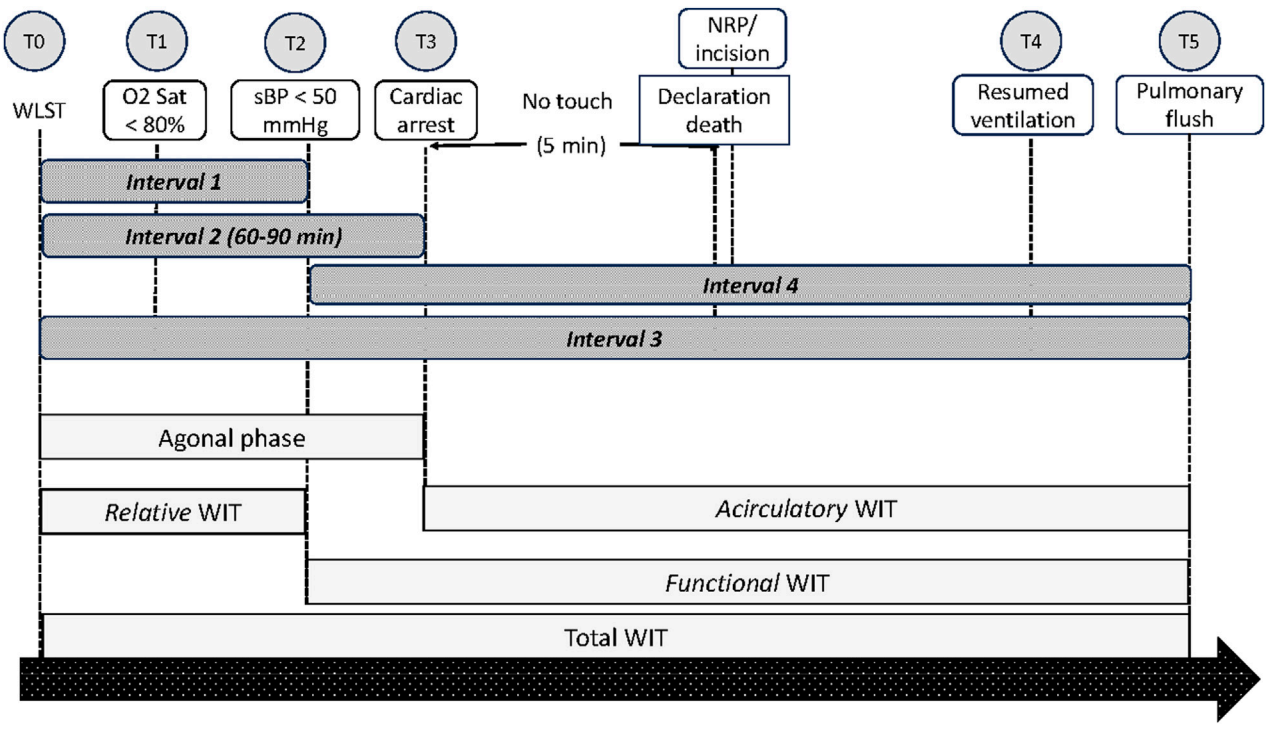

FIGURE 1 | Schematic presentation of time points and intervals as suggested by the ISHLT DCD Working Group.

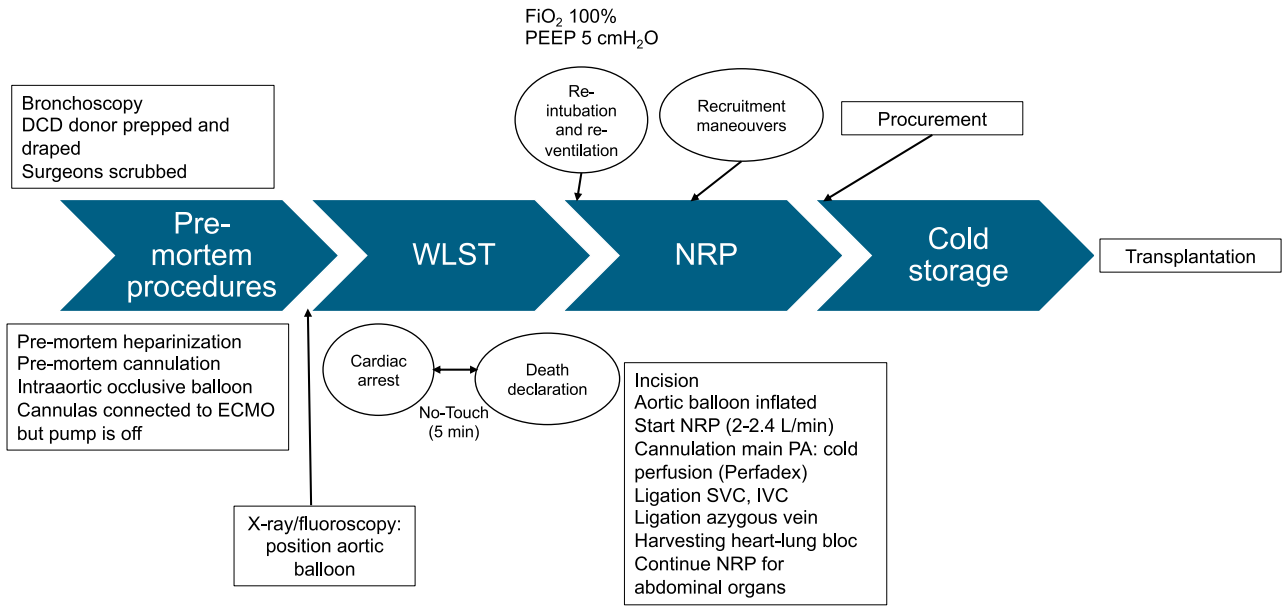

FIGURE 2 | Step by step schematic presentation of DCD organ procurement under NRP. WLST, Withdrawal life sustaining therapies; NRP, normothermic regional perfusion. ECMO, extracorporeal membrane oxygenation; PA, pulmonary artery; SVC, superior vena cava; IVC, inferior vena cava. FiO2, Fraction of inspired oxygen; PEEP, Positive end expiratory pressure.

DONOR SELECTION CRITERIA

The DCD donor selection criteria are the same as for DBD. It is of paramount importance to adequately select DCD donors to reduce the risk of primary graft dysfunction (PGD) after LT. Assessment of lung function in DCD is performed by bronchoscopy, chest x rays, arterial blood gases, and inspection. Potential cDCD donors are critically ill patients with irreversible brain injury or end-stage musculoskeletal disorders who are expected to have circulatory arrest after WLST [25]. Ideally, a cDCD donor should arrest in less than 60 min. Different models have been developed to predict the likelihood of progression to asystole after WLST, like the University of Wisconsin donation after circulatory death evaluation tool [26]. However, they are not 100% accurate, as some donors will not develop cardiac arrest within 120 min, leading to aborted procedures. To date, no reliable models exist to estimate time to circulatory death in DCD donors, and the accuracy of the available ones is modest [26–29].

The rate of aborted procedures (so-called dry-runs) is variable, ranging from 40% [30] to 1% of aborted procedures in Belgium [31]. Possible explanations to this wide variability in the rate of dry-runs are differences in existing legal frameworks, ethical barriers, and lack of technical expertise or required logistics. Moreover, proficiency of the donor coordinator in identifying and selecting a potential DCD donor is associated with low rates of dry-runs [32].

In addition to aborted procedures, DCD donation may be considered as expensive, as it has been reported that the cost per organ from a DCD is 60% higher compared to DBD [33]. Transplant groups may be reluctant to send a team to a donor hospital for fear the donor does not progress into cardiac arrest in an acceptable time, considering the high costs of travel, personnel, and the surgical procedure, added to complex logistics. Further, DCD procurement requires specialized training, especially when joined thoracic and abdominal procurement is planned. In addition, the exceeded costs in DCD organ procurement can be attributable to the higher rate of dry-runs in the earlier experience, mainly secondary to transportation and surgeon fees for declined organs [34] It is expected that, with the expansion of DCD programs, the costs will be reduced to almost those of DBD donation. Likewise, DCD organ donation is cost-effective, as the costs of maintaining the potential candidates on the waiting list expecting to receive a suitable DBD organ donor are probably higher than the benefits on survival and quality of life after receiving an organ from a DCD donor.

Another issue of concern is organ retrieval rate from DCD donors. Organ yield after DCD is lower than DBD. Although the activity has increased significantly worldwide, DCD donors are still underutilized, representing only 2% of LT in the United States and 5% in Europe. This contrasts with higher rates in Australia (28%), the Netherlands (40%), England (25%), and Canada 32% [35–37].

A recent study using data from the United Network for Organ Sharing (UNOS) revealed that, from 30,916 lungs, only 3.8% (1158) were used for transplantation between 2005 and 2009, and nearly 73% were discarded, mainly due to poor lung function [38]. PaO2/IO2 ratio below 250, smoking history, or clinical infection with a blood source were identified as predictors of non-use.

Protocols for DCD donation may differ depending on several factors, including the location of WLST, premortem interventions, withdrawal of tracheal tube, duration of the no-touch period and WIT, and the possibility of *ex-vivo* lung perfusion (EVLP). The consensus document of the ISHLT covers essential aspects of the DCD donor procurement process, including NRP, *ex-vivo* evaluation, and declaration of circulatory death [39].

a) The location of WLST: Operating Room vs. Intensive Care Unit. The location of WLST has a critical impact on DCD organ recovery rate and post-transplant outcomes as, when performed in the ICUs, it lengthens WIT. Consequently, WLST is preferably performed in the operating room (OR) to minimize WIT. Alternatively, it can be done at the intensive care unit followed by transport of the donor to the OR and reintubation.
b) Premortem interventions: These include comfort therapy, pre-arrest heparinization, or premortem bronchoscopy. These practices vary among transplant centers due to ethical considerations. The administration of comfort therapy during WLST could indirectly affect the duration of the agonal period and this topic remains debatable. However, current evidence about the role of sedatives in accelerating death does not provide definitive results [40].
c) The allowed WIT: The interval from WLST to declaration of death is variable and ranges from 60 to 180 min. However, most groups accept 60 min of WIT.
d) Withdrawal of tracheal tube. Protection against aspiration by avoiding extubation and just stopping ventilation is preferred at our Institution. On the contrary, withdrawal of the tracheal tube followed by re-intubation and re-ventilation after declaration of death and the no-touch period can be performed.
d) Placement of nasogastric tube during WLST is routine practice to prevent aspiration of gastric contents and to facilitate dissection around the esophagus.
e) Time of re-ventilation: Re-ventilation is initiated after median sternotomy is performed. This time is important as the maneuver may be associated with autoresuscitation. Thus, many groups wait for at least 5 min after surgical incision, or up to 15 min after cardiac arrest, like in Australia [21].
f) The duration of the no-touch period: as stated before, most LT groups use no-touch periods that range from 2 to 5 min, although they may be as long as 20 min, such as in Italy.
g) The possibility of *ex-vivo* lung perfusion (EVLP): according to the ISHLT report published in 2015, EVLP was used in only 15% of DCD donors, reflecting that EVLP technology is not available in many LT groups. The majority of EVLP runs were reported by the Toronto group [19]. Excellent outcomes of LT from cDCD donors without EVLP have been reported [31]. On the contrary, the use of EVLP is strongly recommended by the ISHLT to evaluate uDCD donors.
h) Heparinization: In the DCD setting, heparin can be administered either pre-mortem or post-mortem. However, there are some ethical concerns, as pre-arrest heparinization is not allowed in all countries due to the possibility of accelerating death in the potential donor. Whereas Oto and others reported a 38% incidence of unexpected donor thromboembolism which was associated with increased rates of PGD after LT [41], other groups have found that delayed heparin administration after cardiac arrest or even not administering it at all are associated with good results. According to the ISHLT DCD Registry, pre-mortem heparin was given in 54% of DCD donors. Interestingly, this not correlated with adverse outcomes after LT [19]. **Table 3** shows some features of the existing legal framework and invasive procedures used in controlled DCD donation in Europe.

TABLE 3 | Available legal framework and invasive procedures used in controlled DCD donation [24].

Country	Ante-mortem medication	Ante-mortem cannulation	Location of WLST	No-touch period (min)
Austria	Yes	Yes but not practiced	OR	10
Belgium	Yes	Yes	OR	5
Czech Republic	No	No	ICU	5
France	Yes	Yes (only guidewires)	ICU	5
Ireland	No	No	OR	10
Italy	Yes	Yes (only guidewires)	ICU	20
Netherlands	No	No	ICU	5
Norway	Yes	Yes (only guidewires)	ICU	5
Spain	Yes	Yes	OR	5
Sweden	No	No	ICU	5
Switzerland	Yes	No	ICU	5
United Kingdom	No	No	ICU	5
USA	Yes	Yes	No standard practices	5
Australia	Variable	NRP not allowed	ICU	5

ICU, Intensive Care Unit; OR, Operating Room; WLST, Withdrawal of life-sustaining therapies.

JOINT THORACIC AND ABDOMINAL ORGAN PROCUREMENT PROM DCD DONORS

Combined thoracic and abdominal organ procurements from DCD donors are standard practice worldwide. Traditionally, standard organ procurement from DCD donors has involved simultaneous super rapid recovery (SSR) of lungs and abdominal grafts, by means of cold thoracic and abdominal perfusion. This technique continues to be the procedure of choice in some countries. Advantages of SSR from DCD donors are the reduced costs, availability, reproducibility, and the familiarity with the procedure [42]. Nevertheless, SSR of organs from DCD donors require expertise and surgical skills to minimize the risks of organ injury or even graft loss secondary to surgical accidents [43].

Ideally, WLST is performed in the operating room with the potential DCD donor prepped and draped in a sterile fashion and

the surgical instruments on the table, ready to be used and, therefore, shortening WIT. In addition, both the thoracic and abdominal teams are scrubbed, and all instruments required are prepared on the instrument table. A separate side table for the thoracic team is desirable, and pulmoplegia is prepared by adding 500 micrograms of Prostaglandin E1 (Alprostadil) to the first bag of Perfadex solution. If the legal framework allows for it, a single bolus of 500–100 Units of heparin sodium is administered. Alternatively, heparin can be added to flush solutions.

Normothermic Regional Perfusion (NRP)

Whereas the outcomes of LT from DCD donors using the technique of SRR are similar or even better than from DBD, as the negative factors associated with brain death are avoided, DCD livers face higher rates of primary non-function, early graft dysfunction, and biliary complications [44]. In addition, DCD kidneys show a higher incidence of delayed graft function compared to DBD [45]. The negative results of abdominal organ transplantation from DCD donors rely on the warm ischemic damage during the hypotensive phase after WLST and, thereafter, during cold ischemia prior to organ reperfusion in the recipient. Thus, normothermic regional perfusion using extracorporeal membrane oxygenation (ECMO) has gained increased interest in recent years [46].

Normothermic regional perfusion (NRP) consists of the use of extracorporeal membrane oxygenation (ECMO) to perfuse organs at normothermia after death declaration prior to organ recovery [47]. Perfusion can be limited to the abdominal cavity (A-NRP) or both the thorax and abdomen (TA-NRP). TA-NRP has facilitated heart procurement from DCD donors, reducing warm ischemic time and allowing for *in situ* evaluation of donor graft function, and its use has expanded in the United States and Australia [48, 49].

NRP restores the flow of oxygenated blood after cardiac arrest, to reverse warm ischemic injury of abdominal organs following the determination of death and before organ recovery. NRP enables assessment of organ function, as opposed to *in situ* cooling and rapid procurement [46]. Furthermore, the use of A-NRP is associated with a lower incidence of ischemic cholangiopathy, liver graft dysfunction, and delayed kidney graft function [44, 50]. Santander's group in Spain reported the largest single center study reporting on combined lung and liver procurement from DCD donors using normothermic abdominal perfusion. In this study, the authors included 60 lung transplants from cDCD and compared the results with 209 LT from DBD donors [51]. In Europe, NRP is applied for DCD organ procurement in France, Italy, Spain, the UK, Belgium, the Netherlands, Norway, and Switzerland [24]. In Spain NRP is used routinely, whereas it is mandated for liver procurement in France and Italy.

DCD Donor Cannulation

Cannulation of the donor for NRP can be performed either before or after WLST, depending on the existing national legal framework. In Spain, pre-mortem cannulation and heparinization are allowed to reduce WIT [52]. In the Spanish setting, appropriate consent is obtained from the next of kin for any pre-mortem interventions, including heparinization and femoral cannulation for NRP. Antemortem heparinization is allowed in Belgium, France, Norway, Spain, and Italy. Whereas antemortem cannulation is allowed only in Spain and Belgium, in other countries like France, Italy, and Norway antemortem vessel localization by guidewires can be performed. On the contrary, no antemortem interventions are allowed in the UK or the Netherlands [53].

Ante-Mortem Vessel Cannulation

Peripheral femoral vessel cannulation is the preferred method for ante-mortem NRP. In some countries, the femoral vessels are identified prior to WLST but cannulation is performed after the declaration of death [24]. Preparation, management of the potential cDCD donor, and surgical technique have been reported previously [47, 52]. The process starts by verifying that the left radial artery is already catheterized. The next step is cannulation of the femoral artery (15-Fr to 19-Fr) and vein (18-Fr to 21-Fr), either surgically or using the Seldinger technique, under proper sedation and analgesia. After guidewires are placed, 500–600 units/Kg of heparin are administered prior to vessel cannulation. The cannulas are de-aeriated and connected to the ECMO circuit, with the heater/cooler at 37°C but the pump off. Through the contralateral groin, an aortic occlusion balloon is placed and advanced until empty and reaching the supraceliac aorta, to ensure the thoracic aorta is adequately blocked during A-NRP to avoid the possibility of autoresuscitation [54]. Alternatively, the descending thoracic aorta can be directly occluded above the diaphragm by a vascular clamp immediately after median sternotomy, to ensure the heart and the brain are not perfused during A-NRP.

Two arterial lines (left radial artery and another from the femoral artery cannula) are monitored during A-NRP to ensure the thoracic aorta is appropriately blocked [52, 54]. For this purpose, just before WLST the aortic occlusion balloon is filled for 4 s and its position checked by X-ray or fluoroscopy, after which the balloon is immediately emptied. The tip of the catheter should be 6-7 cm above the xiphoidal process, between the left subclavian artery and the celiac trunk. The thoracic surgeon performs a fiberoptic bronchoscopy as in DBD donors, if premortem interventions are allowed. Otherwise, this can be performed after the declaration of death, while one of the thoracic surgeons performs median sternotomy.

When the preparation of the potential DCD donor has finished, the surgical field is prepped, and the team proceeds with WLST including donor extubation, according to local protocols. Some groups prefer to leave the tracheal tube on site, instead of removing it, to reduce the risk of aspiration. Separate trolleys for thoracic and abdominal teams are set up with the necessary instruments and flush solutions. The surgical teams are scrubbed in an adjacent OR. After 5 min of the no-touch period, death is certified by the permanent cessation of circulation (pulseless arterial line, continuous apnea, unresponsiveness). At this point, the aortic occlusion balloon is filled, and ECMO is started, and flow is progressively increased until reaching 2–2.4 L/min. At this time, organ procurement can proceed. The thoracic and abdominal teams enter the OR and perform median

sternotomy and laparotomy, respectively. The donor is then re-intubated and mechanical ventilation resumed at FiO_2 100%, PEEP of 5 cm H_2O and a tidal volume of 6–8 mL/kg. For lung perfusion, the pericardium is opened, and the main PA cannulated. 50–60 mL/kg of Perfadex primed with Prostaglandin E1 is delivered and the left atrial appendage is opened for drainage. Meanwhile, both pleurae are opened widely and cold Perfadex or cold saline are used for moderate topical cooling of both lungs. Macroscopic inspection of the lung grafts is performed to anticipate suitability (**Figure 2**).

To prevent ascending flow secondary to mispositioning or displacement of the aortic occlusive balloon, the descending aorta above the balloon can be isolated to allow the placement of an aortic clamp. When the pulmonary flush is finished, the ascending aorta is isolated and transected preferably using staplers rather than placing ligatures, as well as both superior and inferior vena cava. This technique shortens lung procurement, minimizing WIT, more so than placing ligatures or vascular clamps. Moreover, meticulous hemostasis is better achieved, which is of paramount importance for adequate functioning of the A-NRP. Before transecting the inferior vein cava, the position of the return cannula within the right atrium is checked, and the tip is withdrawn below the diaphragm. About 1–1.5 L of fluid are administered intravenously to avoid a decrease in blood flow to the ECMO circuit. A minimum of 30 min and a maximum of 4 h are stipulated for NRP, but 90–120 min are routine practice nowadays [52, 55]. Nevertheless, the optimal duration of NRP remains debatable.

Once lung cold preservation has finished, the heart-lung bloc is removed. The heart is removed first in a standard fashion, followed by the double-lung graft. Care should be taken when dissecting the azygous vein, which is transected using staplers. Once the heart-lung bloc is removed, the thoracic cavity is checked for bleeding. Once on the back table, retrograde flush perfusion through each pulmonary vein is made until the effluent runs clear from the PA as with DBD donors. Finally, the lung bloc is triple-bagged stored in cold Perfadex and transported to the transplant center.

Target parameters of NRP are flow of 2–2.4 L/min, pH 7.35–7.45, temperature of 37°C, and hematocrit >25%. Blood samples from the ECMO circuit are obtained immediately after starting A-NRP and every 30 min, monitoring liver enzymes, lactate levels, urea, electrolytes, and blood gases. For liver graft acceptance, alanine transaminase or aspartate transaminase levels at 30 or 60 min after the initiation of NRP should be <4 times the normal values, together with normal macroscopic appearance and declining lactate levels.

Post-Mortem Vessel Cannulation

Post-mortem cannulation can be performed either in the abdomen or in the common femoral vessels.

The technique of post-mortem cannulation in NRP has been reported previously [56, 57]. After death declaration, a rapid laparotomy is performed with aortic and inferior vena cava cannulation (or iliac vein and artery). The descending thoracic aorta is occluded either by a vascular clamp or by an occluding intra-aortic balloon. Of note, pre-mortem heparin administration is prohibited in the UK. In case of combined lung and liver procurement, the thoracic team performs a rapid median sternotomy for lung harvesting, while the abdominal team performs laparotomy and vessel cannulation for A-NRP [58]. In this case, fiberoptic bronchoscopy is performed once death has been declared, while one thoracic surgeon opens the thorax. Post-mortem cannulation for A-NRP increases functional WIT by 10–23 min [57].

Preparation and Priming of ECMO

A basic ECMO circuit for NRP consists of a centrifugal pump, a membrane oxygenator, the heat exchanger, a central unit controller, tubing and cannulas. For priming, 1.5 L of crystalloid solution and 30 mg of 1% heparin sodium are added to the perfusate to maintain activated clotting time between 180 and 200 s. In addition, bicarbonate is usually needed to maintain pH between 7.35 and 7.45, as donor acidosis is frequent. Packed red blood cells are also added if hemoglobin levels fall below 8 g/dL.

Problems and Solutions During A-NRP

Main problems during A-NRP for organ procurement from DCD donors are reaching very low flows while on NRP, which can lead to organ loss, and the possibility of restoration of perfusion to the heart or the brain, the so-called autoresuscitation of the donor. Sudden loss of venous return is usually observed during lung procurement, secondary to bleeding or due to the collapse of the inferior vena cava. Thus, meticulous hemostasis and ligation of the azygous vein is of paramount importance. Moreover, care should be taken while dissecting the inferior vein cava to avoid compromising venous return.

Autoresuscitation

One of the major concerns is the possibility of autoresuscitation of the donor during NRP. A correctly placed occlusive aortic balloon during NRP should translate into an absence of pulsatile wave form from the left radial artery and into a continuous, non-pulsatile pressure from the femoral arterial line. On occasion, a non-pulsatile wave form from the left radial artery can be observed after A-NRP has started, due to incorrect positioning or inadequate filling of the aortic balloon. In this case, ECMO flow is immediately stopped, the position of the aortic balloon is checked, and an additional 5 min of the no-touch period are observed before A-NRP is re-started [54].

Thoraco-Abdominal NRP

In situ thoraco-abdominal regional perfusion (TA-NRP) has emerged recently as an alternative method to direct heart procurement (DP) with *ex-situ* machine perfusion. The technique was used for the first time in the UK [59] to allow heart transplantation from DCD donors and has increased dramatically in the USA [60]. Following declaration of death and 5 min of the no-touch period, a rapid median sternotomy is performed, the pericardium is opened, and the brachiocephalic trunk, left carotid artery, and left subclavian artery are either clamped or divided to avoid brain perfusion. TA-NRP is initiated once the right atrium and ascending aorta have been cannulated and the intra-aortic occlusive balloon deflated. Either standard

cardiopulmonary bypass (CPB) or veno-arterial ECMO can be used for extracorporeal perfusion. TA-NRP is gradually weaned after 30–60 min once the heart returns to sinus rhythm. Validity of the heart is clinically assessed, as it should be able to perfuse thoracic and abdominal organs. Swan-Ganz catheter, trans-esophageal echocardiography, and visual inspection and palpation are used for that purpose.

Simultaneously, the donor is re-intubated and mechanical ventilation resumed. Targeted parameters during TA-NRP are medium systolic arterial pressure >50 mmHg, flows >2.5 L/min/m^2, and normothermia. According to the Papworth group, median duration of TA-NRP is 45 min (27–190 min) and the donor heart is routinely placed on the OCS Heart Device for transportation [61].

The use of TA-NRP raises some ethical issues, as the permanence principle of death might be violated if the brain is perfused while on NRP. Leaving the distal ends of the aortic vessels vented to atmosphere or cannulating each of them and connecting them to the venous return, as well as inserting a cannula in the ascending aorta to ensure absence of flow to the brain [62], are some of the suggested measures in this regard. Measurement of mean intracranial pressures at the circle of Willis in DCD donors during TA-NRP has clearly demonstrated that these specific measures are effective in avoiding perfusion to the brain [63].

OUTCOMES

NRP in DCD is a technically demanding procedure with more complexity and costs than DBD. Early experience in LT from cDCD using abdominal normothermic perfusion for combined lung and liver procurement came from single case reports by the Newcastle and Birmingham groups in the UK [58]. Later, Miñambres and others from Santander proposed a modified technique including pre-mortem interventions, as the legal framework exists in Spain [64]. In Spain, abdominal *in-situ* NRP with super rapid recovery of the lungs has become the standard technique of DCD organ procurement [65]. Looking at the last report of the Spanish Transplant Organization, 36% of LT performed in 2022 had cDCD donors [66]. The Puerta de Hierro group in Madrid reported for the first time the results of LT from DCD donors under A-NRP compared to classic SRR [67]. The authors did not find any difference in the incidence of PGD, hospital mortality, or one-year survival. Moreover, the complexity of the procedure did not impact negatively on the procurement rate of abdominal organs. Recently, a multicenter study including all LT centers in Spain has analyzed the outcomes of simultaneous lung and liver recovery using A-NRP compared to those of contemporary DBD donors [68]. The rates of grade 3 PDG at 72 h and lung transplant survival at 1 and 3 years were similar between recipients from DCD and DBD donors. Moreover, the incidence of liver graft dysfunction, ischemic cholangiopathy, or liver graft survival did not differ between DBD and DCD donors.

Organ Recovery

Combined lung and liver procurement from cDCD donors using regional perfusion is more complex due to the use of ECMO and dual temperature (cold in the thorax, normothermia in the abdomen) which may have deleterious effects on the grafts.

The Santander group has reported fantastically high recovery rates for both lungs (97.4%) and livers (84.2%) [64]. The multicenter Spanish experience reported recently showed lower recovery rates for livers (78% compared to 85.5% in DBD), but similar percentages for the lungs (73.9% vs. 75.8%) [68]. A systematic review and meta-analysis or regional perfusion in DCD solid organ transplantation has reported 25%–100% organ utilization rates for the liver and 0%–60% for the lungs [69]. A French multicenter study published recently has reported a DCD lung transplantation rate of 76% [70]. From 100 controlled DCD lung grafts offered, 10 were not retrieved due to prolonged agonal phases, failure of cannulation for NRP, or poor *in-situ* evaluation. The remaining 90 were subjected to *ex-vivo* lung perfusion (EVLP) and finally 76 were accepted for transplantation. In the same period, 412 livers from DCD donors were transplanted. Combined lung and liver procurement was performed in 59 cases, and the remaining were isolated liver procurements. In combined procurements, no difference in overall survival for livers and kidneys were observed, compared to isolated abdominal organ procurement.

Graft Quality and Organ Dysfunction

No differences on PaO2/FiO2 at ICU have been observed compared to DBD donors. What is more, A-NRP seems to decrease the rate of grade 1 PGD (4.8% vs. 7.4%) and grade 2 PGD (4.8% vs. 9.6%). On the contrary, a higher incidence of grade 3 PGD has been reported (19% vs. 7.4%) [64]. In contrast, the Spanish multicenter study has not found a higher incidence of grade 3 PGD [68]. Similarly, a single-center retrospective study of DCD lung transplantation from 2013 to 2019 comparing A-NRP with SRR has found a 21% incidence of PGD in both groups (P-1.0) [67].

Mortality and Survival

In most DCD LT series, 30-day mortality and midterm survival rates are similar between cDCD and DBD LT. Identical results have been reported when A-NRP is used. Campo-Cañaveral De la Cruz and others from Spain have reported a 30-day mortality rate of 5.6% in DCD and 6.3% in DBD [68]. Recipient survival at 1 and 3 years for cDCD LT was 79.9% and 66.4% vs. 82% and 69.7% in DBD respectively (p = 0.403) in this Spanish cohort. Two-year survival was 84% in cDCD compared to 90% in DBD according to the Santander experience [64].

SUMMARY

Scarcity of suitable lung donors and the low usage rates of donor lungs remain major limitations to increasing the number of LT performed. Traditionally, the main source of lung allografts has been DBD donors. However, a resurgence in DCD LT has occurred to alleviate lung donor shortage. DCD LT has increased exponentially since the mid-90 s, with comparable results to DBD LT. The ISHLT has provided a consensus document for heart and lung procurement from DBD and

DCD donors, standardizing the procedure, terminology, time-points, and intervals. The Maastricht classification of DCD donors has been subsequently updated to include the location of cardiac arrest and category V, consisting of euthanasia.

Recent expansion of regional normothermic perfusion for abdominal organ recovery from DCD is a growing approach in increasing the number of LT performed. Although adding complexity to the procedure of multiorgan procurement, A-NRP improves abdominal organ recovery rates and organ function.

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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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REVIEW
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Controlled Hypothermic Storage for Lung Preservation: Leaving the Ice Age Behind

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Controlled hypothermic storage (CHS) is a recent advance in lung transplantation (LTx) allowing preservation at temperatures higher than those achieved with traditional ice storage. The mechanisms explaining the benefits of CHS compared to conventional static ice storage (SIS) remain unclear and clinical data on safety and feasibility of lung CHS are limited. Therefore, we aimed to provide a focus review on animal experiments, molecular mechanisms, CHS devices, current clinical experience, and potential future benefits of CHS. Rabbit, canine and porcine experiments showed superior lung physiology after prolonged storage at 10°C vs. ≤4°C. In recent molecular analyses of lung CHS, better protection of mitochondrial health and higher levels of antioxidative metabolites were observed. The acquired insights into the underlying mechanisms and development of CHS devices allowed clinical application and research using CHS for lung preservation. The initial findings are promising; however, further data collection and analysis are required to draw more robust conclusions. Extended lung preservation with CHS may provide benefits to both recipients and healthcare personnel. Reduced time pressure between procurement and transplantation introduces flexibility allowing better decision-making and overnight bridging by delaying transplantation to daytime without compromising outcome.

Keywords: controlled hypothermic storage, lung preservation, lung transplantation, mitochondrial health, static ice storage

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INTRODUCTION

Preservation of organs remains a key area for research in transplantation medicine. Several strategies to reduce organ injury during preservation have been explored. Over the past decades, organ-specific preservation solutions and *ex-vivo* perfusion platforms have been developed [1–4]. Currently, the standard of care for preservation remains static ice storage (SIS). Traditionally, temperature in this setting has been estimated to be around 4°C [1, 5]. However, actual temperature with SIS might be lower and result in freezing injury due to the organ contact with ice [6]. On a cellular level, the low temperatures reached with SIS decrease metabolic demand, but has also been associated with progressive mitochondrial dysfunction [7]. Interestingly, lungs are privileged because the oxygen repleted air in the expanded alveoli continues to support aerobic metabolism during preservation [8].

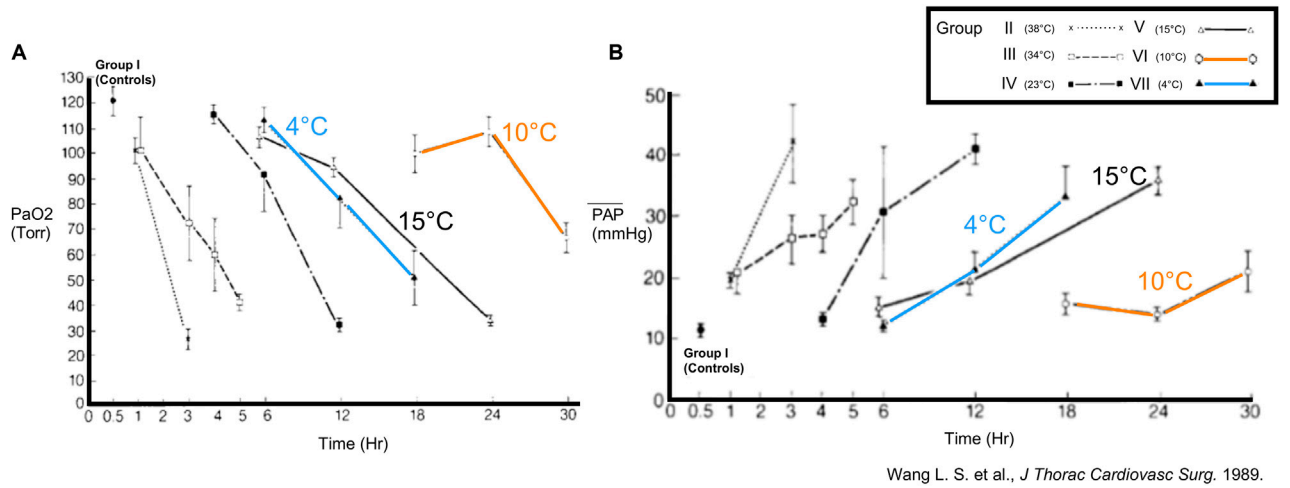


FIGURE 1 | Findings in a rabbit model of *ex-vivo* lung preservation and reperfusion (Toronto, 1989). Physiology in lungs inflated with room air was assessed after different intervals of ischemia and preservation temperatures. Groups with 4°C, 10°C and 15°C are highlighted. **(A)** Partial arterial oxygen pressure (PaO_2) and **(B)** mean pulmonary artery pressure (mPAP) were measured after 10 min of reperfusion. Reprinted from The Journal of Thoracic and Cardiovascular Surgery, Vol. 93(3), Liang-Shun Wang, Koichi Yoshikawa, Shinichiro Miyoshi, Kembu Nakamoto, Chia-Ming Hsieh, Fumio Yamazaki, Paulo F. Guerreiro Cardoso, Hans-Joachim Schaefers G, Julio Brito, Shafique H. Keshavjee, Alexander Patterson, Joel D. Cooper, The effect of ischemic time and temperature on lung preservation in a simple ex vivo rabbit model used for functional assessment, pp. 333-342, Copyright (1989), with permission from Elsevier.

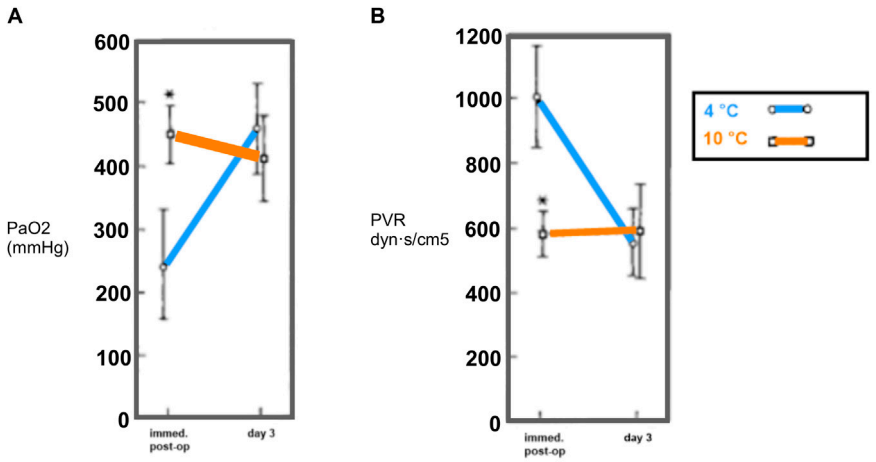
Recently, it has been shown that controlled hypothermic storage (CHS) of lungs at 10°C better maintains mitochondrial health during preservation [9, 10]. Therefore, the optimal temperature for hypothermic lung preservation might be found by balancing aerobic metabolism and oxygen consumption while preventing cellular exhaustion [7, 11, 12]. With SIS, cold ischemia time (CIT) for lungs is advised to be kept as short as possible to minimize cellular injury [1, 13]. The limited preservation time forces teams to perform transplant procedures at night, and restraints long-distance procurement. Overnight transplantation is associated with worse post-transplant outcomes, possibly due to limited resources and expertise, and sleep-deprived personnel [14, 15]. Lung preservation with CHS at a higher temperature has the potential to safely extend preservation time and thereby overcome logistical challenges [9, 16]. In this article we aim to provide a focus review of preclinical research, the cellular mechanisms, and the clinical experience explaining the benefits of CHS in the setting of lung transplantation (LTx).

PRECLINICAL DATA ON CONTROLLED HYPOTHERMIC LUNG STORAGE

Over the past decades, concerns regarding optimal temperature for lung preservation have been raised due to cold-induced lung injury provoked by SIS. In 1989, Wang and colleagues (Toronto, Canada) developed an *ex-vivo* rabbit lung preservation model with the aim to accurately assess post-ischemic lung function [17]. For lungs with different CIT and preservation temperatures (4°C, 10°C, 15°C, 23°C, 34°C, 38°C), they assessed post-ischemic functional variables like partial pressure of arterial oxygen (PaO_2), pulmonary artery pressure (PAP) and oxygen uptake (**Figure 1**). Of note, the 4°C group was performed in a temperature-controlled room, and not by immediate ice contact. They hypothesized that lower temperature would improve post-ischemic lung function. However, after 12 h CIT followed by 10 min reperfusion, lungs preserved at 10°C were functionally superior to those preserved at 4°C. The 10°C group tolerated CIT periods of 18–24 h with PaO_2 and PAP similar to 30 min preservation at 4°C. The extension of CIT to 30 h deteriorated gas exchange (**Figure 1**). These observations raised questions regarding optimal lung preservation temperature and led to a revision of the dogma advocating cold storage of lungs at 4°C.

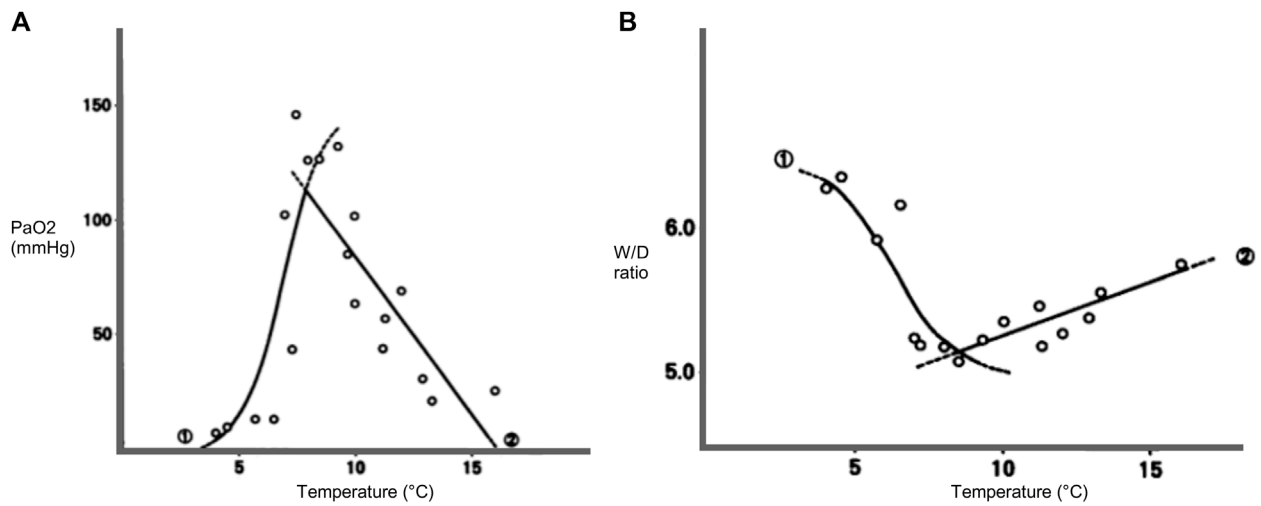
In 1992, Date et al. (St. Louis, Missouri) set up a canine model of orthotopic left-LTx to compare pulmonary function in lungs filled with room air after 18 h of preservation at 10°C vs. 4°C [18]. Furthermore, they conducted a pulmonary metabolic study evaluating adenosine triphosphate (ATP) levels. Functionally, gas exchange and perfusion were superior in the 10°C group with higher PaO_2 and lower pulmonary vascular resistance (PVR) immediately post-LTx. No important difference in PaO_2 or PVR was observed on day 3 (**Figure 2**). In addition, ATP levels remained stable during the 18 h preservation at 10°C and 4°C. Impaired lung physiology at 4°C preservation could hence not be explained by cellular ATP depletion. Therefore, it was suggested that sodium/potassium (Na+/K+) ATPase might be impaired at 4°C, leading to intracellular Na + accumulation and cellular swelling. The St. Louis researchers (1992) concluded that optimal lung preservation temperature is near 10°C [18].

Also in 1992, Nakamoto et al. (Kagawa, Japan) preserved rabbit lungs for 18 h at predetermined temperatures ranging from 4°C to 15°C [19]. Physiology was assessed with PAP, PaO_2 and wet-to-dry (W/D) ratio as a measure for edema. Pulmonary vasculature was assessed with perfusion of blood containing



Date H. et al., *J Thorac Cardiovasc Surg.* 1992.

FIGURE 2 | Findings in a canine model of orthotopic left-lung transplantation (St. Louis, 1992). The left lung was inflated with room air and preserved at 10°C or 4°C for 18 h. During occlusion of the contralateral pulmonary artery, partial arterial oxygen pressure (PaO_2) **(A)** and pulmonary vascular resistance (PVR) **(B)** were measured immediately post-LTx (n = 6/group) and at day 3 post-LTx (n = 4/group). Mean values are shown and * indicates $p < 0.05$. Reprinted from The Journal of Thoracic and Cardiovascular Surgery , Vol. 103(4), Hiroshi Date, Oriane Lima, Akihide Matsumura, HiroharuTsuji, D. André d'Avignon, Joel D. Cooper, In a canine model, lung preservation at 10° C is superior to that at 4° C. A comparison of two preservation temperatures on lung function and on adenosine triphosphate level measured by phosphorus 31-nuclearmagnetic resonance, pp. 773-780, Copyright (1992), with permission from Elsevier.



Nakamoto K. et al., *Ann Thorac Surg.* 1992.

FIGURE 3 | Findings in a rabbit model of lung preservation and *ex-vivo* reperfusion. Regression lines and curves during high-flow perfusion with ventilation and lung preservation temperature. Every dot represents a measurement. **(A)** Partial arterial oxygen pressure (PaO_2) (n = 19) and **(B)** wet-to-dry weight (W/D) ratio (n = 16) of lungs inflated with room air and preserved for 18 h in function of different preservation temperatures. Mathematically projected significant regression equations 1 and 2 intersect at 7.9°C for PaO_2 and at 8.4°C for W/D ratio. Reprinted from The Annals of Thoracic Surgery, Vol. 53(1), Kembu Nakamoto, Masazumi Maeda, KiyohideTaniguchi, Noriyuki Tsubota, Yasunaru Kawashima, A study on optimal temperature for isolated lung preservation, pp. 101-108, Copyright (1992), with permission from Elsevier.

indocyanine green (ICG) and histopathological evaluation was performed using colloidal carbon black perfusion.

Based on mathematical regressions of W/D and PaO_2 (**Figure 3**), optimal temperature for lung preservation was found between 8°C and 9°C. At 8°C, superior physiology was observed, and microvasculature was intact in contrast to other groups. Each fitted sigmoid curve for W/D and PaO_2 showed an inflection point at 6°C–7°C, which seems to be a critical temperature for vascular obstruction. After 4°C preservation, carbon colloid particles were absent in capillaries and massive alveolar hemorrhage with

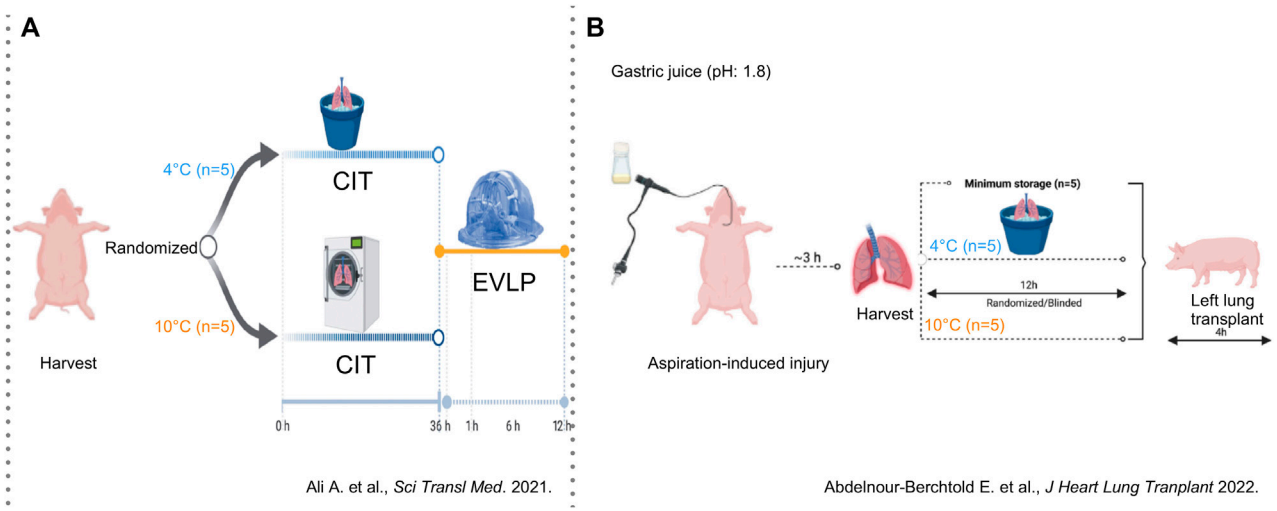


FIGURE 4 | Findings in a porcine model of lung preservation followed by *ex-vivo* perfusion (Toronto, 2021) or orthotopic left lung transplantation (Toronto, 2022) **(A)** Lungs were inflated with inspired oxygen (FiO_2) of 50% and preserved for 36 h in a cooler at 4°C or in a cooler at 10°C (n = 5/group) followed by 12 h of normothermic *ex-vivo* lung perfusion (EVLP). Lung physiology and molecular patterns in lung tissue and EVLP were assessed. From Aadil Ali et al., Static lung storage at 10°C maintains mitochondrial health and preserves donor organ function. Sci. Transl. Med.13,eabf7601(2021). DOI:10.1126/scitranslmed.abf7601. Reprinted with permission from AAAS. **(B)** Lungs were injured with instillation of gastric juice and procured 3 h later with 50% FiO2. Preservation was done for 1 h (n = 5) or for 12 h on ice (n = 5) or at 10°C in a cooler (n = 5). Lung graft physiology and molecular patterns of injury were assessed after orthotopic left lung transplantation followed by 4 h of reperfusion. Reprinted from The Journal of Heart and Lung Transplantation, Vol. 41(12), Etienne Abdelnour-Berchtold, Aadil Ali, CristinaBaciu, Erika L. Beroncal, Aizhou Wang, OliviaHough, Mitsuaki Kawashima, Manyin Chen, YuZhang, Mingyao Liu, Tom Waddell, Ana C. Andreazza, ShafKeshavjee, Marcelo Cypel, Evaluation of 10°C as the optimal storage temperature for aspiration-injured donor lungs in a large animal transplant model, pp. 1679-1688, Copyright (2022), with permission from Elsevier.

arteriolar obstruction was seen, suggesting impaired physiology was related to vascular obstruction. However, at 15°C, carbon colloid particles were present, and centrilobular alveolar hemorrhage was observed, suggesting deterioration at 15°C was due to increased injury to the alveolocapillary membrane rather than vascular obstruction [19].

Other extended preservation experiments were performed in canine left LTx models by Keshavjee et al. (Toronto, 1989), Mayer et al. (Toronto, 1991) and a porcine left LTx model by Steen et al. (Lund, 1993) [20–23]. However, further translation of CHS to clinical practice was considered a major challenge due to lack of insights into the underlying mechanisms and logistical demands.

Three decades later, in 2021, the concept of 10°C preservation with the intention of better preserving lung physiology and metabolism re-emerged in Toronto where Ali, Cypel and colleagues developed a porcine model of lung preservation followed by *ex-vivo* lung perfusion (EVLP) (**Figure 4A**). Pig lungs were randomized to storage at 4°C (walk-in cooler) or 10°C (thermoelectric cooler) for 36 h followed by 12 h normothermic EVLP [9].

CHS at 10°C resulted in higher compliance, better oxygenation, and less edema. Furthermore, CHS was associated with upregulated expression of cytoprotective anti-oxidative metabolites (itaconate, glutamine, N-acetyl glutamine) as well as decreased perfusate protein levels of pro-inflammatory cytokines and cell-free mitochondrial DNA (mtDNA) (**Figure 5**) [9].

In a porcine model of orthotopic left LTx, Abdelnour-Berchtold et al. (Toronto, 2022) studied potential benefits of 10°C storage in marginal lungs injured by 5 mL gastric juice (**Figure 4B**) [10]. Three hours after instillation, lungs were procured and randomized to 10°C or ice for 12 h followed by LTx. As control group, five injured lungs were placed on ice for 1 h followed by LTx.

Four hours post-Tx, 10°C preservation resulted in superior selective left-lung PaO_2, compliance, and histopathological evidence of reduced acute lung injury. Based on metabolomic analysis, they found no metabolic activity for SIS. However, 10°C preservation showed active metabolism and higher levels of anti-oxidative metabolites (glutathione, ascorbate). Tissue levels of interleukin-1β were significantly lower for 10°C preservation. Compared to controls, free mtDNA was expressed less at 10°C.

These findings suggest that intentional delay of LTx by using CHS might trigger cell protective mechanisms and result in better post-LTx outcome.

ICE IS NOT 4°C

The International Society for Heart and Lung Transplantation (ISHLT) consensus statement on organ preservation advises to avoid contact of organs with ice to limit cold-induced cellular injury ([6]). In transplantation, a misconception exists regarding temperatures reached by SIS, wherein it is commonly assumed that organs are maintained at 4°C. However, no temperature measurements of donor lungs during SIS were available. This misconception of 4°C might

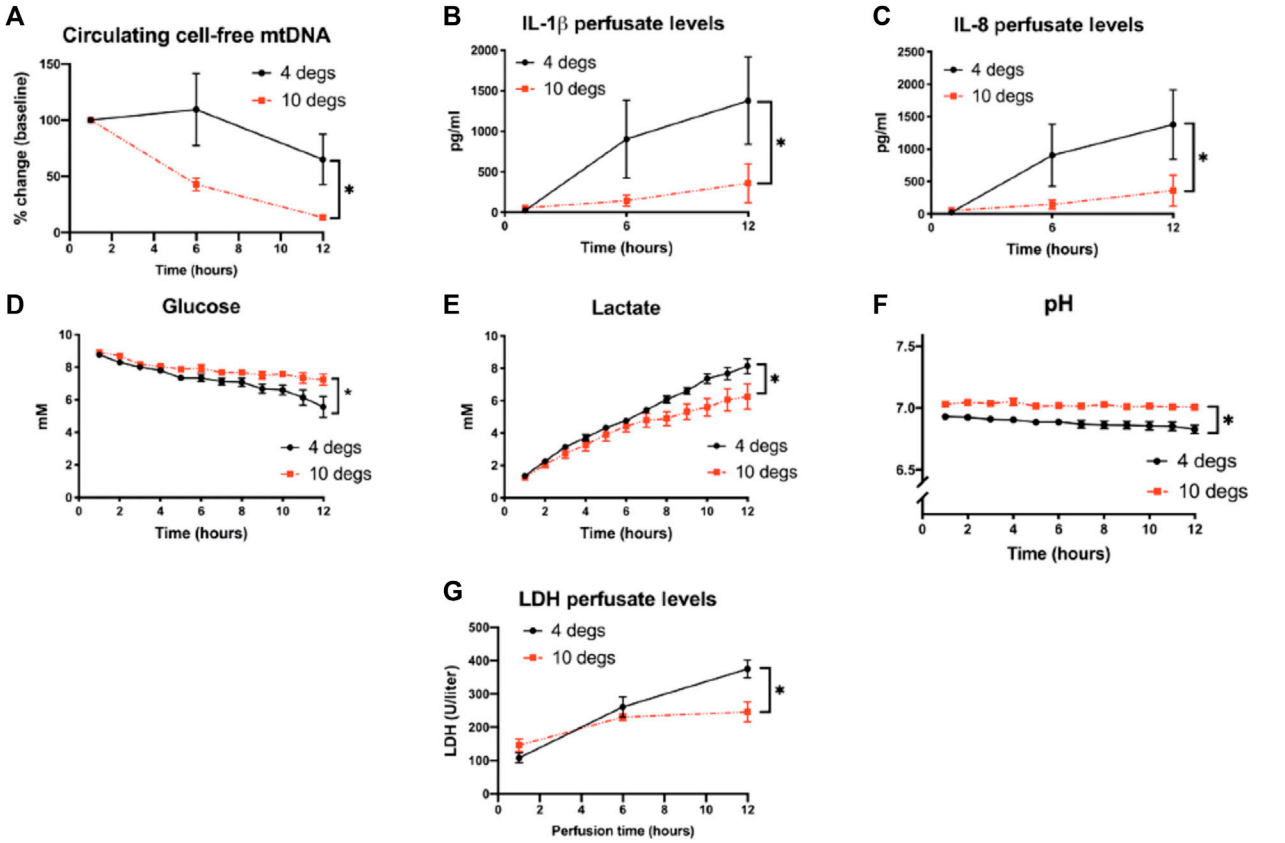


FIGURE 5 | Assessment of mitochondrial health between pig lungs stored at 10°C versus 4°C. Pig lungs were filled with 50% inspired oxygen (FiO_2) and preserved at 10°C vs. 4°C for 36 h. **(A)** Circulating cell-free mitochondrial DNA (mtDNA) extracted from normothermic *ex vivo* lung perfusion (EVLP) perfusate and quantified using qPCR during lung perfusion. Fold changes from baseline concentrations (1-h perfusion). **(B–G)** Perfusate **(B)** Interleukin 1β (IL-1β) **(C)** IL-8 levels, **(D)** glucose, **(E)** lactate and **(F)** pH. **(G)** LDH activity within EVLP perfusate, a measure of cellular necrosis. *$p < 0.05$, two-way ANOVA; data expressed as means ± SEM. From Aadil Ali et al., Static lung storage at 10°C maintains mitochondrial health and preserves donor organ function. Sci. Transl. Med.13, eabf7601(2021). DOI:10.1126/scitranslmed.abf7601. Reprinted with permission from AAAS.

refer to the temperature at which water reaches its maximum density in a soluble state. The actual freezing temperature of water at atmospheric pressure (1013 hPa) is 0°C, at which water is present in the form of solid ice (**Figure 6**). Interesting to note is that slushed ice used in organ preservation is derived from 0,9%NaCl physiologic saline with a freezing point below 0°C, approximating −0,59°C. Furthermore, a saline cooling model by Robicsek et al. noted temperature drops in clinical setting to −7,1°C and osmolality differences between the center and periphery of the solid ice [25].

In the earlier described canine LTx model, temperature dropped below 1°C after 2 h of SIS [18]. In a recent preliminary study of porcine lung preservation thermodynamics, Patel et al. showed that preservation and tissue temperature reached around 0°C after 3 h of SIS (**Figure 7**) [26].

In a clinical study at our center (Leuven, Belgium) donor lung temperatures were measured after preservation with SIS on ice [27]. Median parenchymal surface temperature measured with a thermal camera (**Figure 8**) and core temperature measured with a probe (3 mm) wedged in a lower lobe bronchus was close to 0°C with SIS.

COLD-INDUCED CELLULAR INJURY AND THE MECHANISMS BY WHICH CONTROLLED HYPOTHERMIC STORAGE PROTECTS CELLULAR VIABILITY

Static Ice Storage Causes Cold-Induced Cellular Freezing Injury

Cellular structure and metabolic function are altered at low temperatures (**Figure 9**). Cold-induced protein denaturation is caused by changes in hydrophobic amino-acid interactions at near-freezing temperatures and are responsible for loss of stability and misfolding of globular structural proteins [28]. In tissues exposed to temperatures around 0°C, formation of ice crystals causes electrolyte and

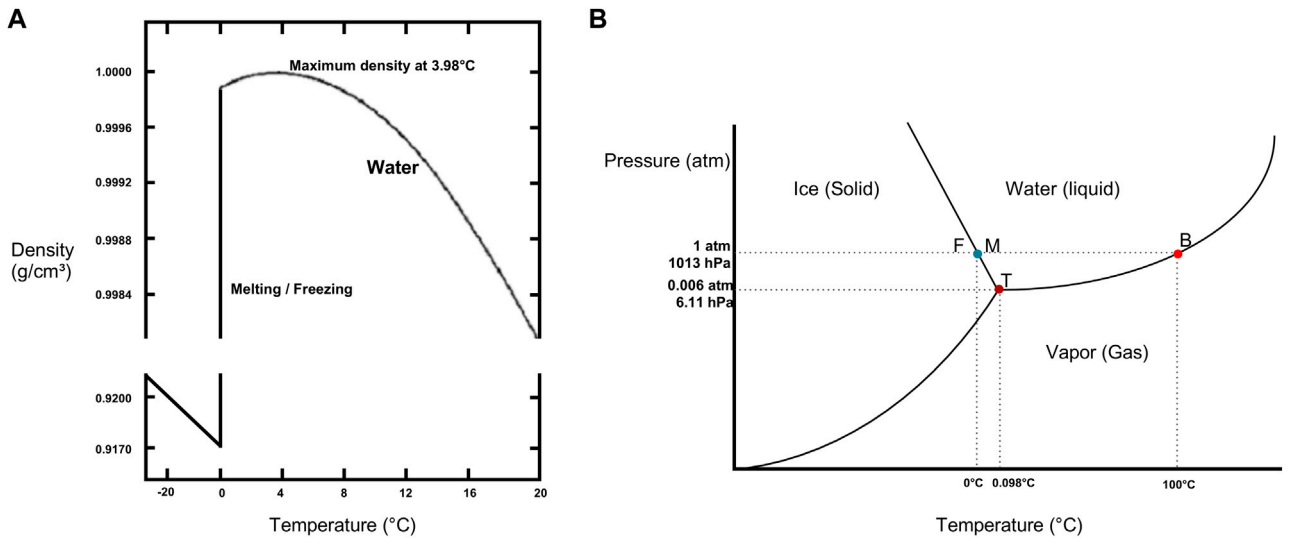


FIGURE 6 | Physical properties of water **(A)** Density of water/ice as a function of temperature. Note maximum density of water at 4°C and freezing (F) or melting (M) point of water at 0°C. Data from the CRC Handbook of Chemistry and Physics [24]. **(B)** Phase diagram of water illustrating triple point (T), boiling point (B) and freezing (F) or melting (M) point in function of pressure (P) and temperature. For 1,013 hPa corresponding to the atmospheric pressure (atm), freezing point of water is at 0°C and boiling point of water is at 100°C. Data obtained from CRC Handbook of Chemistry and Physics [24].

osmotic imbalances leading to mitochondrial swelling and dysfunction [29].

Cellular survival depends on ATP metabolism and the main source for ATP is aerobic metabolism in the mitochondria. Furthermore, mitochondrial injury and subsequent cell death results in the release of mtDNA which triggers inflammatory pathways [9, 30]. Mitochondrial dysfunction is also associated with increased levels of reactive oxygen species (ROS) which further propagate mitochondrial disintegration and oxidative stress [31]. Taken together, safeguarding mitochondrial function is essential to maintain organ quality during preservation.

The Role of the Na+/K + ATPase During Static Ice Storage and Controlled Hypothermic Storage

The Na+/K + ATPase maintains cell membrane potential. Dysfunction of Na+/K + ATPases results in extramitochondrial accumulation of Na+ and Ca+2 followed

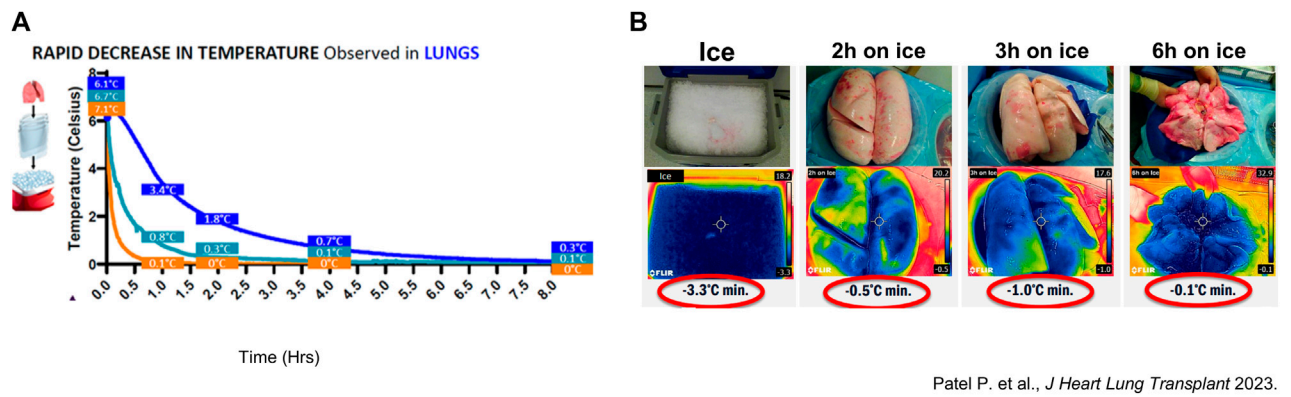


FIGURE 7 | Thermodynamic assessment of porcine and clinical lung temperature during static ice storage **(A)** Average temperature of porcine lungs (n = 3) preserved on ice (blue). Measurements were performed with a probe in the left lung lower lobe. Another probe measured the preservation solution (Perfadex) in contact with the lungs (cyan) and saline solution (n = 3) (orange). Temperatures were measured every 30 s. **(B)** Thermal images of ice used for standard static ice storage (SIS), and surface temperature of clinical lungs preserved for 2-, 3- and 6-hours.

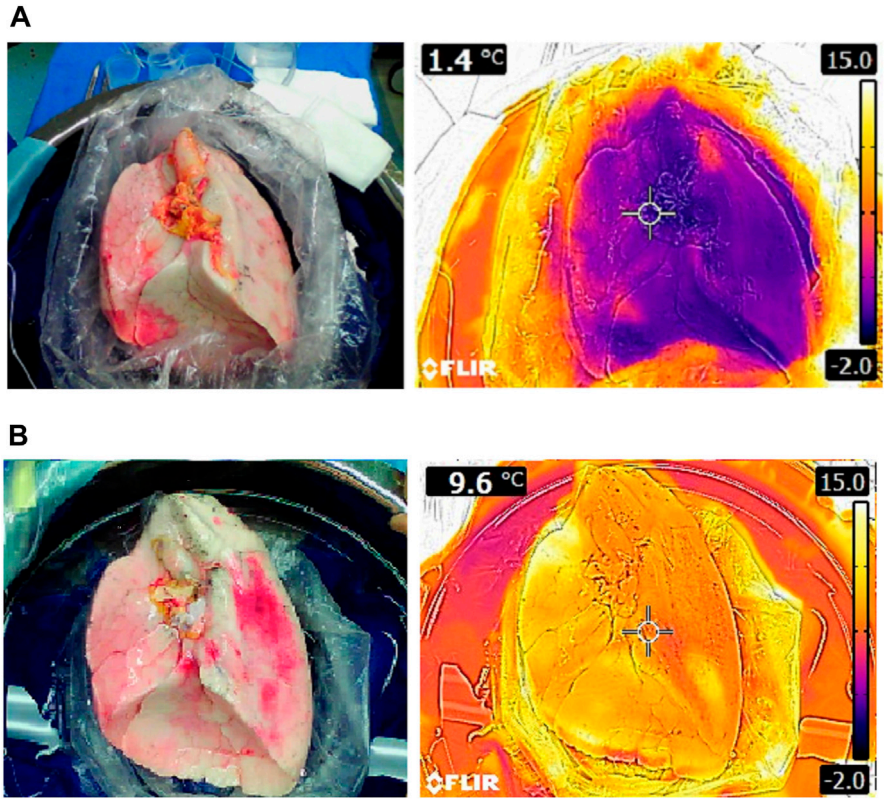


FIGURE 8 | Thermal images of left lungs preserved with static ice storage versus controlled hypothermic storage in clinical lung transplantation: **(A)** Right lung after 228 min of SIS (ice cooler) and **(B)** right lung after 276 min of CHS (LUNGguard).

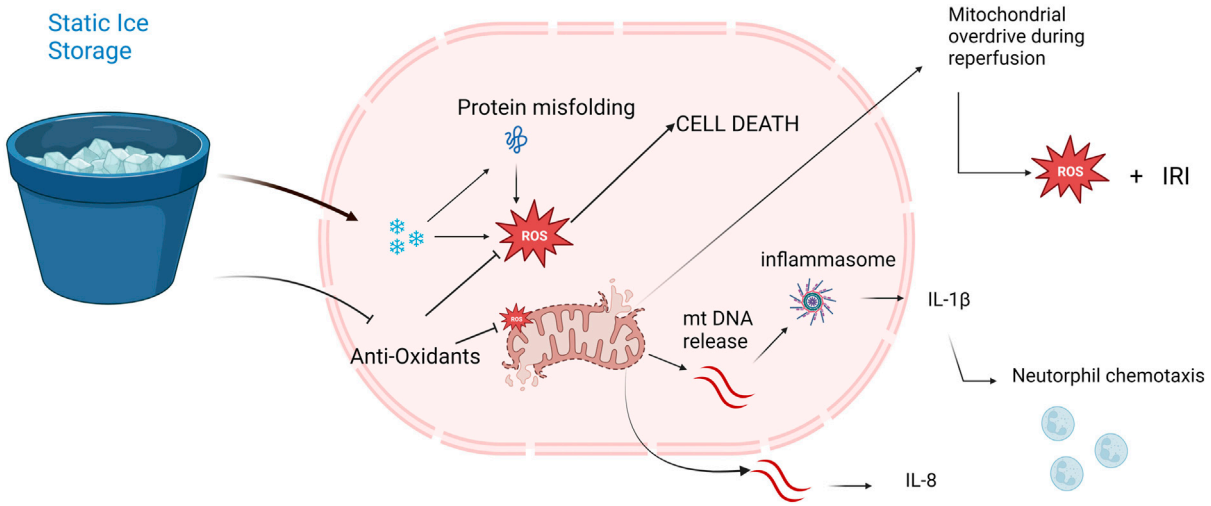


FIGURE 9 | Potential mechanisms of freezing injury and mitochondrial dysfunction provoked by static ice storage. Freezing injury is mediated via formation of miniscule ice crystals disturbing electrolyte-and homeostatic balances. Near freezing temperatures destabilize hydrogen bonds resulting in misfolding of globular proteins. Halted metabolism decreases the levels of anti-oxidative metabolites like glutathione and ascorbate. Damaged mitochondria release mitochondrial DNA (mtDNA) which triggers the inflammasome pathway resulting in neutrophil chemotaxis and increased inflammatory cytokine release (e.g., interleukin-8, interleukin-1β). At reperfusion, depletion of mitochondria results in a state of mitochondrial overdrive during which excessive amounts of reactive oxygen species (ROS) are produced causing extensive ischemia reperfusion injury (IRI).

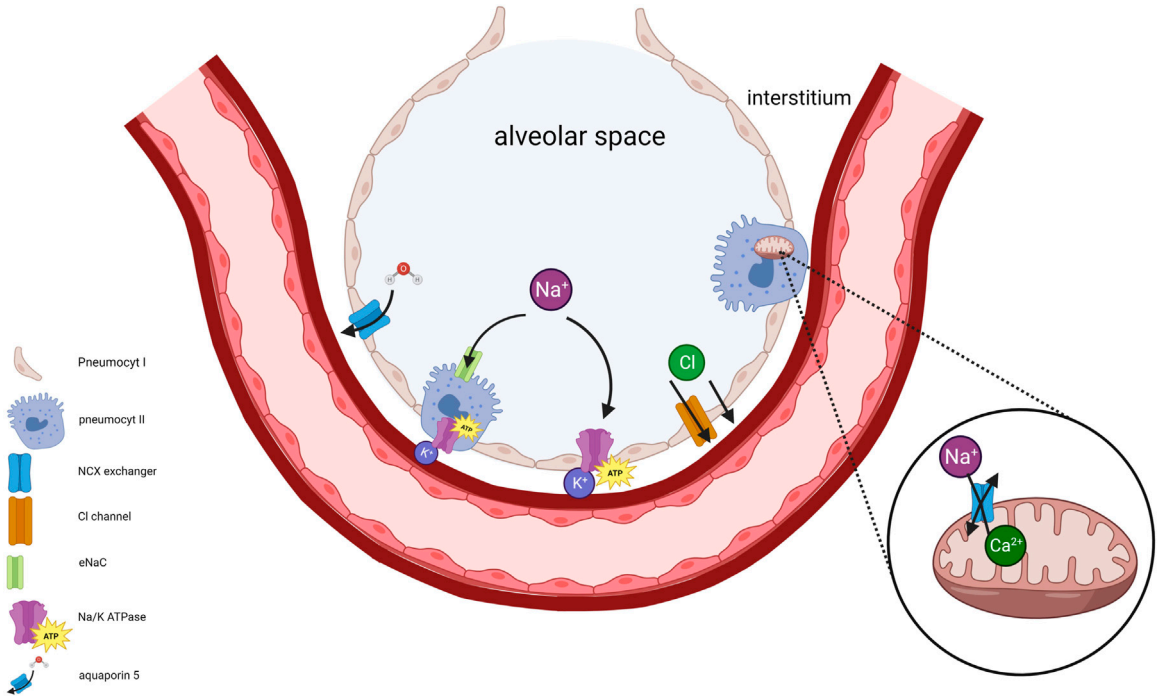


FIGURE 10 | The significance of maintaining Na^+/K^+ ATPase activity in pneumocytes and mitochondria is illustrated. Disruption of intracellular Na^+ concentration, resulting from impaired Na^+/K^+ ATPase, disturbs membrane potentials. This disruption triggers an elevation of cytosolic Ca^{+2} concentration followed by inhibition of mitochondrial Ca^{+2} efflux primarily due to impaired Na^+/Ca^{+2} exchanger (NCX) activity. Consequently, intramitochondrial retention of Ca^{+2} occurs accompanied by mitochondrial swelling. Type I and II pneumocytes provide alveolar fluid clearance (AFC) through vectorial ion transport, generated by an osmotic gradient towards the interstitium. Na^+ enters the pneumocyte via apical epithelial Na^+ channels, subsequently exiting through the basolateral Na^+/K^+ ATPase. Cl^- moves towards interstitium via the cystic fibrosis transmembrane conductance regulator (CFTR). Water follows along the osmotic gradient.

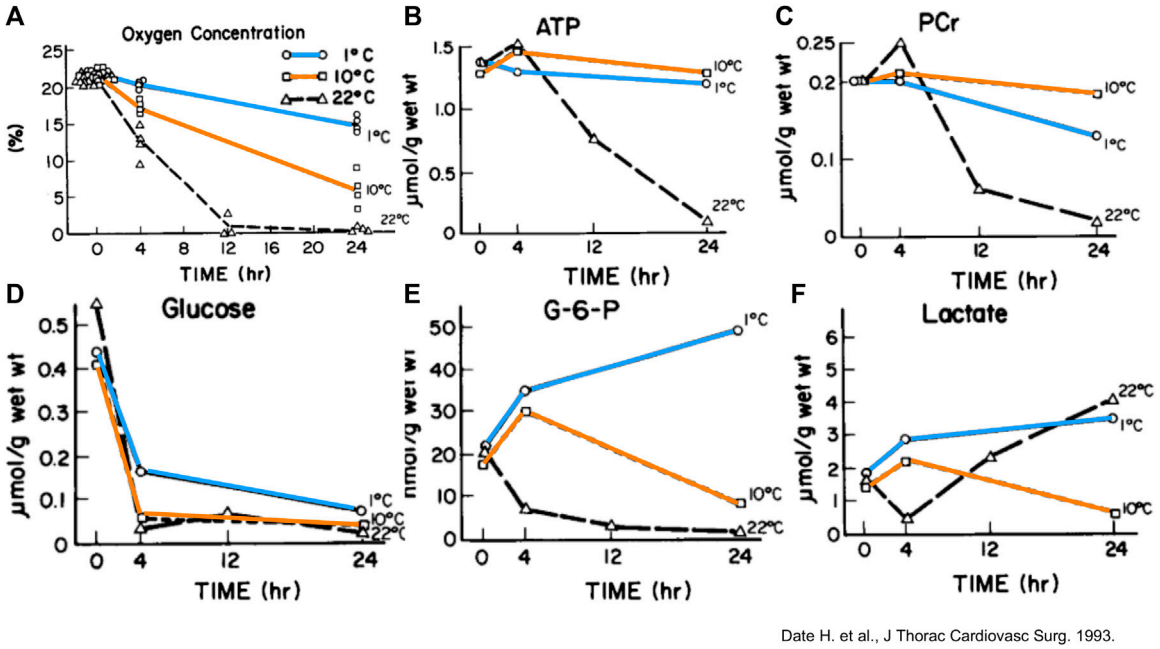
by impairment of the mitochondrial Na+/Ca+2 pump (NCX). ATP depletion and impaired Na+/K + ATPase function therefore cause accumulation of intramitochondrial Ca+2 resulting in mitochondrial swelling and dysfunction [32]. The intracellular accumulation of Ca+2 activates proteases which leads to an irreversibly conversion of xanthine dehydrogenase to xanthine oxidase. Xanthine oxidase and increased hypoxanthine is responsible for ROS formation during anoxic ischemia reperfusion injury (IRI) [33]. Alveolar fluid clearance (AFC) via type I and II pneumocytes depends on an osmotic gradient towards the interstitium created by Na + transport across the apical membrane through epithelial sodium channelss and basolateral Na+/K + ATPase (**Figure 10**).

In rabbit and canine models of lung preservation, it was shown that aerobic mitochondrial metabolism is sustained during preservation. In contrast to other solid organs, the lung is indeed privileged by its supply of oxygen in the air-filled alveoli. Interestingly, the analyses showed that ATP levels were equal during 1°C/4°C and 10°C preservation [8, 18]. Therefore, improved organ preservation with CHS cannot be explained by improved Na+/K + ATPase function. In the porcine model of lung preservation, comparative analysis of Na+/K + ATPase activity during preservation showed no significant difference between 4°C and 10°C [9]. However, Na+/K + ATPase is essential to maintain organ quality. During an experiment where Na+/K + ATPase was inhibited by ouabain during 10°C preservation and subsequent EVLP, lung physiology was significantly impaired [9].

Controlled Hypothermic Storage Favors Aerobic Mitochondrial Metabolism

It must be recognized that the mechanisms of ROS formation are different in lung tissue in comparison to other solid organs due to the presence of oxygen during ischemia. In other solid organs like the liver or heart, ischemia during preservation is coupled to anoxia, a process called anoxic ischemia. Depletion of oxygen in the alveoli impairs aerobic cellular respiration in the mitochondria. ATP synthesis is consequently shifted towards anaerobic glycolysis and phosphocreatine [8].

In a rabbit model of 24 h lung preservation, metabolic characteristics were compared at 1°C, 10°C and 22°C (**Figure 11**) [8]. Oxygen consumption and metabolic need increased with higher temperatures. Energy stores were depleted fastest at 22°C. 1°C preservation resulted in minimal ATP consumption with preserved glucose and glucose-6-phosphate levels over time. However, 1°C preservation beyond 4 h led to increased lactate levels in the 1°C group suggesting cell death. Conversely, lactate levels decreased over time during 10°C preservation [8, 34]. Furthermore, ATP and phosphocreatine levels were highest after 24 h of 10°C preservation.



Date H. et al., J Thorac Cardiovasc Surg. 1993.

FIGURE 11 | Measurements and/or means of gas concentration and metabolic parameters in rabbit lungs in function of time (Hr) preserved at different temperatures. **(A)** Oxygen concentration in the airway. Each point represents one experiment. **(B)** Mean Adenosine triphosphate (ATP). **(C)** Mean phosphocreatine (PCr). **(D)** Mean glucose. **(E)** Mean glucose-6-phosphate (G-6-P). **(F)** Mean lactate. Reprinted from The Journal of Thoracic and Cardiovascular Surgery, Vol.105(3), Hiroshi Date, Akihide Matsumura, Jill K. Manchester, Joshua M. Cooper, Oliver H. Lowry, Joel D. Cooper, Changes in alveolar oxygen and carbon dioxide concentration and oxygen consumption during lung preservation The maintenance of aerobic metabolism during lung preservation, pp. 492-501, Copyright (1993), with permission from Elsevier.

Controlled Hypothermic Storage Favors Anti-oxidative Metabolism

In the porcine model of lung preservation after gastric juice instillation, a shift towards anti-oxidative metabolism was found for 10°C preservation [10]. Higher lung tissue levels of glutathione and ascorbate were detected after 10°C compared SIS, suggesting that CHS favors anti-oxidative metabolism. Glutathione and ascorbate are scavenger molecules that neutralize oxygen radicals and are consumed at ischemia-reperfusion injury (IRI) [35]. Higher levels of anti-oxidative metabolites reduce cellular oxidative stress and in turn better preserve mitochondrial health [9, 10, 36].

Metabolic profiling after 36 h preservation of porcine lungs showed increased tissue levels of glutamine, n-acetyl glutamine and itaconate at 10°C vs. 4°C [9]. Accumulation of succinate during ischemia acts as a potential electron store which in turn drives superoxide formation with the introduction of oxygen during reperfusion (**Figure 12**) [37–39]. Itaconate inhibits succinate dehydrogenase (Complex II) and decreases succinate-derived formation of ROS at reperfusion and can therefore explain the association between higher itaconate levels in lung tissue after 10°C CHS with reduced lung injury during EVLP after 36 h preservation [9, 38].

Taken together, CHS of oxygen repleted lungs appears to achieve the right balance of preventing freezing injury and cellular exhaustion, ideally between 8°C and 10°C where cellular metabolism is preserved producing anti-oxidative metabolites, while maintaining efficient oxygen consumption.

HYPOTHERMIC STORAGE DEVICES FOR DONOR LUNG PRESERVATION

myTEMP 65HC Incubator (Benchmark Scientific)

An overview of the different controlled hypothermic storage devices is summarized under **Table 1**. The myTEMP 65HC Incubator allows storage at an accurate and stable temperature ranging from 0°C to 60°C. A large viewing window is available for visual monitoring (**Figure 13A**). This device meets the electrical and technical specifications for use in an operating theatre (cf. infra). It is not portable and does not allow CHS during organ transport.

LUNGguard™ (Paragonix)

LUNGguard (Paragonix, Boston, MA, United States), the first commercially available portable device was first used in clinics in 2021 (Duke Hospital, Durham, United States) and focuses primarily on preventing ice-contact related injury during lung CHS [40]. It received Food and Drug Administration (FDA) clearance and a Conformitéee Européenne (CE) mark (**Figure 13B**). LUNGguard is designed for CHS at a temperature ranging from 4°C to 8°C. Sherpa Cooling technology with phase-changing material must be cooled at −20°C for 48 h and provides CHS up to 40 h. A smartphone application connected to a logger and thermometer in the LUNGguard allows remote real-time monitoring of location and storage temperature. Also, CHS devices exist for heart (SherpaPak) and liver (LIVERguard).

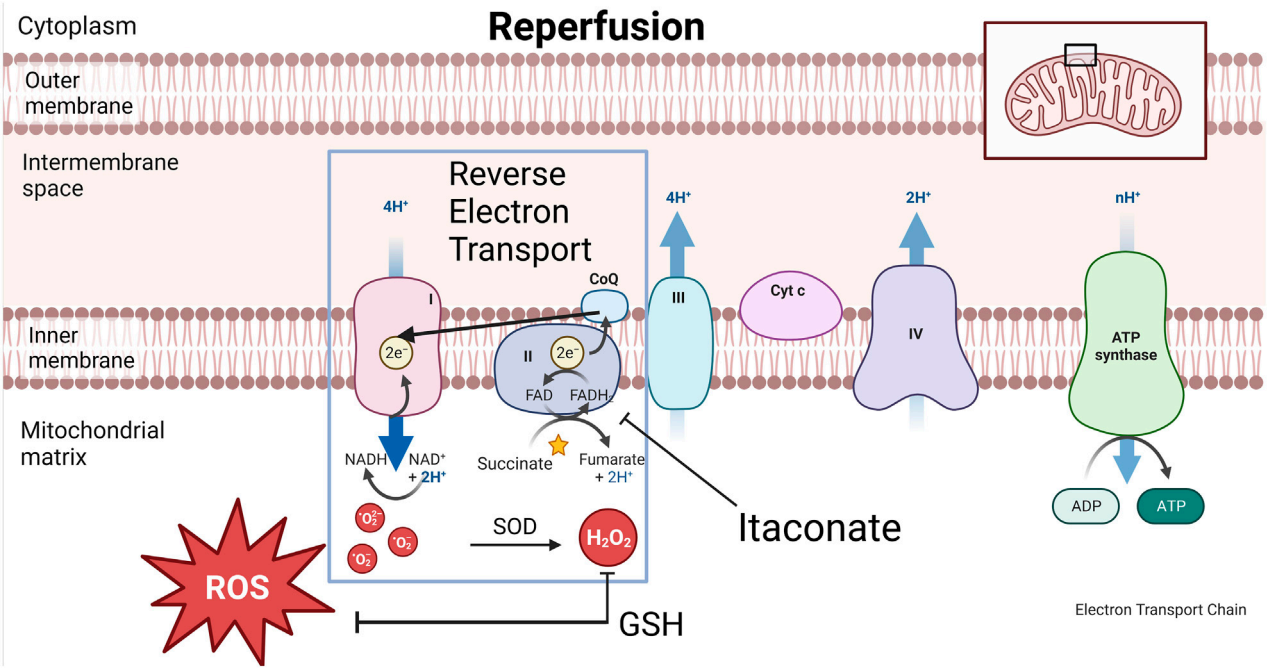


FIGURE 12 | Graphical representation of cytoplasm, mitochondrial inner-and outer membrane and electron transport chain (ETC) during reperfusion. During reperfusion, the electron transport chain suddenly awakens with increasing temperature. Reactive oxygen species (ROS) formation via reverse electron transport (RET) is associated with ischemia reperfusion injury (IRI). During this process, Complex II or succinate dehydrogenase (SDH) in the ETC is overwhelmed by the amount of electron passage presented by succinate. Electrons slip away from the CoQ pool back to complex I, a process called RET during which oxygen radicals are formed. This process is inhibited by itaconate which enables a gradual awakening of the ETC and reduced ROS formation. Superoxide dismutase (SOD) transforms oxygen radicals into H_2O_2 which the clearance of partially depends on the antioxidative metabolite glutathione (GSH).

Moreover, recently, BAROguard received FDA-clearance and its first clinical utilization has been reported [41]. In addition to the features of LUNGguard, BAROguard automatically controls airway pressure of donor lungs during preservation within the recommended range, which is relevant during air transport to avoid pressure-related injury.

VITALPACK EVO (E3 CORTEX)

The Class IIa Medical Device VITALPACK® EVO is an organ transport container that complies with the Directive 93/42/EEC, used for packaging, transport and preservation of organs (**Figure 13C**). VITALPACK provides storage of all organs at 2°C–8°C. The source of cooling consists of four gel packs which

TABLE 1 | Overview of hypopothermic preservation devices used for/or available for controlled hypothermic storage (CHS) of donor lungs. (A) The myTEMP 65HC Incubator (Benchmark Scientific) used in preclincial CHS studies, proof-of-concept study and prospective non-randomized multicenter trial initiated by the Toronto group. (B) LUNGguard PARAGONIX being used for the ongoing GUARDIAN-LUNG regitsry. (C) VITALPACK® EVO, no data available for the lungs. (D) X°Port Lung Transport Device (Traferox Technologies Inc.) being used in an ongoing prospective randomized controlled multicenter trial initiated by the Toronto group.

	MyTemp 65HC incubator	LUNGguard	Vitalpack Evo	X°Port lung transport device
Cooling source	Electrical	Sherpa cooling	Eutectic plates	Gel packs
Preparation	Custom temperature by user	48 h: −20°C storage	24 h: −18°C storage	48 h on 4°C
Temperature range	10°C	4°C–8°C	2°C–8°C	10°C
Time between temperature range	Continuous	40 h	40 h	36 h
FDA	X	✓	X	X
CE-Mark	X	✓	✓	x
Monitoring	Temperature	Temperature and location	VITALTRACK Temperature and location	Temperature and location
Data on lung storage	Preclinical and clinical - proof-of-concept - prospective multicenter non-randomized trial	Ongoing clinical - Guardian lung registry	No data available	Ongoing clinical - Prospective multicenter randomized controlled trial

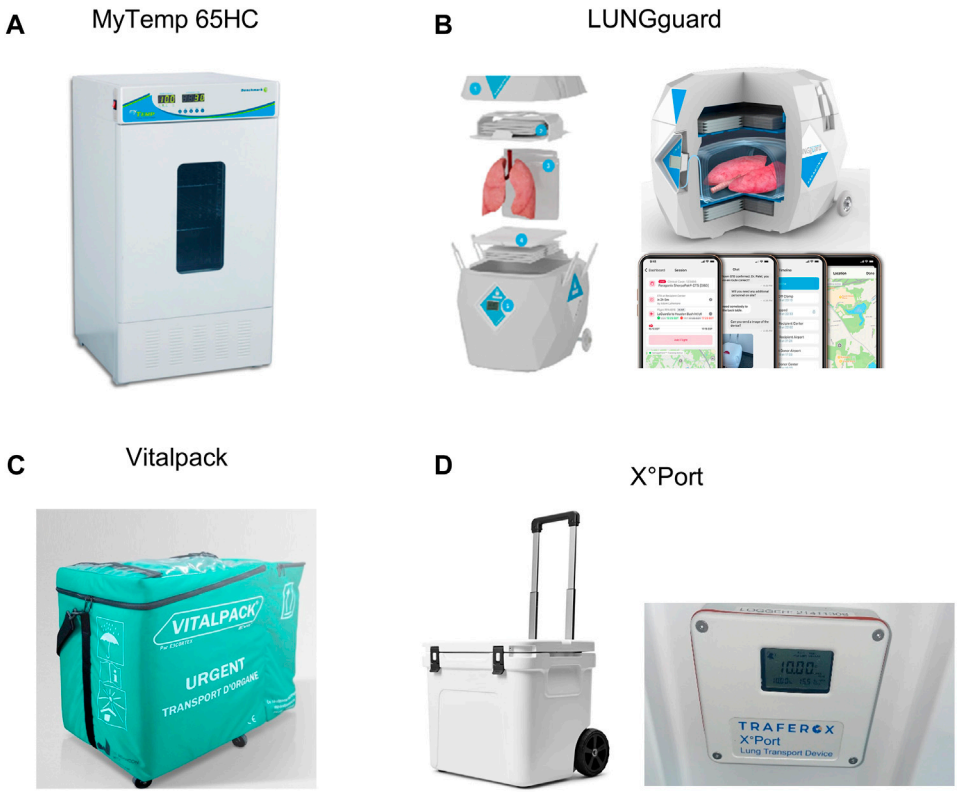


FIGURE 13 | Images of hpothermic preservation devices used for/or available for controlled hypothermic storage (CHS) of donor lungs. **(A)** The myTEMP 65HC Incubator, Benchmark Scientific. **(B)** LUNGguard, Paragonix. **(C)** VITALPACK® EVO, E3 CORTEX. **(D)** X °Port Lung Transport Device (Traferox Technologies Inc.).

are to be frozen at −18°C for at least 24 h and placed at room temperature for 30 min before inserted into the box. VITALPACK ensures a start temperature inside the box of 8°C, decreasing to >2°C with a preservation duration of minimal 40 h (tests provided by company on website) [42]. It is designed and guaranteed for 10 uses. VITALTRACK is an extra service in the form of a device that allows location and temperature monitoring. No data has been reported on lung preservation. VITALPACK is standard use for all organ preservation in France since 2017, and has recently been implemented in Switzerland (personal communication E3 CORTEX).

X°Port Lung Transport Device (Traferox Technologies Inc.)

X°Port Lung Transport Device (Traferox Technologies Inc.) has been developed by the Toronto Lung Transplant Program (**Figure 13D**). Its design was inspired by the animal and preliminary clinical research. X°Port has not yet received FDA-clearance or a CE-mark. The device was designed for donor lung storage at 10°C and is being utilized in a multicenter randomized trial with Northern American and European centers [43].

CLINICAL STUDIES

The Proof-Of-Concept for 10°C CHS in Lung Transplantation With the myTEMP 65HC Incubator

In 2021, the first clinical proof-of-concept study of donor lung CHS was reported by Toronto. In five bilateral LTx cases, extended 10°C CHS in the myTEMP 65HC incubator was performed to avoid overnight transplantation [9]. After procurement, donor lungs were first transported by SIS. In the transplant center, lungs were transferred to the incubator at 10°C. Total ischemic time was extended up to 16h30. No incidence of primary graft dysfunction grade 3 (PGD3) was observed at 72 h and all patients were reported to be alive at post-LTx day 330. This study was the first to show the possibility of safely bridging the night using CHS.

Multicenter Prospective Non-randomized Trial of 10°C CHS With the myTEMP 65HC Incubator

In 2023, the results of a multicenter prospective non-randomized trial on 10°C CHS were published by Toronto,

Vienna, Madrid and Florida [16]. CHS at 10°C in 70 LTx cases was compared to 140 propensity matched SIS cases. Donor lungs were transported with SIS to the transplant center and transferred to the incubator set at 10°C if cross-clamp time was expected between 6 PM and 4 AM. Recipient anesthesia was induced after 6 AM. Authors aimed to study if extended preservation with CHS at 10°C is safe, and to compare outcome with LTx after SIS.

Total ischemic time of the second implanted lung was extended to a median of 14h08. There was no significant difference for PGD3 at 72 h (5.7% for CHS vs. 9.3% for SIS), 30-day -and 1-year survival, showing that extending lung preservation time up to 12 h with 10°C CHS is safe.

GUARDIAN-LUNG Registry for Post-market Registration of LUNGguard Utilization

The Global Utilization and Registry Database for Improved preservAtion of doNor LUNGs (GUARDIAN-LUNG) aims to collect real-world data on LTx with CHS in LUNGguard. In a preliminary analysis, 86 LUNGguard and 90 SIS cases were compared [44]. Incidence of PGD3 at 72 h was not significantly different but a trend with 54% reduction after LUNGguard preservation was observed. Kaplan-Meier analysis revealed a significantly improved survival for LUNGguard vs. SIS with a one-year survival rate of 92.7% vs. 82.2%, respectively.

Another preliminary study compared short-term outcome and costs after LTx with LUNGguard vs. SIS [45]. Total ischemic times were similar for both preservation techniques. The incidence of PGD3 at 72 h and rejection rate prior to discharge and survival were similar.

Interestingly, in 2023, results from the GUARDIAN-heart registry including 569 patients (255 SherpaPak vs. 314 ice) in heart transplantation were reported [46]. Data indicated a favorable outcome after CHS of donor hearts in SherpaPak with significantly lower incidence of PGD and a trend towards reduced need for post-transplant mechanical circulatory support and improved 1-year survival.

Multicenter Randomized Controlled Trial With X°port

In 2023, a multicenter prospective clinical randomized controlled trial (RCT) was set up by Toronto comparing 10°C CHS in the X°Port vs. SIS [43]. The aim of this study is to show non-inferiority of extended preservation at 10°C in X°Port. End-points are PGD3 at 72 h, postoperative recovery, acute rejection, performance status and 1-year survival.

OVERNIGHT BRIDGING OF LUNG PRESERVATION WITH CHS

Conventionally, there has been an emphasis on restricting CIT to within the acceptable range of 6–8 h using traditional ice storage [13]. Nevertheless, there have been reports of successful LTx cases exceeding the 8 h timeframe, and retrospective studies revealed inconclusive correlation between ischemia time and post-LTx outcomes [47–49]. Furthermore, within the framework of SIS, the transplant team contends with persistent time constraints and a tendency for overnight transplantation. This situation presents logistical challenges, especially in the context of remote and distant procurements.

The effect of performing transplantation outside regular working hours has been studied for different organs, and available data show potential negative impact on outcome [50]. In a retrospective cohort of 563 LTx cases, a higher incidence of PGD3 was observed within 72 h when reperfusion was between 4 AM and 8 AM [51]. In a propensity matched study on LTx, 187 overnight cases were compared with 187 daytime cases. Overnight transplantation was associated with higher rates of postoperative major events, worse 5-year survival and shorter freedom from bronchiolitis obliterans syndrome [14].

Safely extending ischemic times with CHS can overcome the challenges associated with time pressure. Longer transport time allows procurement in hospitals located further away. Limited capacity at the operating theatre or multi-organ transplants can be managed more easily [52]. Shorter intervals between procurement allow consecutive LTx procedures. Hence, CHS introduces more flexibility for transplant teams without compromising outcome, which benefits healthcare personnel. Daytime transplantation improves overall fitness of transplant teams favoring cognitive and psychomotor skills and reducing the likelihood of errors. An anonymous survey of 7,900 correspondents reported an association of burnout and decreased career satisfaction with overnight surgery. A significantly higher proportion of surgeons working >80 h/week or >2 nights on call/week indicated that they would not become a surgeon again [53]. Furthermore, daytime LTx provides more technical expertise and personnel available. Also, providing a scheduled daytime LTx would attract young professionals and retain more surgeons and transplant personnel.

LUNGGUARD UTILIZATION AND EXPERIENCE AT UNIVERSITY HOSPITALS LEUVEN

CHS with LUNGguard was introduced in our center (Leuven, Belgium) in November 2022. Our current policy states that CHS with LUNGguard is used for cases with donor cross-clamp after 6 PM with recipient anesthesia starting at 7.30 AM, in cases of logistical limitations to accept valid donor offer, and if a complex recipient procedure is anticipated. By the end of February 2024, CHS with LUNGguard was performed in 24 cases. The average donor age was 60 years (12M/12F), 7 DCD cases were performed. In 18 (75%) cases, LUNGguard was used to extend preservation until daytime. The ischemic time of the second implanted lung exceeded 12 h in 18 (75%) and 15 h in 15 (63%) cases. The average preservation temperature was 6.8°C. Median total ischemia time for the second implanted lung was 15 h with a maximum of 22 h. The incidence of PGD3 at 72 h was 0% and median postoperative time on ventilator was 39 h. One patient died 1 week after LTx, suffering from the consequences of intraoperative ECMO-failure [54, 55].

LEAVING THE ICE AGE BEHIND: THE DAWN OF A NEW ERA

Research encompassing historical and recent animal experiments, groundbreaking clinical studies and the development of new preservation devices have shown that CHS of donor lungs is here to stay. The ice box has served transplant teams for decades but an irreversible shift towards use of CHS has set off. Reaching donor hospitals located further away and extending preservation to daytime can increase the number of transplants and improve outcome.

Larger patient numbers and longer follow-up are needed to study the safety of extended CHS preservation with associated reduction of PGD and prolongation of survival. Currently, temperatures for CHS are between 6°C and 10°C, but optimal temperature for CHS has not yet been pinpointed. Another interesting subject is to investigate whether CHS in general or prolonged preservation with CHS improves outcome for extended criteria donor lungs (injury, pneumonia). More research on the optimal preservation duration, temperature and unraveling the mechanism of CHS remain topics for further research. Further investigation is also required to determine whether the advantages of CHS are solely dependent on the presence of oxygen in the alveoli, making them specific to the lung, or if they also apply to other solid organs.

CONCLUSION

Based on preclinical and clinical data, we reviewed the underlying mechanisms of improved lung preservation with CHS. It was shown that preservation of lungs by CHS better maintains mitochondrial health and cellular viability. The current clinical data supports the feasibility of implementing CHS to safely extend preservation time, and to avoid overnight transplantation. Additional experimental, clinical studies and RCTs are necessary to further define the future of preservation in LTx. Furthermore, the potential benefits of CHS for other solid organs also require thorough investigation.

AUTHOR CONTRIBUTIONS

Conceptualization: IC, JVS, A-LP, and LJC. Supervision: JVS and LJC. Literature review: IC, JVS, A-LP, AB, RV, JP, DVR, and LJC. Writing original draft: IC and LJC. Writing second draft and reviewing: IC, JVS, A-LP, and LJC. Review and feedback final draft: A-LP, AB, CV, CB, BV, RV, JP, and DVR. Figure construction: IC and JVS. Contributed important ideas: JVS and JP. All authors contributed to the article and approved the submitted version.

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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GLOSSARY

AFC	alveolar fluid clearance
AsA	ascorbate
ATP	adenosine triphosphate
CE	conformité Européenne
CHS	controlled hypothermic storage
CIT	cold ischemic time
COPD	chronic obstructive pulmonary disease
CT	core temperature
DBD	donation after brain death
DCD	donation after circulatory death
ECMO	extracorporeal membrane oxygenation
ETC	electron transport chain
EVLP	*ex-vivo* lung perfusion system
FDA	food and drug administration
FiO_2	fraction of inspired oxygen
G-6-P	Glucose-6-phosphate
GSH	gluthathione
GUARDIAN	Global Utilization And Registry Database for Improved preservation of DoNor
ICU	intensive care unit
IL-1	interleukin-1
IL-1β	interleukin-1β
IRI	ischemia reperfusion injury
ISHLT	International Society for Heart and Lung Transplantation
LDH	lactate dehydrogenase
LTx	lung transplantation
mtDNA	mitochondrial desoxyribonucleic acid
PaO2	arterial pressure of Oxygen
PAP	pulmonary artery pressure
PCr	phosphocreatine
PGD	primary graft dysfunction
PGD3	primary graft dysfunction grade 3
RCT	randomized controlled trial
RET	reverse electron transport
ROS	reactive oxygen species
SDH	succinate dehydrogenase
SIS	static ice storage
ST	surface temperature
Tx	transplantation
W/D	wet-to-dry ratio

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BRIEF RESEARCH REPORT
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Tobramycin Systemic Absorption in Lung Transplant Recipients Treated With Inhaled Tobramycin: A Cohort Study

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Inhaled tobramycin treatment has been associated with nephrotoxicity in some case reports, but limited data are available about serum levels and its possible systemic absorption in lung transplant recipients (LTR). We conducted a single-center, observational and retrospective study of all adult (>18 years old) LTR treated with inhaled tobramycin for at least 3 days between June 2019 and February 2022. Trough serum levels were collected and >2 µg/mL was considered a high drug level. The primary outcome assessed the presence of detectable trough levels, while the secondary outcome focused on the occurrence of acute kidney injury (AKI) in individuals with detectable trough levels. Thirty-four patients, with a median age of 60 years, were enrolled. The primary indications for treatment were donor bronchial aspirate bacterial isolation (18 patients) and tracheobronchitis (15 patients). In total, 28 patients (82%) exhibited detectable serum levels, with 9 (26%) presenting high levels (>2 µg/mL). Furthermore, 9 patients (26%) developed acute kidney injury during the treatment course. Median trough tobramycin levels were significantly elevated in invasively mechanically ventilated patients compared to non-ventilated individuals (2.5 µg/mL vs. 0.48 µg/mL) ($p < 0.001$). Inhaled tobramycin administration in LTRs, particularly in those requiring invasive mechanical ventilation, may result in substantial systemic absorption.

Keywords: aminoglycosides, drug levels, nebulized, acute kidney injury, nephrotoxicity

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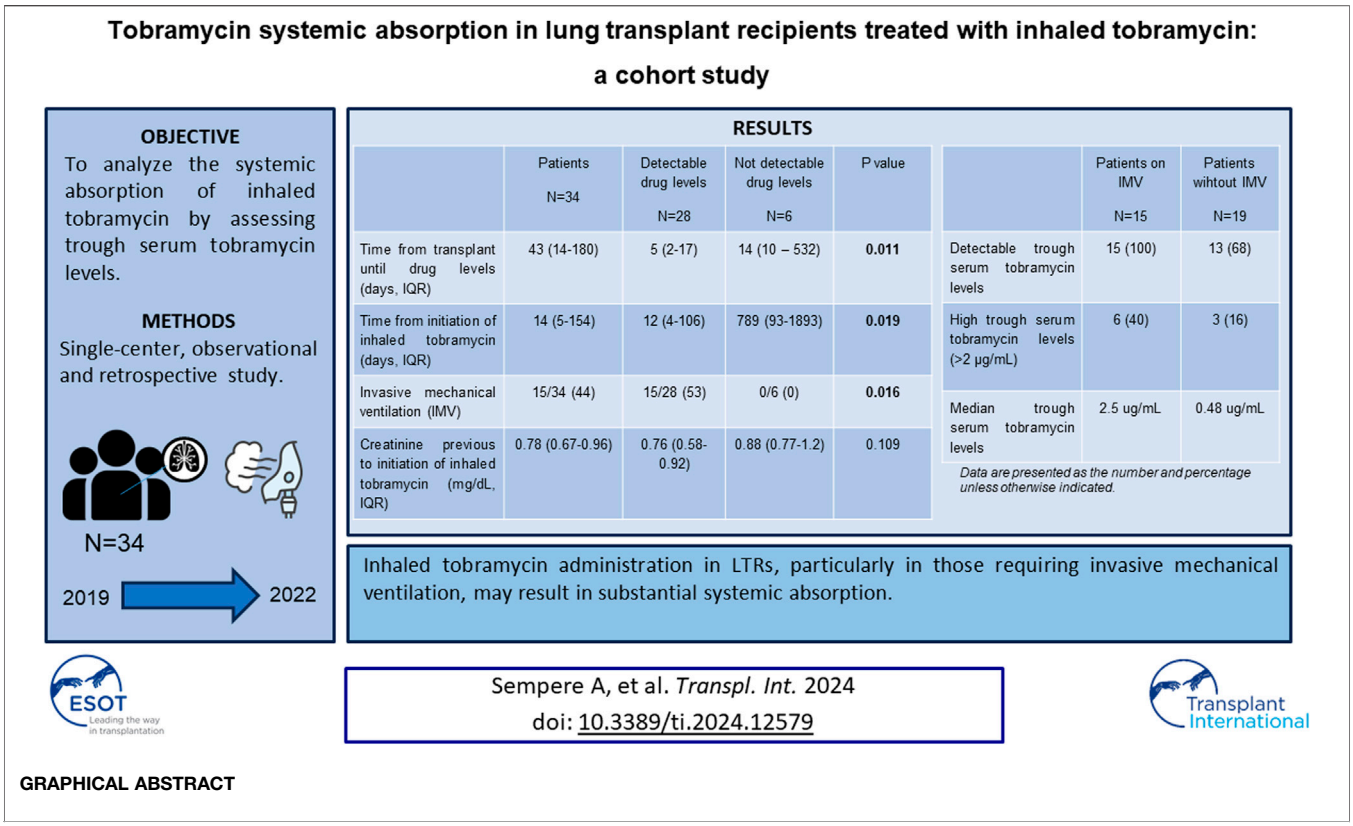
INTRODUCTION

Bacteria are the most frequent cause of infection in lung transplant recipients (LTR) [1], leading to tracheobronchitis or pneumonia and might affect the bronchial suture [2]. To control such infections, inhaled antibiotics are frequently used, although few published data are available [1]. We have previously described the use of inhaled antibiotics to prevent donor-derived infection, even



GRAPHICAL ABSTRACT

in multidrug resistant bacteria [3]. The efficacy of secondary prophylaxis involving nebulized antibiotics during the intensive care unit (ICU) admission of lung transplant recipients has also been documented [4].

The inhalational route facilitates the direct delivery of high antibiotic concentrations to the lungs, exposing bacteria to lethal concentrations while minimizing potential systemic toxicity by limiting absorption [5]. Colistin and tobramycin are very common nebulized antibiotics. Several studies have indicated that plasma levels in patients treated with nebulized colistin for ventilator-associated pneumonia are either undetectable or very low, falling below levels associated with potential nephrotoxicity [6]. Systemic tobramycin is recognized for its adverse effects, encompassing nephrotoxicity and ototoxicity [7]. Consequently, it becomes crucial to ascertain the extent of systemic absorption of inhaled, particularly in LTR who frequently receive other nephrotoxic drugs such as calcineurin inhibitors [8]. However, there are few studies describing the systemic absorption of inhaled tobramycin. Detectable tobramycin levels have been reported, mainly in patients with cystic fibrosis and associated with nephrotoxicity in some cases [9–11]. Nevertheless, these findings have not been confirmed in other studies [12–14]. In the setting of lung transplantation, cases of drug absorption and nephrotoxicity have also been documented [15–19].

The aim of our study is to analyze the systemic absorption of inhaled tobramycin by assessing trough serum tobramycin levels in a cohort of LTR treated with inhaled tobramycin.

MATERIAL AND METHODS

Patients and Setting

This is an observational retrospective study performed at Hospital Universitari Vall d'Hebron, a 1,000-bed teaching hospital in Barcelona (Spain). Our institution is the leading lung transplant center in Spain, conducting approximately 120 lung transplants annually. The study encompassed all consecutive adult patients (≥18 years of age) who underwent lung transplantation and received inhaled tobramycin treatment for a duration of at least 3 days. The study period extended from June 2019 to February 2022.

Following a clinical protocol implemented by the lung transplant unit since June 2019, tobramycin trough levels were systematically assessed in all lung transplant recipients undergoing nebulized tobramycin treatment. The frequency of drug monitoring was determined by the treating physician according to usual clinical practice.

The Clinical Research Ethics Committee of our hospital approved the study (EOM(AG)021/2022(5980)) and waived the requirement for informed consent.

Lung Transplant Antibiotic Protocol

The preventive antibiotic strategy in our center involves the administration of intravenous amoxicillin-clavulanate and ceftazidime during the surgical lung transplant procedure. This intravenous regimen is continued until the results of perioperative cultures are obtained. In cases where intraoperative bacterial isolation is identified in either the

TABLE 1 | Comparison of demographic variables between patients with detectable tobramycin levels vs. patients with undetectable levels.

	Patients (*N* = 34)	Detectable drug levels (*N* = 28)	Not detectable drug levels (*N* = 6)	*p*-value
Male sex	21 (62)	18 (86)	3 (50)	0.848
Age, years, median (IQR)	60 (52–65)	60 (52–64)	62 (51–66)	0.421
Lung disease				0.283
Pulmonary fibrosis	13 (38)	11 (39)	2 (33)	
Chronic obstructive pulmonary disease	12 (35)	10 (36)	2 (33)	
Bronchiolitis obliterans	2 (6)	2 (7)	0	
Bronchiectasis	1 (3)	1 (3.5)	0	
COVID-19 pneumonia	1 (3)	1 (3.5)	0	
Cystic fibrosis	1 (3)	0	1 (17)	
Pleuropulmonary fibroelastosis	1 (3)	0	1 (17)	
Pulmonary alveolar proteinosis	1 (3)	1 (3.5)	0	
Pulmonary lymphangioleiomyomatosis	1 (3)	1 (3.5)	0	
Pulmonary veno-occlusive disease	1 (3)	1 (3.5)	0	
Type of lung transplant				
Single	3 (9)	0	3 (50)	—
Bilateral	31 (91)	28 (100)	3 (50)	0.349

Data are presented as the number and percentage unless otherwise indicated.

recipient or donor, intravenous antibiotics are customized based on antibiotic susceptibility patterns and extended for a duration of 10–14 days. Furthermore, inhaled antibiotics such as tobramycin (300 mg every 12 h) or colistin (2–5 million units every 8 h) are introduced, again guided by the antibiotic susceptibility profile. Tobramycin was mainly used when bacterial isolates were resistant to colistin. This inhaled antibiotic regimen is typically continued for a period of 2–4 months. The nebulized antibiotic is maintained on an outpatient basis assessing the risk-benefit ratio according to the criteria established by the treating physician.

Data Collection

Patients were identified through the pharmacy database. Demographic, clinical, and microbiological data were collected from electronic medical records and entered anonymously into a database, specifically created for the study.

Definitions

Respiratory tract infections were defined as outlined by the multidisciplinary working group of The International Society for Heart and Lung Transplantation [2]. Donor lung bacterial isolation was based on the isolation of any amount of bacteria in a selected and protected bronchial aspirate performed after opening the bronchial suture just prior the implantation.

Acute kidney injury (AKI) was defined as a reduction in renal function within 48 h characterized by an absolute increase in the serum creatinine level exceeding 0.3 mg/dL or a 50% increase above the baseline value [20].

Tobramycin was prescribed at the discretion of the treating physician and administered over 30 min. Treatment was nebulized via Aeroneb Pro in mechanically ventilated patients and via vibrating mesh-nebulizer nebulizer in the other patients. The prescribed dosage was 300 mg/5 mL (TOBI®) every 12 h and the treating physician determined the duration of treatment. To monitor tobramycin serum levels, blood samples were collected 30 min before each dosing to ensure measurement at trough concentration. We considered high tobramycin serum levels those concentrations exceeding 2 μg/mL [21, 22].

The primary outcome was the presence of detectable trough serum levels of tobramycin and high tobramycin levels (>2 μg/mL). The secondary outcome was to describe the presence of acute kidney injury in patients with detectable trough serum levels of tobramycin.

Tobramycin drug levels were measured in serum with homogeneus particle-enhanced turbidimetric immunoassay (QMS-ThermoFisher) and laboratory lower limit of detection was 0.1 μg/mL.

Statistical Analysis

Categorical values were expressed in absolute numbers and percentages, while quantitative variables were reported as medians and interquartile ranges (IQRs). Wilcoxon rank sum test was used to compare quantitative variables and Fisher exact test to compare categorical variables. Undetectable (<0.1 μg/mL) drug levels were computed as 0, and in patients with more than one detectable drug level the higher drug level was selected for the analysis.

RESULTS

A total of 34 patients, with a median age of 60 years, were enrolled in the study. Most patients, 31 (91%), underwent a bilateral lung transplantation. Baseline characteristics are summarized in **Table 1**. All patients except one received a tacrolimus-based immunosuppressive treatment.

Inhaled tobramycin was primarily initiated by bacterial isolation in donor bronchial aspirate (n = 18), tracheobronchitis (n = 15), pneumonia (n = 4) and bronchial suture infection (n = 1). Four patients presented

TABLE 2 | Comparison of main variables between patients with detectable tobramycin levels vs. patients with undetectable levels.

	Patients *N* = 34	Detectable drug levels *N* = 28	Not detectable drug levels *N* = 6	*p*-value
Time from transplant until drug levels (days, IQR)	43 (14–180)	5 (2–17)	14 (10–532)	**0.011**
Time from initiation of inhaled tobramycin (days, IQR)	14 (5–154)	12 (4–106)	789 (93–1893)	**0.019**
Invasive mechanical ventilation	15/34 (44)	15/28 (53)	0/6 (0)	**0.016**
Creatinine previous to initiation of inhaled tobramycin (mg/dL, IQR)	0.78 (0.67–0.96)	0.76 (0.58–0.92)	0.88 (0.77–1.2)	0.109
Calcineurin inhibitor trough levels previous to initiation of inhaled tobramycin (mg/dL, IQR)	9.4 (8.1–12.1)	9.8 (8.1–12.8)	8.9 (8–10.1)	0.634
Calcineurin inhibitor first trough levels after initiation of inhaled tobramycin	8.4 (6.4–12.1)	8.2 (7.7–11.1)	12.1 (6.6–13.5)	0.594
Hypoalbuminemia previous to initiation of inhaled tobramycin	23/34 (67)	22/28 (78)	1/6 (17)	0.0921
Hypoalbuminemia at first measure after initiation of inhaled tobramycin	18/34	18/28	0/6	0.260

Data are presented as the number and percentage unless otherwise indicated.
Bold values indicate p < 0.05.

both donor bronchial aspirate bacterial isolation and lower respiratory tract infection. The main isolated bacteria were *Staphylococcus aureus* (n = 17), Enterobacterales (n = 14) and *Pseudomonas aeruginosa* (n = 5). Additional microbiological data as well as main variables for all patients are provided in **Supplementary Tables S1, S2**.

Twenty-nine patients received tobramycin at a dose of 300 mg/12h, and an additional 5 LTR patients were administered 300 mg/24 h (adjusted by the lung transplant physician in the outpatient setting). No patients received intravenous tobramycin, other intravenous aminoglycosides, intravenous colistin, vancomycin or other nephrotoxic agents concurrently, other than calcineurin inhibitors.

Tobramycin trough levels were determined at least twice in 18 patients (53%). Tobramycin was detected at least once in 28 patients (82%), with a median value of 0.76 μg/mL (IQR 0.38–2.2). Nine patients (26%) presented high tobramycin levels, with a median value of 3.81 μg/mL (IQR 2.39–6.65). All patients on IMV (n = 15) had detectable serum tobramycin levels after a median of 5 days (IQR 4–9) of inhaled treatment.

The median creatinine value before initiating nebulized tobramycin was 0.78 mg/dL (IQR 0.67–0.96) and two patients (5%) had a history of previous renal failure.

Inhaled tobramycin treatment was discontinued in 11 patients (32%) due to either high drug levels or acute kidney injury. Nine patients developed AKI after a median of 28 days (IQR 4–125) of nebulized tobramycin, with a median peak creatinine of 1.8 mg/dL (IQR 1.6–2.1). The median drug level in these patients was 2.8 μg/mL (IQR 1.9–6.3). Three of these patients did not recover their baseline renal function by 6-month.

Variables among patients with detectable and undetectable tobramycin levels are compared in **Tables 1**, **2**. Patients with detectable levels exhibited a shorter time from both transplantation (12 vs. 789 days, p = 0.019) and antibiotic initiation until drug levels (5 vs. 14 days, p = 0.011). Additionally, patients with detectable levels were more frequently subjected to invasive mechanical ventilation (53% vs. 0, p = 0.016) Moreover, the median trough drug levels were significantly higher in invasively mechanically ventilated patients compared to those not ventilated (2.5 μg/mL vs. 0.48 μg/mL) (p < 0.001).

DISCUSSION

In our study, 82% of LTR exhibited detectable tobramycin drug levels, with 26% of patients demonstrating high tobramycin levels and 26% developing acute kidney injury. Notably, all recently transplanted patients on mechanical ventilation had detectable tobramycin levels.

A prior retrospective study, including both amikacin and tobramycin treatments, reported 39% detectable drug levels in LTR [18]. In a multivariate analysis from that study, factors such as cystic fibrosis, lung transplantation, chronic kidney disease, mechanical ventilation, and use of tobramycin instead of amikacin were associated with detectable drug levels. In our study, mechanical ventilation was also associated with higher trough drug levels in LTR. Additionally, in a recent study involving non-transplant patients on mechanical ventilation treated with inhaled tobramycin, 66% of patients had detectable drug levels [23]. Our study results also suggest that IMV, could promote the systemic absorption of tobramycin. Variables such as the time from transplantation until performing drug levels or the time from initiation of nebulized tobramycin are likely influenced by invasive mechanical ventilation. The systemic absorption of inhaled tobramycin during mechanical ventilation could be attributed to improved aerosol delivery, changes in pulmonary physiology, and increased vascular permeability due to lung tissue damage [18, 23]. These two studies [18, 23] did not demonstrate a statistically significant association between detectable levels of tobramycin and acute renal failure. However, in one of the studies, the levels were not analyzed as a continuous variable, and in both cases, the drug levels were not trough drug levels. Some reported cases of suspected nephrotoxicity due to inhaled tobramycin systemic absorption have involved non-mechanically ventilated LTR [15–17]. In these cases, high trough tobramycin levels were detected (8.7 mg/mL [16] and 2.7 mg/mL [17]), with recovery of previous renal function observed after discontinuation of inhaled tobramycin.

In our study, nine patients (26%) with detectable levels presented with renal failure. Of interest, six out nine patients who experienced AKI recovered their baseline renal function, while three (33%) did not. LTR possess numerous risk factors

for nephrotoxicity, including immunosuppressive drugs (mainly calcineurin inhibitors), haemodynamic instability, exposure to other nephrotoxic drugs, or diabetes mellitus, among others. Consequently, the etiology of renal failure in these patients is often multifactorial and challenging to discern. AKI is highly prevalent after lung transplantation (39%–62%) and is associated with increased mortality [24]. Given the need of calcineurin inhibitors, minimizing exposure to other nephrotoxic agents is recommended [8]. Elevated trough levels of systemic aminoglycosides are correlated with a heightened risk of nephrotoxicity [7]. Therefore, the potential for systemic absorption of inhaled tobramycin in lung transplant recipients should be considered. Vestibular toxicity has also been reported in some cases [16], with one suspected case in our cohort that might have gone undetected.

Several limitations characterize our study, including its retrospective nature, limited sample size, and variability in the timing of tobramycin level measurements according to daily clinical practice. Furthermore, different doses of tobramycin (300 mg every 24 h) were administered to some patients. Nevertheless, our study provides insights into a cohort of lung transplant recipients undergoing nebulized tobramycin treatment at various stages of lung transplantation.

In conclusion, our findings suggest that inhaled tobramycin in LTR, particularly those on invasive mechanical ventilation, may undergo significant systemic absorption. Monitoring of tobramycin trough levels seems advisable to mitigate potential drug absorption.

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 studies involving human participants were reviewed and approved by Clinical Research Ethics Committee of Vall d'Hebron University Hospital (EOM(AG)021/2022(5980)). The ethics committee waived the requirement of informed consent.

AUTHOR CONTRIBUTIONS

JV tested tobramycin levels. LS CrB, DC-H, MM-G, LS, DM-G, CaB, OL, and JG participated collecting and managing these patients. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

IL-A has received honoraria for speaking at educational events from MSD and Pfizer and has received travel support from Gilead, Merck and Menarini for scientific purposes. OL has received research grants from Pfizer and Merck, and has been a speaker for Pfizer, Astellas, Novartis and Merck.

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.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/ti.2024.12579/full#supplementary-material

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ORIGINAL RESEARCH
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Anaesthesiologic Considerations for Intraoperative ECMO Anticoagulation During Lung Transplantation: A Single-Centre, Retrospective, Observational Study

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Background: Extracorporeal membrane oxygenation (ECMO) is frequently used during lung transplantation. Unfractionated heparin (UFH) is mainly used as part of ECMO support for anticoagulation. One of the most common perioperative complications is bleeding, which high-dose UFH can aggravate.

Methods: We retrospectively analyzed (*n* = 141) patients who underwent lung transplantation between 2020 and 2022. All subjects (*n* = 109) underwent central cannulated VA ECMO with successful intraoperative ECMO weaning. Patients on ECMO bridge, postoperative ECMO, heart-lung transplants and transplants without ECMO were excluded. The dose of UFH for the entire surgical procedure, blood loss and consumption of blood derivatives intraoperatively and 48 h after ICU admission were recorded. Surgical revision for postoperative bleeding were analyzed. Thrombotic complications, mortality and long-term survival were evaluated.

Results: Lower doses of UFH administered for intraoperative ECMO anticoagulation contribute to a reduction in intraoperative blood derivates consumption and blood loss with no thrombotic complications related to the patient or the ECMO circuit. Lower doses of UFH may lead to a decreased incidence of surgical revision for hemothorax.

Conclusion: Lower doses of UFH as part of intraoperative ECMO anticoagulation might reduce the incidence of complications and lead to better postoperative outcomes.

Keywords: ECMO, UFH, anticoagulation, lung transplantation, anesthesiology

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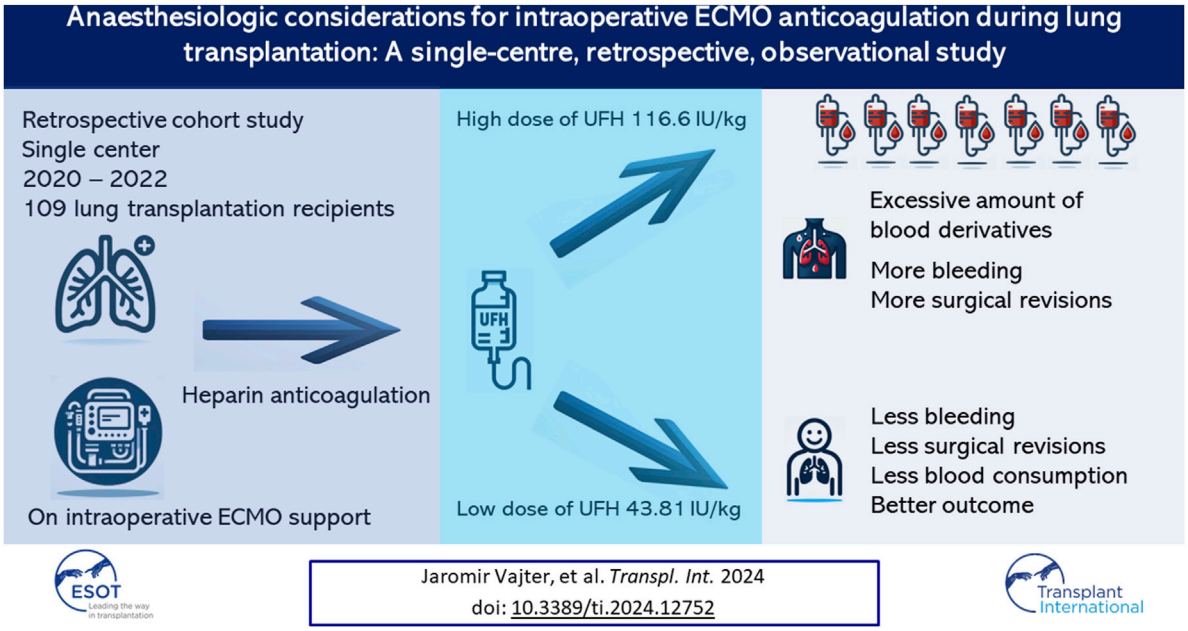
Abbreviations: ACT, activated clotting time; APTT, activated partial thrombin time; BMI, body mass index; CONSORT, Consolidated Standards for Reporting Trials; CPB, cardiopulmonary bypass; ECMO, extracorporeal membrane oxygenation; FFP, fresh frozen plasma; HLTx, heart-lung transplant; ICU, intensive care unit; LUTx, lung transplant; MCS, mechanical circulatory support; NSAID, non-steroidal anti-inflammatory drugs; PEEP, positive end-expiratory pressure; PFA, platelet function assay; RBCs, red blood cells; ROTEM, rotational thromboelastometry; Tx, transplantation; UFH, unfractionated heparin.



GRAPHICAL ABSTRACT |

INTRODUCTION

Intraoperative extracorporeal membrane oxygenation (ECMO) is routinely used during lung transplant surgery to provide the patient with temporary respiratory and circulatory support [1–3]. Over the past two decades, many centres have switched from intraoperative support with cardiopulmonary bypass (CPB) to ECMO [1, 2, 4, 5]. The intraoperative ECMO approach allows surgeons to perform the procedure with greater precision and efficiency while minimizing the risk of lung injury [2, 4]. Most centres that use intraoperative ECMO, to a greater extent, support the idea that ECMO helps to control lung graft reperfusion [4, 6, 7]. Another advantage of intraoperative ECMO support is the possibility of transplanting more patients with comorbidities who would not be able to undergo surgery on selective unilateral ventilation without extracorporeal support. For example, patients with severe pulmonary hypertension have a high risk of left-sided heart failure, pulmonary fibrosis, and very low pulmonary compliance [8, 9]. ECMO has a much lower pro-inflammatory potential than CPB [1]. However, ECMO also carries risks of bleeding, infection, and thrombotic complications [10–13]. The entire team should decide to use intraoperative ECMO on a case-by-case basis, considering the patient's medical history and condition [14, 15]. Our study aims to highlight that lower doses of anticoagulants administered during intraoperative ECMO support do not lead to increased risks and may be beneficial for the patient.

Intraoperative ECMO Anticoagulation During Lung Transplants

A certain amount of anticoagulation is crucial to prevent thrombotic complications during lung transplantation with intraoperative ECMO support [1, 2, 11]. Thrombotic events, such as clot formation, can cause significant harm to the patient and negatively impact the transplant's success. However, the use of intraoperative anticoagulation is associated with a risk of bleeding, which can be problematic during surgery. Full heparinization with high activated clotting time (e.g., ACT >400 s) values are no longer necessary from CBP to ECMO support transition (also, thanks to heparin-coated ECMO cannulas and circuits). In contrast, the more knowledge we have about ECMO issues in the context of understanding coagulopathy, the more we strive for significantly lower doses of anticoagulation [4]. According to the current guidelines, the recommended procedure for ECMO cannulation is to administer a certain UFH bolus, usually 2000–5000 IU or 25–100 IU/kg, and then control the anticoagulation level using ACT [1, 11]. The ACT should be maintained in the range of 180–220 s [1, 11]. Other options (increasingly used over ACT) for anticoagulation monitoring are activated partial thrombin time (APTT) in the range of 60–90 s [16] and APTT ratio of 1.5–2.5 [16]. The anti-Xa assay is also a possible method for monitoring anticoagulation. The target values of the anti-Xa assay are 0.3–0.7 IU/mL [16]. According to the literature, it is also possible to perform anticoagulation monitoring using viscoelastic methods such as rotational thromboelastometry (ROTEM), explicitly using the CT INTEM/HEPTEM ratio [17].

MATERIALS AND METHODS

This study was approved by the Local Ethics Committee (reference number EK-786/23) and was registered in the clinical trial database at ClinicalTrials.gov (identifier number

TABLE 1 | Standard, essential post-transplant ICU care.

Mechanical ventilation

• Maximum effort to achieve early extubation
• Upon admission to the Intensive Care Unit
  • Pressure control mode mechanical ventilation (initially PEEP 8–10 cmH_2O)
  • Pressure support mode mechanical ventilation (PEEP 5 cmH_2O)
  • extubation

Vasopressoric support • First choice—Norepinephrine • Additional—Vasopressin
Immunosuppressives medication • Tacrolimus • Mycophenolate-mofetil • Methylprednisolone • Basiliximab
Antibiotics and antivirotics • Piperaciline-Tazobactam i.v. • Amphotericin inh. • Ganciclovir i.v.
Analgesia combination, according to a visual analogue scale and patient needs • Bilateral ESPB • Sufentanil • Paracetamol • NSAID • Dexmedetomidine • LMWH (Enoxaparine) s.c. based on antiXa assay • Ketamin
Thrombosis prevention • LMWH (Enoxaparine) s.c. based on antiXa assay
Specific examination and procedures • Chest X-ray every 24 h • Physiotherapy 4 times/day or based on the needs of the patient • Microbiology findings every 24 h • Pulmonary artery pressure continual monitoring • Continual hemodynamic monitoring • Early enteral feeding

Abbreviations: PEEP, Positive end-expiratory pressure; cmH_2O centimeter of water; i.v. intravenous; inh. inhalation; NSAID, non-steroidal anti-inflammatory drugs; LMWH, low molecular weight heparin, s.c. subcutaneous.

NCT06054997). This study was designed as a single-centre, retrospective, observational study that included all lung transplants performed between January 2020 and December 2022 within the Prague Lung Transplant Program Motol University Hospital. A total of 141 patients underwent transplantation during the study period. The exclusion criteria were lung transplantation without intraoperative ECMO support, block heart-lung transplantation, ECMO bridge-to-lung transplant, and planned postoperative ECMO. The inclusion criteria were lung transplantation performed with intraoperative ECMO support, central ECMO cannulation, and successful ECMO support termination at the end of surgery. According to the study inclusion criteria, only lung transplants performed under intraoperative central ECMO cannulation with successful ECMO weaning were included. In total, 109 patients fulfilled the inclusion criteria. Thirty-two patients were excluded based on the following criteria: heart-lung transplant (HLTx), n = 4; ECMO bridge, n = 4; transplantation (Tx) without ECMO, n = 8; and planned prolonged ECMO, n = 16. UFH was used for ECMO anticoagulation in all the patients. The subjects were divided into two groups for further analysis according to the UFH dose administered during the entire surgical procedure. In the first group, the UFH dose was ≤60 IU/kg/surgery. In the second group, the UFH dose was greater than 61 IU/kg/surgery. A cutoff value of 60 IU/kg was determined based on the available literature review. Values ≤60 IU/kg/surgery were considered relatively lower doses, and values >61 IU/kg/surgery were considered higher doses of UFH [2, 4, 10–13, 16–19]. The UFH effect was monitored using activated clotting time (ACT) values. The intraoperative haemoglobin level target for red blood cell (RBC) substitution was 100 g/L. Coagulopathy was managed according to the clinical experience of the anesthesiologist and viscoelastic Point of care methods (ROTEM, PFA). The parameters followed up intraoperatively in both groups were total blood loss in milliliters and related to the patient's weight during the surgery (assessed by the amount of blood in a calibrated suction device); the total amount of UFH administered to the patient during surgery in the international unit (IU) and related to patient weight; and the consumption of blood derivatives during the surgical procedure, such as RBC, fresh frozen plasma (FFP), and platelets (PLT). In both groups, ACT values were monitored after the administration of UFH before ECMO cannulation and then every 60 min. In both groups, protamine was administered at the end of the surgical procedure until physiological ACT values below 120 s were achieved (if needed). No type of biological glue to seal the anastomosis was used. In both groups, intraoperative VA ECMO was implanted electively, and no patient underwent urgent ECMO cannulation due to cardiac or pulmonary reasons. The Maquet Rotaflow RF-32 centrifugal pump provided Intraoperative VA ECMO support. Heparin-coated cannulas and a heparin-coated tubing system were used for cannulation. According to internal guidelines, the ECMO flow was maintained at 1/2 to 2/3 of the calculated cardiac output. In the postoperative period, we followed up on the development of hemothorax requiring surgical revision. We considered surgical revision for hemothorax to be a significant bleeding complication. We also evaluated blood product consumption in the period 48 h after ICU admission, PGD grade three in 72 h after LUTx, 30-day and 90-day mortality, long-term survival and ECMO circuit-related and patient-related thrombotic complications. The basic, standard points of care in the Intensive care unit (ICU) are displayed in **Table 1**.

Recipient and Donor Characteristics

Both groups of recipients were relatively homogenous, even though the number of subjects was not the same in both groups (lower dose UFH/kg group, n = 44; higher dose UFH/kg group, n = 65). The p-values for sex, age, height and mean pulmonary artery pressure (mPAP) were above the significance level of 0.05. The lower dose UFH/kg group recipients had a slightly higher weight (p = 0.048) and a higher BMI (p = 0.0093). The distribution of diagnoses for which the recipients were transplanted was also homogeneous

TABLE 2 | Recipient and donor characteristics.

Recipient-characteristic variable	≤60 IU/kg UFH group (*n* = 44)	>61 IU/kg UFH group (*n* = 65)	*p*-value
Male sex, n (%)	26 (59.09%)	44 (67.69%)	0.36
Age (years; mean ± SD)	54.14 ± 11.53	52.09 ± 12.47	0.59
Weight (kg; mean ± SD)	79.91 ± 15.47	73.93 ± 15.26	0.048
Height (cm; mean ± SD)	172.1 ± 7.39	173.4 ± 7.72	0.39
Body mass index (mean ± SD)	26.9 ± 4.43	24.6 ± 4.57	0.0093
mPAP (torr; mean ± SD)	26.3 ± 9.6	27.8 ± 13.4	0.528
thoracotomy prior LUTx (sum)	1	1	0.78
Transplant indication			
COPD, n (%)	15 (34%)	26 (40%)	0.59
Pulmonary fibrosis, n (%)	18 (40.9%)	23 (35.38%)	0.50
Cystic fibrosis, n (%)	3 (6.8%)	5 (7.69%)	0.89
Others, n (%)	8 (18.18%)	11 (16.92%)	0.82
Donor-characteristic variable	**≤60 IU/kg UFH group (*n* = 44)**	**>61 IU/kg UFH group (*n* = 65)**	***p*-value**
Male sex, n (%)	21 (41.72%)	37 (56.92%)	0.34
Age (years; mean ± SD)	47.41 ± 16.05	44.78 ± 16.32	0.41
Weight (kg; mean ± SD)	70.9 ± 13.62	76.7 ± 17.12	0.059
Height (cm; mean ± SD)	170.3 ± 10.12	172.6 ± 10.95	0.26
Body mass index (mean ± SD)	24.1 ± 3.64	25.6 ± 4.7	0.074
Cause of death			
Subarachnoid haemorrhage, n (%)	10 (22.7%)	14 (21.5%)	0.88
Intracerebral bleeding, n (%)	8 (18.2%)	14 (21.5%)	0.67
Traumatic brain injury, n (%)	15 (34.1%)	16 (24.6%)	0.28
Anoxic brain injury, n (%)	8 (18.2%)	13 (20.0%)	0.81
Others, n (%)	3 (6.8%)	8 (12.3%)	0.35

Abbreviations: IU, international unit; UFH, unfractionated heparin; SD, standard deviation; n, Number of Subjects; kg, Kilogram(s); COPD, chronic obstructive pulmonary disease; mPAP, mean pulmonary arterial pressure.

($p > 0.05$). These numbers are listed in **Table 2**. Donor characteristics were completely homogeneous in both groups. The *p*-values of sex, age, weight, height, BMI, and cause of death were above the significance level of 0.05 (**Table 2**).

Statistical Analysis

Statistical analyses were performed with version 8.0.1 (244) of GraphPad Prism statistical software. Statistical significance was set at $p < 0.05$. The unpaired *t*-test was used to statistically evaluate blood loss, UFH dose, consumption of blood derivatives, and ACT values. For the statistical evaluation of surgical revision for hemothorax and PGD, we chose chi-square test. Kaplan-Mayer curve and log-rank test have been performed for 30-day and 90-day" and long-term survival assessment (long-term survival time endpoint October/2023).

RESULTS

Patients were recruited between January 2020 and December 2022, and based on the exclusion criteria, a total of 32/141 patients were excluded from the study. A flow diagram based on the Consolidated Standards of Reporting Trials (CONSORT) is shown in **Figure 1**. The lower dose of UFH (≤60 IU/kg/surgical procedure) and higher dose of UFH (>61 IU/kg/surgical procedure) groups ultimately consisted of 44 and 65 patients, respectively. For most parameters, we obtained surprising results, clearly in favour of the administration of lower doses of UFH (≤60 IU/kg). Total blood loss during surgery was significantly lower in the group treated with lower doses of UFH (≤60 IU/kg). The mean total intraoperative blood loss was 753 and 1,470 mL, respectively ($p < 0.0001$) (**Table 3**). Blood loss related to body weight was also significantly lower in the UFH group ≤60 IU/kg). The mean intraoperative blood loss/patient body weight was 9.628 mL/kg and 20.97 mL/kg, respectively ($p < 0.0001$) (**Table 3**). The total UFH dose was significantly lower in the UFH group (≤60 IU/kg). The mean total intraoperative UFH doses were 3491 IU and 8694 IU, respectively ($p < 0.0001$) (**Table 3**). The total UFH dose, based on body weight, was significantly lower in the UFH group (≤60 IU/kg). The mean dose of UFH/patient bodyweight intraoperatively was 43.81 IU/kg and 116.6 IU/kg, respectively ($p < 0.0001$) (**Table 3**). We also noticed a significant difference in favour of reducing the consumption of blood derivatives in the group with lower doses of UFH ≤60 IU/kg. The consumption of RBCs during the surgical procedure was 0.5581 and 1.908 units, respectively ($p = 0.0009$) (**Table 3**). The FFP consumption during surgery was 0.4186 and 1.862 units, respectively ($p = 0.0009$) (**Table 3**). The platelet consumption during surgery was 0.1628 and 0.4154 units, respectively ($p = 0.1461$) (**Table 3**). The mean ACT values before ECMO cannulation and 3 min after the administration of UFH were lower in the UFH group ≤60 IU/kg (156.3 and 209.1) ($p < 0.0001$) (**Table 3**). There was a significant reduction in bleeding complications in terms of surgical revision for hemothorax in the lower UFH dose group ≤60 IU/kg, with only one revision (2.27%) and nine revisions (13.85%), respectively ($p = 0.040$) (**Table 3**). In the postoperative period 48 h after admission to the ICU, we did not

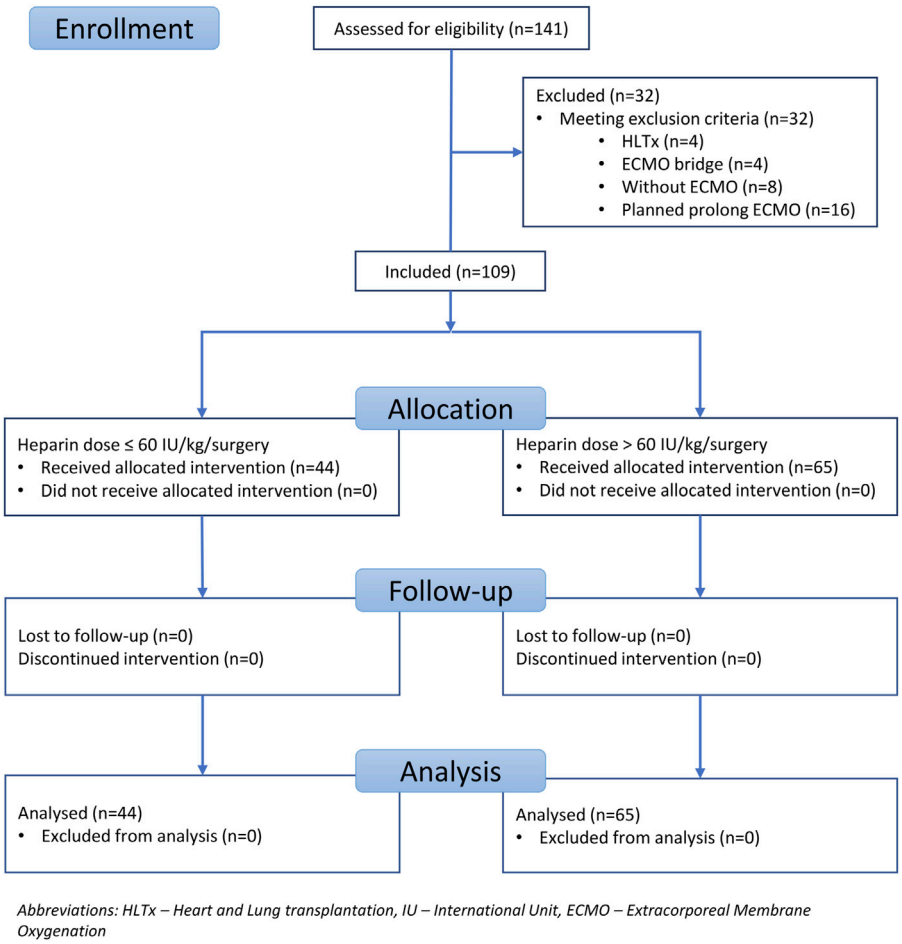


FIGURE 1 | Flow chart of the study population.

TABLE 3 | Variables blood loss, intraoperative blood product consumption, UFH dose, and surgical revision for haemothorax.

Variables	≤60 IU/kg UFH group (*n* = 44)	> 61 IU/kg UFH group (*n* = 65)	*p*-value
Total blood loss (ml; mean ± SD)	753.5 ± 522.5	1,470 ± 1,014	**<0.0001**
Blood loss per kg (ml/kg; mean ± SD)	9.63 ± 6.59	20.97 ± 15.64	**<0.0001**
Total dose of heparin (IU; mean ± SD)	3,491 ± 1,088	8,694 ± 3,790	**<0.0001**
Dose of heparin per kg (IU/kg; mean ± SD)	43.81 ± 12.08	116.6 ± 44.98	**<0.0001**
RBC consumption (unit)	0.5581 ± 1.119	1.908 ± 2.435	**0.0009**
FFP consumption (unit)	0.4186 ± 1.349	1.862 ± 2.543	**0.0009**
PLT consumption (unit)	0.1628 ± 0.5314	0.4154 ± 1.044	0.1461
Surgical revision due to haemothorax (% of revision)	2.27	13.85	**0.040**

Abbreviations: IU, international unit; UFH, unfractionated heparin, mL, millilitre; kg, kilogram; SD, standard deviation; RBC, red blood cells; FFP, fresh frozen plasma; PLT, platelets. Bold values are statistically significant.

observe a significant decrease in the consumption of blood derivatives (**Table 4**). However, there was a significantly lower incidence of third-degree PGD 72 h after LUTx in the group where a lower dose of UFH was administered (p = 0.038) (**Table 4**; **Figure 2**). The 30-day, 90-day, and long-term survivals were not different (**Figure 3**). The log-rank test was p = 0,6879 (**Figure 3**). Mortality rates were not different in either group. We did not record any thrombotic complications arising from the ECMO circuit or patient-related complications in any group.

DISCUSSION

Intraoperative ECMO support during lung transplantation is a routine method frequently used to facilitate surgical procedures.

TABLE 4 | Variables post LUTx (PGD 3 in 72hours post LUTx), (FFP, RBC, PLT 48 h post LUTx).

Variables	≤60 IU/kg UFH group (*n* = 44)	> 61 IU/kg UFH group (*n* = 65)	*p*-value
PGD grade 3 (total, percentage)	**0 (0%)**	**6 (9.23%)**	**0.038**
RBCs consumption mean (unit)	1.43 ± 2.0	1.55 ± 2.4	0.7996
FFP consumption mean (unit)	0.41 ± 1.4	1.12 ± 3.0	0.1567
PLT consumption mean (unit)	0.23 ± 1.1	0.35 ± 1.1	0.5615

Abbreviations: IU, international unit; UFH, unfractionated heparin; LUTx, lung transplantation; PGD, primary graft dysfunction; FFP, fresh frozen plasma; RBCs, red blood cells; PLT, platelets; IU, international unit.
Bold values are statistically significant.

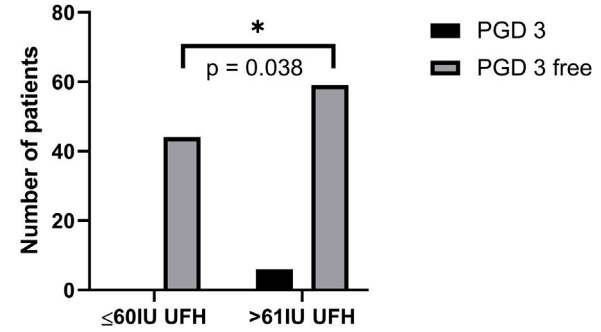


FIGURE 2 | PGD grade 3 in 72 h after LUTx. Abbreviations: PGD, primary graft dysfunction; UFH, unfractionated heparin; LUTx, lung transplantation.

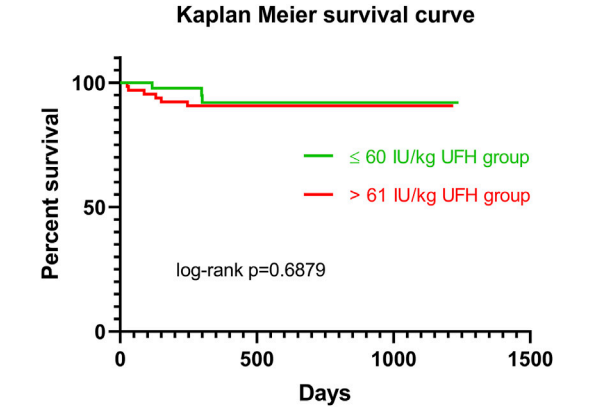


FIGURE 3 | Kaplan Meier survival curve for patients in ≤60 IU/kg UFH group (green line) vs >61 IU/kg UFH group (red line). Abbreviations: IU, international unit; UFH, unfractionated heparin.

This enables the procedure to be performed in significantly more polymorbid patients who cannot handle selective ventilation. Another indisputable advantage of ECMO is the possibility of controlled reperfusion of lung grafts. During ECMO support, the blood is in contact with allogeneic materials such as cannulas, circuits, and oxygenators. All of these factors can cause potential complications, including bleeding and thrombosis. Therefore, balancing the edge between anticoagulation and procoagulation is very important to minimize complications during intraoperative ECMO support. UFH remains the most widely used drug for ECMO anticoagulation. From the available recommendations of thoracosurgery societies, we know the recommendation for UFH dosing, most often between 25–100 IU/kg and effect control with ACT in the range of 180–220 s. However, the literature and the guidelines more frequently report the possibility of reducing the dose of UFH and, very importantly, without an increase in thrombotic complications. According to the recommendations of the thoracosurgery societies, in the case of bleeding complications or anticipated intraoperative bleeding (for example, significant intrapleural adhesions), it is recommended to minimize the dose of UFH or completely eliminate UFH and perform heparin-free ECMO. For example, Bernhardt et al. mentioned in The International Society for Heart and Lung Transplantation/ Heart Failure Society of America Guidelines on Acute Mechanical Circulatory Support that "bleeding complications in acute mechanical circulatory support (MCS) are common and frequently necessitate withdrawal of anticoagulation" and stated that "in the settings of life-threatening bleeding, full discontinuation of all anticoagulation may be necessary" [20]. Hartwig et al. stated in The American Association for Thoracic Surgery guidelines that "low or no heparin regimes are recommended for patients with significant adhesions and impaired coagulation status" [1]. Additionally, Lorusso et al., in the EACTS/ELSO/STS/AATS expert consensus, mentioned that "anticoagulation is required during prolonged ECLS to prevent circuit thrombus formation with embolization and/or circuit failure. However, bleeding remains the most frequent complication associated with ECLS. UFH infusion is typically delayed until haemostasis is achieved, often within 24–48 h. Reports that suggest the safety of prolonged withdrawal of anticoagulation for as long as 3 days when faced with bleeding are important" [21]. This trend was also confirmed by our study, which suggests that a lower dose of UFH as part of intraoperative ECMO support may not pose a risk but, on the contrary, can have substantial benefits for the patient. Using lower doses of UFH below 60 IU/kg (43.81 IU/kg based on our study) can benefit the patient and the entire perioperative period. Nonetheless, these results must be interpreted with caution, and numerous limitations should be considered. The major limitation is that the dosage of the UFH used in the study (high or low dose) has been based on subjective criteria, i.e., the clinical experience of the

anesthesiologist guides its dosage. Another possible limitation of this study may be the division of subjects according to UFH into groups below and above 60 IU/kg since the available literature does not define the exact dose of UFH but only the range. Therefore, it was necessary to set a limit for dividing the patients into groups. However, the medians of the two groups were very far apart 43.81 IU/kg vs. 116.6 IU/kg; we presume this is more of a minor bias. Furthermore, having a more significant number of investigated subjects would be advisable, which would add even more weight to the entire study. Similarly, a particular bias may have been introduced into this study because the primary hemostasis disorder was not investigated; primary hemostasis can be disturbed as part of ECMO support during lung transplantation, thereby potentiating intraoperative bleeding [22]. The authors hypothesize that the significantly higher incidence of PGD grade 3 in 72 h postop in the group with higher doses of UFH may be caused by the need to administer a larger number of blood derivatives, which is one of the possible reason for the development of PGD. The anesthesiologist plays a crucial role in the decision-making process and is mostly responsible for anticoagulation management of intraoperative ECMO support [15, 23, 24]. Such management depends not only on their experience but also on their knowledge of the latest findings and recommended practices. The decision to withhold anticoagulation therapy or decrease its dosage involves balancing the competing risks between bleeding and clotting.

Conclusion

The results of this study generates a hypothesis that lower doses of UFH (mean dose of UFH: 43.81 IU/kg) administered for intraoperative ECMO anticoagulation may contribute to a reduction in intraoperative blood loss and decrease the incidence of surgical revisions for haemothorax. Furthermore, lower doses of UFH may reduce the intraoperative consumption of blood derivatives such as RBCs and FFP. Notably, the concept of lower doses of UFH did not have a negative effect on 30-day, 90-day and long-term survival. No thrombotic complications of the ECMO circuit or thrombotic complications related to the patient were observed. Further investigation in this area is needed to provide deeper insights into the potential use of lower doses of UFH during ECMO.

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 studies involving humans were approved by the Local Ethics Committee (reference number EK-786/23). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

AUTHOR CONTRIBUTIONS

JaV designed the study; JaV and GH collected the study data; JaV analyzed the data and wrote the major part of the manuscript; GH, JiV, MS, RN, TV, and RL participated in the research performance and/or substantially contributed to the writing of 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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BRIEF RESEARCH REPORT
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A Split-Lung *Ex Vivo* Perfusion Model for Time- and Cost-Effective Evaluation of Therapeutic Interventions to the Human Donor Lung

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With the ongoing shortage of donor lungs, *ex vivo* lung perfusion (EVLP) offers the opportunity for objective assessment and potential therapeutic repair of marginal organs. There is a need for robust research on EVLP interventions to increase the number of transplantable organs. The use of human lungs, which have been declined for transplant, for these studies is preferable to animal organs and is indeed essential if clinical translation is to be achieved. However, experimental human EVLP is time-consuming and expensive, limiting the rate at which promising interventions can be assessed. A split-lung EVLP model, which allows stable perfusion and ventilation of two single lungs from the same donor, offers advantages scientifically, financially and in time to yield results. Identical parallel circuits allow one to receive an intervention and the other to act as a control, removing inter-donor variation between study groups. Continuous hemodynamic and airway parameters are recorded and blood gas, perfusate, and tissue sampling are facilitated. Pulmonary edema is assessed directly using ultrasound, and indirectly using the lung tissue wet:dry ratio. Evans blue dye leaks into the tissue and can quantify vascular endothelial permeability. The split-lung *ex vivo* perfusion model offers a cost-effective, reliable platform for testing therapeutic interventions with relatively small sample sizes.

Keywords: donor lungs, *ex vivo* lung perfusion, organ assessment, lung transplantation, split-lung perfusion model

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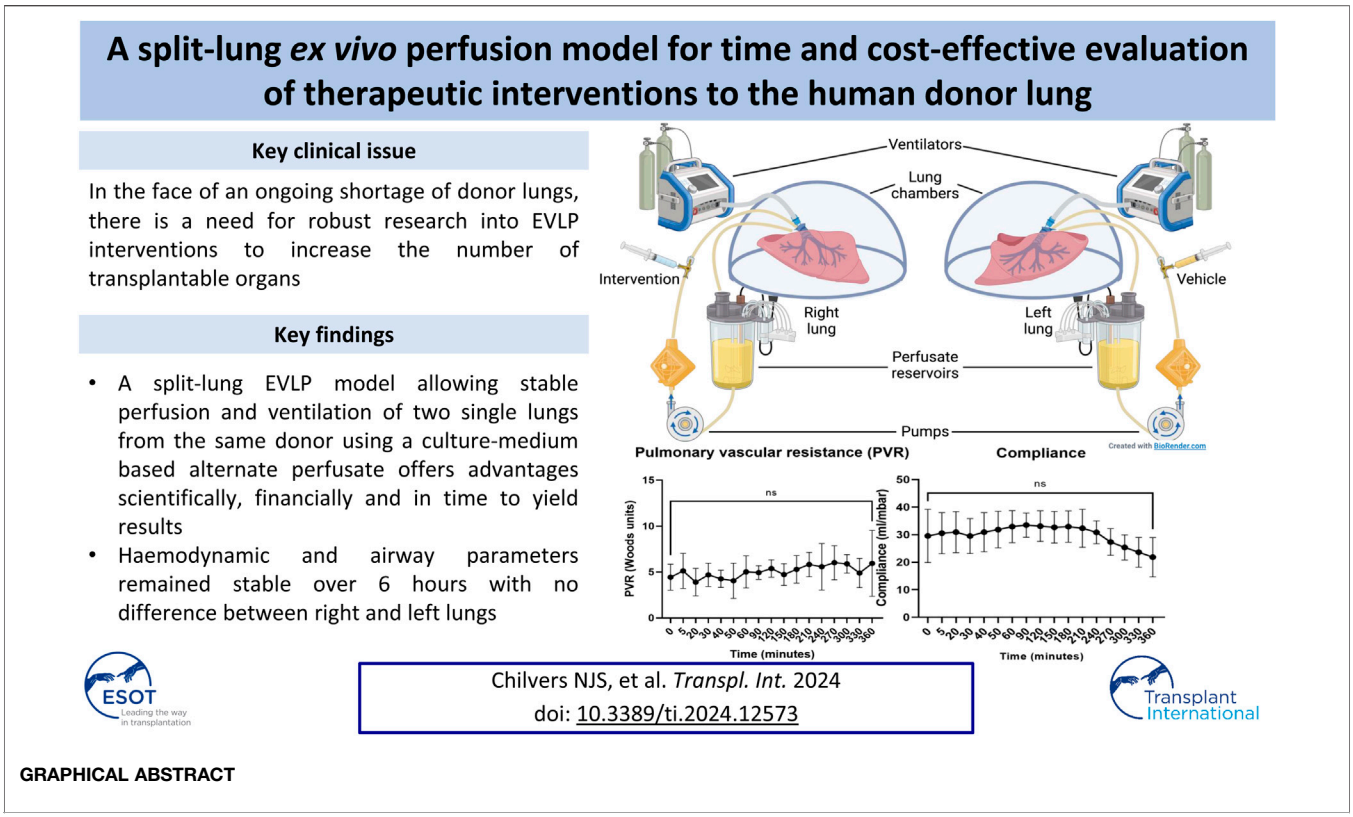
INTRODUCTION

Despite a significant demand for organs, more than 80% of donor lungs from brain-dead donors offered for transplantation are currently declined as unsuitable in the United Kingdom [1]. This is largely because the lungs are highly susceptible to injury, which impairs function and negatively affects transplant outcomes. Marginal or "extended criteria" organs, for example, from older donors >65 years old, are rarely used. The impact for patients is that there continues to be a large discrepancy between the number of patients on the waiting list and the number of organs accepted for transplant and hence the number of lung transplants performed [1].

Research efforts have focused on preventing or minimizing donor lung injury and optimizing the quality of marginal donor organs through assessment and preservation. One such method is *ex vivo* lung perfusion (EVLP) which provides the opportunity for objective organ assessment and a time



GRAPHICAL ABSTRACT

period for therapeutic repair [2, 3]. Steen et al. in Sweden performed the first clinical transplant following EVLP in 2001 using lungs from an uncontrolled Donation after Circulatory Death donor [4]. Subsequently, this group demonstrated that lungs initially rejected for transplantation could be successfully transplanted following reconditioning using EVLP [5]. Multiple studies have now shown that marginal donor lungs transplanted after EVLP have similar outcomes to standard donor organs preserved by static cold storage on ice [3, 6]. Furthermore, EVLP allows for longer preservation times, which offers logistical benefits.

EVLP was initially developed for assessment/preservation but there is increasing interest in EVLP as a platform for therapeutic intervention. This is particularly attractive as, in the isolated lung, there is no renal/hepatic excretion or risk of off-target toxicity [2]. While early research studies have focused on determining optimal perfusion parameters, further research is required to overcome the hurdles that limit wider usage and to ensure clinical translation that will maximize the potential of EVLP as a therapeutic platform [3].

In the clinical setting, EVLP is almost exclusively performed on intact double lungs, but in the research setting this approach presents challenges due to inherent variability and confounding factors between different donors in the treatment and control groups, requiring larger sample sizes. We have therefore developed a split-lung human EVLP model consisting of two identical independent perfusion circuits, each with its own ventilator. Recently mechanisms of IL-1β-mediated Inflammation [7]. Since then, we have further optimized this setup, including more comprehensive pressure monitoring, and tested Dulbecco's Modified Eagle Medium (DMEM)-based perfusate, previously described by Shaver et al. [8]. This model offers a cost-effective, reliable platform for testing therapeutic interventions with relatively small sample sizes.

MATERIALS AND METHODS

Research Approval and Ethics

Donor organs, declined for transplantation, are only utilised where consent has been obtained for research by Specialist Nurses in Organ Donation. The NIHR Blood and Transplant Research Unit laboratory in Newcastle has ethical approval from the NHS North East Research Ethics Committee, reference 16/NE/0230, and NHSBT (Study 66).

Donor Lung Retrieval and Preparation

The UK benefits from the national "Increasing the Number of Organs Available for Research" (INOAR) framework, established by National Health Service Blood and Transplant (NHSBT), through which the next of kin of organ donors are approached for consent for research if organs are not suitable for transplantation. Patient history, clinical findings, and diagnostic investigations, including chest imaging, are reviewed to assess suitability for EVLP studies. Due to our split-lung model, we excluded lungs with unilateral pathology, e.g., trauma, infection, or consolidation, in addition to lungs with significant trauma/contusions, blood-borne viruses, or significant/untreated infection. Donor lungs were assessed and explanted by the NHSBT National Organ Retrieval Service

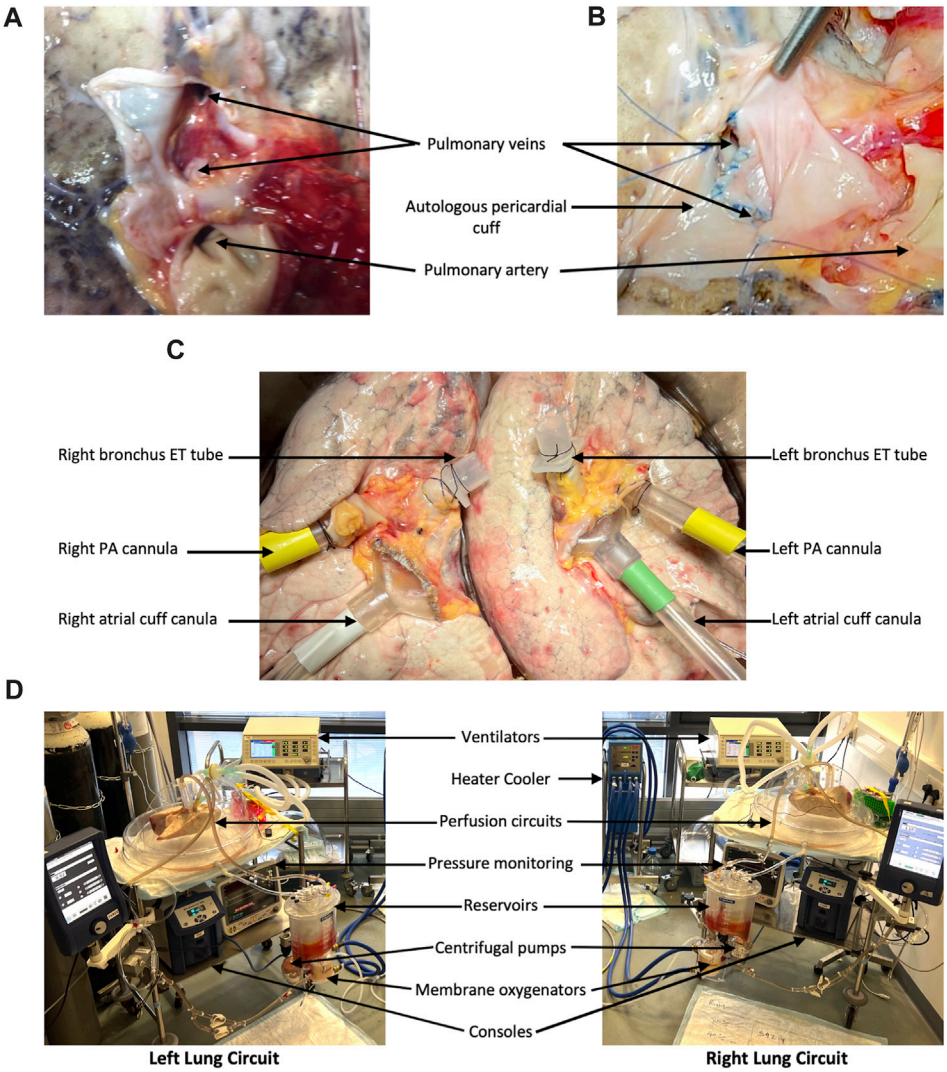


FIGURE 1 | Lung preparation and split-lung circuits. In order to have a closed left atrium (LA) in the circuit, donor lungs without a sufficient LA cuff **(A)** require reconstruction with autologous pericardium **(B)**. The lungs are separated before EVLP cannulae are sutured to the LAs and pulmonary arteries (PAs) and shortened ET tubes are secured in the left and right main bronchi **(C)**. Our split-lung model consists of two separate identical circuits attached to two ventilators **(D)**.

Cardiothoracic Teams in a standard fashion, including antegrade and retrograde flushing with Perfadex (XVIVO Perfusion AB, Sweden) and inflation with 50% O_2, prior to static cold storage in an icebox. Certified medical couriers transported the lungs from the donor hospital to our research laboratory.

Upon arrival in the laboratory, the lungs were placed in cold 0.9% saline. Excess tissue and pericardium were excised and dissection was carried out to prepare the pulmonary arteries, veins, and bronchi for cannulation/intubation. The posterior wall of the left atrium (LA) was divided in the midline to create two LA cuffs, left and right, each receiving a superior and inferior pulmonary vein. On occasion, the received lungs may have had very little LA tissue (**Figure 1A**) and the cuffs had to be reconstructed with autologous pericardium (**Figure 1B**). Two XVIVO EVLP LA cannulae (XVIVO Perfusion AB, Sweden) were cut to size and sutured to the LA cuffs using 4-0 Prolene (**Figure 1C**). The pulmonary artery (PA) was divided at its bifurcation and two XVIVO PA cannulae were inserted into the left and right PAs and secured with 2-0 silk purse-string sutures (**Figure 1C**). Finally, the left main bronchus was clamped and divided proximally (keeping, initially, the left lung inflated). This bronchial stump was then securely closed, flush with the trachea, allowing separate intubation of the main trachea (well clear of the right upper lobe orifice) and then separately of the left main bronchus. The endotracheal tubes were secured with 2-0 silk sutures (**Figure 1C**).

DMEM Perfusate

A 5% bovine serum albumin (BSA) solution was prepared by adding 25 g of BSA (Melford Laboratories Ltd., United Kingdom)

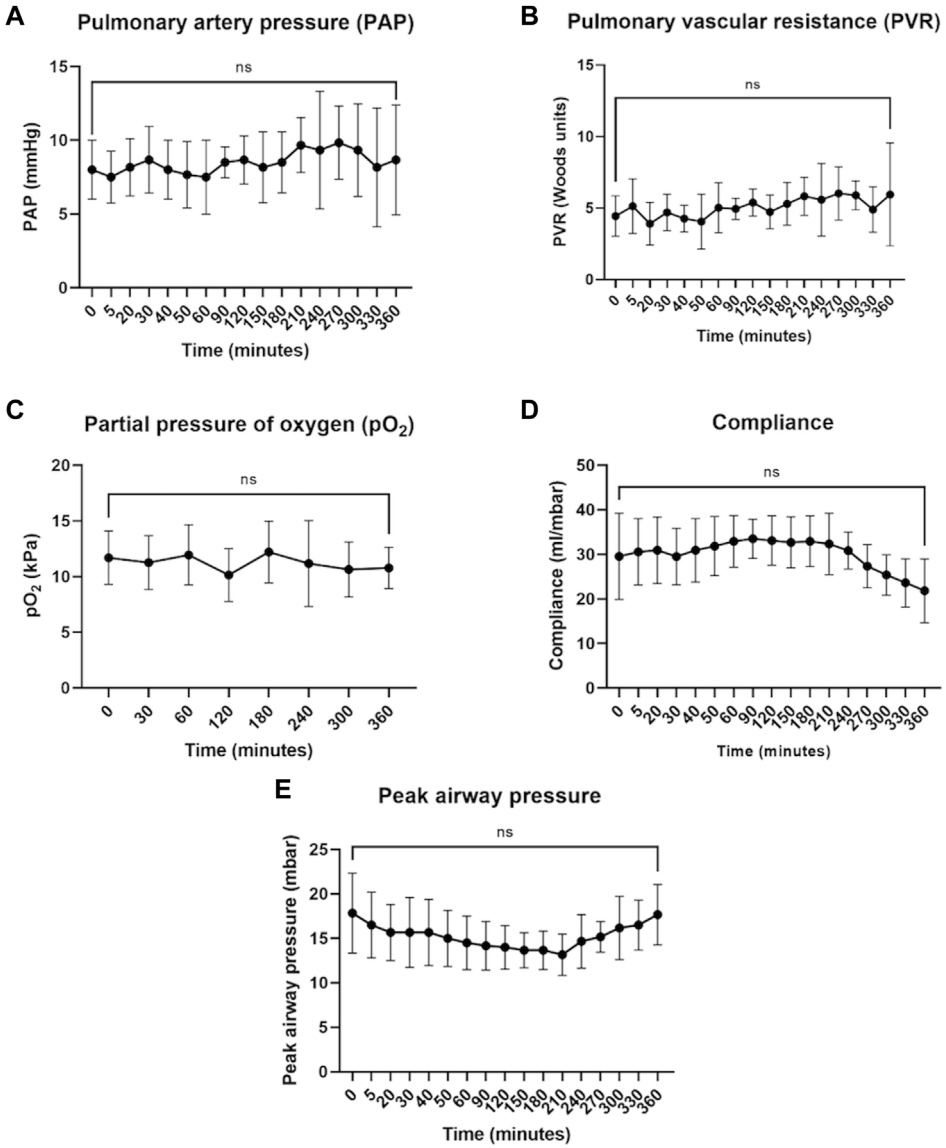


FIGURE 2 | Hemodynamic, blood gas, and airway parameters during 6 h of perfusion. Pulmonary artery pressure **(A)**, pulmonary vascular resistance **(B)**, and partial pressure of oxygen **(C)** remained stable throughout perfusion. Compliance **(D)** and peak airway pressure **(E)** showed some improvement but by the end of 6 h returned to values similar to time 0 (defined as the point at which full flow and ventilation were established). The data are expressed as mean ± SD, and analyzed with one-way repeated measures ANOVA and with Dunnett's multiple comparisons test to determine statistical significance compared to time 0. The data presented are n = 6 unpaired control lungs from six independent split-lung perfusions.

to each 500 mL of DMEM-high glucose, without phenol red (Sigma-Aldrich, United States). The solution was mixed with a magnetic stirrer until dissolved before purification using a vacuum filter unit. DMEM perfusate was stored at 4° until required.

EVLP

The EVLP setup (**Figure 1D**) and protocol for this model are based on those described in the Toronto protocol [9]. Two separate but identical circuits (Medtronic Limited, United Kingdom) consisted of outflow tubing to a reservoir from which perfusate was pumped, by a centrifugal pump, through a membrane oxygenator (attached to a heater-cooler) to the inflow tubing. Circuits were primed with 1500 mL of perfusate and 7500 IU of heparin and heated initially to 32°. Cardiac output was calculated based on the ideal donor body weight and divided between the left and right lungs with a 45%/55% split. Flow was started at 20% and the PA cannulae were attached to the lungs followed by the LA cannulae. Pressure monitoring was attached to the PA and LA cannulae. The LA pressure was maintained between 3 and 5 mmHg by adjusting a gate clamp on the outflow tubing, the LA pressure was maintained between 3 and 5 mmHg. Over 15 min, flow was increased to 40%.

Once the lung temperature reached 32°, two separate ventilators were attached to the ET tubes. The protective ventilation protocol (**Supplementary Table S1**) slowly increased tidal volume with each further degree of temperature increase until tidal volumes of 7 mL/kg (split 45%/55%) were reached. Regular blood gas analyses were carried out. CO_2 flow to the membrane oxygenator was adjusted to maintain pCO_2 between 3 and 5 kPa. Tris (hydroxymethyl)aminomethane was added to maintain a pH between 7.35 and 7.45.

Assessment of Lung Physiology

Hemodynamic parameters including PA pressures and ventilation parameters such as compliance and peak airway pressures were measured continuously throughout the perfusion. Regular blood gas and perfusate samples were taken according to a comprehensive established protocol. Depending on the research question, tissue samples were also collected for histological and transcriptomic analysis.

Assessment of Pulmonary Vascular Leak

Pulmonary edema/extravascular lung water were assessed by lung weight, tissue sampling for wet-to-dry ratio, and ultrasound assessment using the DireCt Lung Ultrasound Evaluation (CLUE) score [10]. Additionally, after 2 h of perfusion, 0.05% Evans blue (0.75 g/1500 mL) was added to each circuit, and tissue and bronchioalveolar lavage (BAL) samples were taken 2 and 4 h later. Evans blue concentration is measured spectrophotometrically as a marker of endothelial permeability.

Statistics

Data are presented as mean ± standard deviation (SD). Weight increase was compared using a Student's unpaired *t*-test, whereas one-/two-way repeated measures ANOVAs or mixed-effects analyses with multiple comparisons were used for hemodynamic/ventilation parameters. Evans blue concentration was analyzed using a Student's paired t-test or one-way ANOVA. Statistical tests were performed using GraphPad Prism 9.5.0 and p values less than 0.05 were considered statistically significant.

RESULTS

The Split-Lung Model With DMEM-Based Perfusate Provides Stable Perfusion for 6 h

Lungs were retrieved from 6 donors (5 men and 1 woman, 1 DCD donor, and 5 DBD donors) with a mean age of 34.8 ± 10.2 years. The cause of death was either hypoxic brain damage or intracranial hemorrhage and the lungs were rejected for clinical transplantation due to poor function (n = 3), lack of suitable recipients (n = 2), or infection (n = 1). During 6 h of normothermic perfusion of control single lungs with prolonged cold ischemic times (11.5 ± 1.8 h), there was no significant change in hemodynamic parameters, including pulmonary artery pressure (**Figure 2A**) and pulmonary vascular resistance (**Figure 2B**), blood gas parameters such as pO_2 (**Figure 2C**) and compliance (**Figure 2D**). Peak airway pressure varied over time (ANOVA p = 0.02), but multiple comparisons were not significant. At 6 h there was no significant difference from time 0 (**Figure 2E**). There was no difference in parameters between the left and right lungs (**Supplementary Figure S1**).

In a study of four unpaired single lungs comparing DMEM versus STEEN solution as a perfusate, we found no significant difference in the percentage increase in lung weight (46.7% ± 23.2% vs. 99.4% ± 36.3%, p = 0.226).

The Split-Lung Model Provides a Platform for Comprehensive Lung Assessment

Pulmonary edema/extravascular lung water could be readily evaluated using several techniques. The ultrasound CLUE score uses a non-invasive technique to quantify edema (**Figure 3A**). Evans blue could be appreciated visibly, and quantified at multiple time points, from both tissue (**Figure 3B**) and BAL (**Figure 3C**) samples. Both increased during perfusion, reaching significance in BALs. Additionally, lung weights were measured pre- and post-perfusion and a large tissue sample was taken to calculate a wet-to-dry ratio (data not shown).

Split-Lung Perfusion With DMEM-Based Perfusate Offers a Cost-Effective Research Model

Table 1 displays the costs of consumables for the 2 separate circuits that comprise the split-lung model. Additional costs, not shown, include staff time, specialized courier transport of the organs, and equipment rental/maintenance.

The cost of 4 L of DMEM-based perfusate is £148, which is significantly less expensive than more commonly used options such as STEEN solution. As a result, the total cost of consumables, approximately £2,473/€2879/$3,107, makes this approach affordable in the research setting.

DISCUSSION

There is a growing interest in the optimization and assessment of extended criteria donor lungs using EVLP. Research initially focused on establishing optimal perfusion protocols, parameters for functional lung assessment, and, in clinical studies, their impact on, or correlation with, patient outcomes [2, 3]. Recent studies have proposed more accurate methods of assessment, including the CLUE score [10] and the "PaO_2/FiO_2 ratio difference" (the difference between PaO_2/FiO_2 at a FiO_2 of 1.0 and 0.4) [11]. Lately, research has shifted focus to more sophisticated biomarkers and to assessing the efficacy of interventions on lung function or in reducing risks after implantation. EVLP is particularly attractive for the latter due to the absence of off-target effects [2]. Studies have also suggested that it may even be possible to treat transmissible conditions from the donor, such as hepatitis C and cytomegalovirus [12, 13].

We have described an optimized split-lung EVLP model based on the Toronto perfusion protocol [9], which is generally accepted as being preferable for longer preservation studies [14]. Acellular perfusate removes the risk of infection and

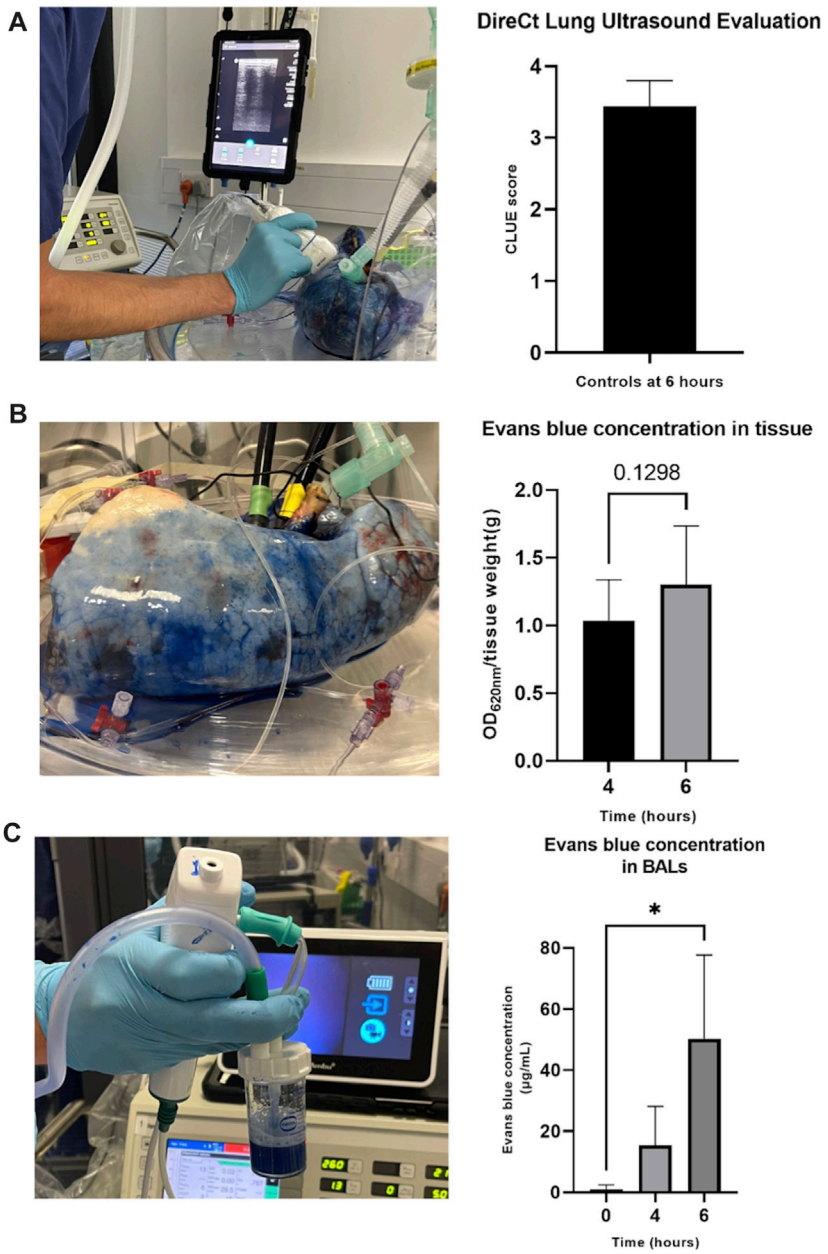


FIGURE 3 | Assessment of pulmonary edema/extravascular lung water during perfusion. DireCt Lung Ultrasound Evaluation (CLUE) was performed by using ultrasound to assess 14 (left) or 16 (right) areas of the lung, providing an average CLUE score **(A)**. Evans blue concentration in tissue was analyzed by placing biopsy samples in formamide and heating at 55°C for 24 h before centrifuging, transferring supernatant samples to a 96-well plate, and reading using a spectrophotometer at 620 nm **(B)**. Evans blue concentration was measured in BAL samples by centrifugation, placing the supernatant in a 96-well plate and reading alongside a standard curve on the spectrophotometer at 620 nm **(C)**. The data are expressed as mean ± SD, and analyzed by a Student's paired t-test **(B)** or one-way repeated measures ANOVA and Dunnett's multiple comparison test **(C)**. The data presented are $n = 6$ unpaired control lungs from six independent split-lung perfusions. *$p < 0.05$.

hemolysis, simplifies logistics, and has been shown to have no detrimental effect on physiological outcomes compared to cellular perfusate [15, 16]. The lower flow rates are associated with improved oxygenation [14, 17] and wet-to-dry ratio [17]. A closed LA offers improved hemodynamic parameters, compliance and oxygenation, and less edema [18]. Our 45%/55% split of donor-specific calculated flow and tidal volumes ensures comparable results between the left and right lungs. Accurate pressure monitoring in both LAs and PAs allows the maintenance of physiological conditions. Recently, we have introduced DMEM-based perfusate [8], although blood or specific cells such as isolated neutrophils could be added depending on the research question. Huang et al. showed that a similar DMEM-based perfusate reduced apoptosis and

TABLE 1 | Costs of consumables for the split-lung model (2 identical circuits) and DMEM-based perfusate. Costs are based on quotes that were correct at the time of manuscript submission.

Consumables	DMEM perfusate protocol
Lung perfusion Circuits × 2	£1,200
Lung cannula set	£705
Perfusate (4 L)	
DMEM	£56
Bovine Serum Albumin	£60
Disposable bottle top filters	£28
Heparin	£4
Plasticware and consumables	£200
Ventilator consumables, suction consumables, pressure monitoring lines, sutures, scalpels, staples, specimen tubes, cryovials	
Medical gas/air	£45
Blood gas analyzer cartridges/consumables	£110
Reagents	£65
Formalin, RNA later, THAM, formamide, Evans blue	
Total cost	£2,473

increased both glutathione and heat shock protein 70 protein levels compared to STEEN, in an EVLP cell culture model [19]. DMEM also has a higher osmolality (317–351 mOsm/kg vs. 275–315 mOsm/kg).

The major benefit of the split-lung model is the intra-donor control. This allows research studies to use smaller numbers of replicates per experiment as each lung pair comes with a treatment and control organ. This is especially important in human research because lungs are such a precious resource, but most importantly it ensures that they are better matched, eliminating the numerous inherent confounding factors of having entirely different donors in the treatment and control groups. The advantages of this approach have been described in the context of hepatitis C treatment trials, as the control lung would have a similar viral load [12]. This methodology has been used in our laboratory to understand the mechanism of interleukin-1β driven inflammation during EVLP [7], and to assess the therapeutic effects of endothelial barrier protection, potential COVID-19 treatments [20], cell therapies, and, currently, extracellular vesicles, with significant results from only five lung pairs. Smaller numbers allow studies to be completed more quickly and mitigate the risk of unbalanced groups in the event of recruitment failure, as both arms are balanced throughout. This model could also be used for large animal research where an experimental transplantation outcome is required as part of the investigation.

The affordability of this set-up, largely due to the modest costs of DMEM perfusate, makes it a more viable research model. The smaller number of experimental replicates more than offsets the cost of the additional consumables per lung pairs through savings in organ transport and personnel costs. Furthermore, cannulae and lung domes can be reused multiple times for pre-clinical research so the costs listed in **Table 1** are overestimates. Strong links through collaborative work between our university laboratory and the hospital provide access to equipment such as ICU standard ventilators retired from clinical service, and expired consumables.

To improve organ utilization, several countries, including the United Kingdom, have either established or are actively considering a system of centralized organ "Assessment and Recovery Centres." Benefits include centralized expertise, as lower-volume centers have been observed to have worse outcomes [21], standardization of protocols, and the ability to conduct multicenter trials. A feasibility study in the US demonstrated similar survival rates following transplantation using organs from a centralized EVLP program compared to conventional lung transplant recipients [22]. The authors suggest that centralization will be particularly important as EVLP is increasingly used for the delivery of therapeutics especially if prolonged perfusion is required to facilitate this. There is therefore a need for concurrent research into accurate markers of organ injury/function and potential therapeutics. Organ assessment and repair centers increase the opportunities for both clinical and preclinical research, as organs turned down before or during clinical EVLP can also be used in preclinical studies.

Considerations/Limitations

Although affordable, there are still significant costs and complex logistics associated with this model. We mitigate the former somewhat by not priming circuits until we have assessed the donor lung pairs on arrival. The split-lung closed LA model requires greater surgical expertise for timely cannulation, particularly if reconstruction is required. Finally, the lung weight data presented here for STEEN vs. DMEM were from unpaired lungs and were not adjusted for donor differences. However, we were satisfied with the performance of DMEM and, in the research setting, STEEN is not a feasible expense for most research centers.

CONCLUSION

In an exciting era where centralized lung assessment and repair may become the norm, this split-lung EVLP model, using a culture medium-based alternate perfusate, offers a cost-effective way to test therapeutic interventions with relatively small sample sizes. The in-built control offered by the contralateral lung affords robust results and we would encourage the adoption of this model for future preclinical EVLP research.

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 studies involving humans were approved by the NHS North East Research Ethics Committee, reference 16/NE/0230,

and NHS Blood and Transplant (Study 66). Through the NHSBT national organ donation programme, donors or their families are consented for organ donation for transplant and research. 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

NC, JG, MB, LB, CP, JD, and AF conducted the experiments, HP provided expertise regarding ventilation. All authors contributed to the article and approved the submitted version.

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AUTHOR DISCLAIMER

The views expressed are those of the author(s) and not necessarily those of the NIHR, NHS Blood and Transplant, or the Department of Health and Social Care.

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.2024.12573/full#supplementary-material

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REVIEW
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The Advent of Semi-Elective Lung Transplantation—Prolonged Static Cold Storage at 10°C

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Since the early days of clinical lung transplantation the preservation of donor organs has become a fairly standardized procedure and most centers do follow similar processes. This includes the use of low-potassium high dextran flush solutions and static cold storage (SCS) in a cooler filled with ice. Depending on the length of SCS, organs usually arrive at the recipient hospital at a temperature of 0°C–4°C. The question of the optimal storage temperature for donor lung preservation has been revisited as data from large animal experiments demonstrated that organs stored at 10°C experience less mitochondrial damage. Thus, prolonged cold ischemic times can be better tolerated at 10°C—even in pre-damaged organs. The clinical applicability of these findings was demonstrated in an international multi-center observational study including three high-volume lung transplant centers. Total clinical preservation times of up to 24 hrs have been successfully achieved in organs stored at 10°C without hampering primary organ function and short-term outcomes. Currently, a randomized-controlled trial (RCT) is recruiting patients with the aim to compare standard SCS on ice with prolonged SCS protocol at 10°C. If, as anticipated, this RCT confirms data from previous studies, lung transplantation could indeed become a semi-elective procedure.

Keywords: 10°C, lung transplantation, semi-elective, preservation, prolonged storage

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INTRODUCTION

Since the initiation of clinical lung transplantation, the procedure has always been defined as an emergent or acute operation, which requires teams to adhere to a strict timeline to minimize ischemic injury to the graft. There is currently a general consensus within the lung transplant community, that the length of graft preservation clinically should not exceed 6–8 h. This practice is reflected in large database analyses including the International Society for Heart and Lung Transplantation (ISHLT) Registry data. These studies have uniformly shown that short-term mortality increases with longer preservation time, with 30-day survival being significantly worse for ischemic times greater than 6 h [1]. Storage on ice has been considered the standard of care for preservation of donor lungs (and all organs for that matter) and has been implemented as such in lung transplant centers around the world. Storage on ice is intended to provide storage temperatures ranging between 0°C and 4°C, which is empirically considered clinically safe.

CURRENT EVIDENCE IN DONOR LUNG PRESERVATION

The current practice in lung transplantation—including optimal donor lung preservation–is mostly based on empirical clinical experience and expert consensus. Due to the low number of procedures

performed per year, it has often been considered difficult to provide robust data in the field of lung transplantation. Moreover, the significant heterogeneity among patients and center practice has represented a major hurdle to design multicenter prospective clinical trials. To the best of our knowledge, only two randomized controlled trials have been published in the field of donor lung preservation. Both of them compared an *ex-vivo* lung perfusion (EVLP) protocol with SCS. The INSPIRE trial [2] tested the Organ Care System in "standard" donor lungs. Before that, a single-center RCT was conducted by the Vienna Lung Transplant Program which compared the Toronto EVLP protocol to SCS, also in standard donor lungs [3]. Both trials showed comparable results of EVLP with the "simpler" and "cheaper" static preservation on ice. Therefore, EVLP did not replace SCS, but was introduced to clinical practice as a tool to evaluate lungs with a questionable quality and to optimize marginal donor lungs [4–8].

LUNG PRESERVATION AT 10°C

As early as 30 years ago, attempts were made to understand the effects of temperature on graft function and to optimize the temperature during donor lung preservation [9]. Subsequently this was confirmed in a study by Kayano et al., using a rat model, that reported an optimal storage temperature of 10°C [10]. Similar findings were observed in a large animal (canine) lung transplant study, published in 1992, comparing three different preservation temperatures [11]. Once again, lungs preserved at 10° degrees showed better oxygenation and decreased pulmonary vascular resistance. However, because of the lack of ability to accurately maintain organs at 10°C at the time (and incomplete understanding of the underlying biologic mechanisms), clinical concern that a temperature increase above this 10°C threshold could have deleterious effects on the lungs, the lower, more convenient target value of 4°C was widely adopted to provide a safety margin. As such, SCS on ice came to be defined as the "standard of care."

One of the main disadvantages of SCS on ice is that the true temperature of the donor lung can significantly diverge from 4°C. A thermographic evaluation of donor lungs transported on ice showed that the surface temperature was non-homogenous and depending on preservation times ranged from 0.2°C to 10.6°C [12]. Consequently, temperature-controlled preservation devices, which use more accurate cool packs instead of ice cubes, have been developed.

Rationale

The rationale underlying hypothermic organ storage is to reduce cellular metabolism, and thus maintain viability during the storage time with limited oxygen and nutrients. Since most of the deleterious effects of hypoxia are caused by simple biochemical reactions, it seemed reasonable to reduce the temperature close to 4°C in order to decrease enzyme activity in the donor organ. However, because this approach is non-selective, vital enzymes, such as Na+/K+ ATPase, are also affected in their function, leading to an ionic imbalance that can result in cell edema and damage [13]. In addition, intracellular calcium accumulation induces further cellular damage and the formation of reactive oxygen species can be promoted during cold ischemia [14]. In recent years, efforts have been made to optimize preservation strategies by revisiting the topic of optimal storage temperature of donor lungs. These efforts aimed to extend preservation times in order to increase the donor pool, optimize the immunological matching between donor and recipient and transition organ implantation from an urgent to a semi-elective procedure.

Pre-Clinical Studies

First, the feasibility of prolonged donor lung storage at 10°C was assessed in a large animal model. Porcine lungs stored for 36 h at 10°C showed lower airway pressures, had a better lung compliance and improved oxygenation after implantation as compared to lungs stored conventionally on ice [15]. Importantly, markers of mitochondrial injury were found to be lower in the study group, which provided a mechanistic insight into the benefit of 10°C storage (**Figure 1**). In a subsequent study, a possible recovery or regenerative effect of 10°C preservation was tested in a model of gastric acid aspiration injury [16]. Moderate lung damage was induced by intrabronchial instillation of gastric juice. Injured donor lungs were harvested and randomly assigned to storage for 12 h on ice or at 10°C. A third group consisted of immediate transplantation after only a short period of SCS on ice. A left single lung transplant was performed, followed by a 4-hour functional assessment. During reperfusion, lungs stored at 10°C showed significantly better oxygenation. Moreover, they had lower tissue levels of IL-1β after reperfusion, histologic evaluation demonstrated lower acute lung injury scores and significantly less apoptosis in the 10°C group. In all measured parameters, storage of lungs at 10°C for 12 h was associated with improved graft quality, even when compared to minimal cold ischemia on ice.

Clinical Studies

These preclinical data were then translated to the clinical setting and a multicenter non-randomized clinical trial was designed. Three high-volume lung transplant centers—Toronto, Vienna, Madrid—recruited patients in a safety and feasibility study [17]. In this study, grafts from donors with cross-clamp times between 6:00 PM and 4:00 AM had an earliest possible implantation start time of 6:00 AM. Lungs were retrieved and transported using a traditional ice cooler. Upon arrival at the transplant hospital, the lungs were transferred to a temperature-controlled 10°C incubator (MYTEMP™65HC, Benchmark Scientific) and were stored until implantation. The primary outcome of this study was the incidence of Primary Graft Dysfunction (PGD) grade 3 at 72 h. Results were compared to a contemporary cohort of recipients, who received donor lungs that had been preserved by SCS on ice, using propensity score matching at a 1:2 ratio. Seventy patients were included in the study arm. Mean cold static preservation was significantly longer in the 10°C study group vs. matched controls for both the first and second implanted lung (**Table 1**). PGD grade 3 at 72 h was 5.7% in the study group vs. 9.3% in matched controls ($p = 0.39$). There were no differences in

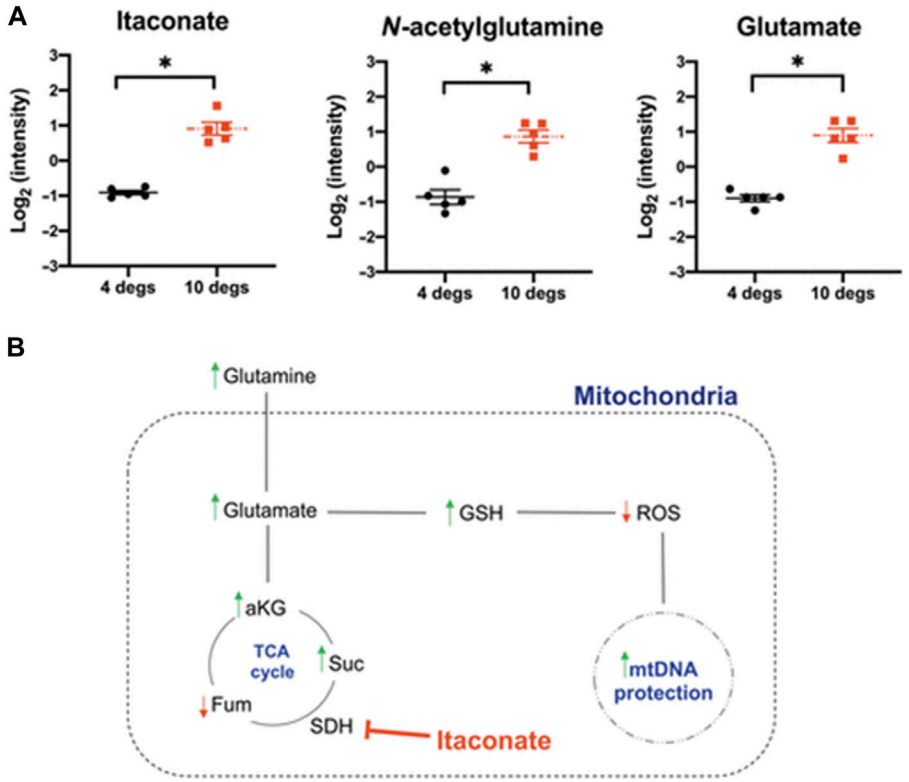

FIGURE 1 | Levels of metabolites relevant for mitochondrial oxidative protection are displayed in **(A)**. **(B)** shows a scheme of how the tricarboxylic acid (TCA) cycle impacts mitochodrial health.

TABLE 1 | Post-transplant outcomes of pilot, prospective, multi-center, non-randomized clinical trial, adapted from Ali et.al. permission to reprint obtained [17].

Outcome	Study cohort (*n* = 70)	Matched controls (*n* = 140)	Difference (95% CI)
Incidence of PGD3 at 72 h, n (%)	4 (5.7)	13 (9.3)	–3.6 (–10.5, 5.3)
Recipient Vent time (hours), median (IQR)	49 (29, 82)	52 (27, 89)	–3 (–15, 7)
ICU LOS (days), median (IQR)	5 (3, 9)	5 (3, 12)	0 (–2, 1)
Hospital LOS (days), median (IQR)	25 (20, 40)	30 (20, 54)	–5 (–8, 2)
Post-LTx ECMO used, n (%)	5 (7.1)	13 (9.3)	–2.1 (–9.3, 7.2)
30-day survival, n (%)	70 (100)	135 (96.4)	3.6 (–2.0, 8.1)

LOS, length of stay; ICU, intensive care unit; PGD3, International Society for Heart and Lung Transplantation Primary Graft Dysfunction Grade 3; LTx, Lung transplantation; ECMO, Extracorporeal membrane oxygenation; Vent, Ventilation.

the need for post-op ECMO, median ICU length of stay (LOS) or median hospital LOS between the two groups. Also, one-year survival was similar between the two groups (p = 0.37) with a median follow-up time of 336 days [17].

Based on the above findings, a multicenter, prospective, randomized-controlled trial was designed, to which recruitment officially started in May 2023 (NCT05898776). This study, involving 15 transplant centers worldwide, is designed as a non-inferiority study that will compare an extended preservation period (time from donor aortic cross-clamp to anesthesia start in the recipient hospital) of up to 12 hrs using a portable 10°C cooler (XPort, Traferox, Toronto, Canada) to conventional preservation (SCS on ice; time from donor aortic cross-clamp to anesthesia start in the recipient hospital of up to 6h). The results of this RCT will hopefully provide the evidence for changing the standard practice of donor lung preservation, which will in turn lead to significant flexibility in clinical lung preservation times.

ADVANTAGES OF PROLONGED STORAGE AT 10°C

Avoid Night Time Transplantation

Multi-organ donation and the subsequent need to coordinate several organ procurement and implantation teams has

shaped transplant medicine into an acute and challenging discipline. As all donor organs have different tolerances to cold ischemia and operating room capacity is often limited at procurement sites, multiorgan procurement is often performed in the evening or night time. As a result, implantation teams are often required to perform complex and exhausting procedures in critical recipients during night time hours. Of interest, several studies in the field of transplantation have linked night time procedures with worse clinical outcomes. It has been demonstrated that night time lung transplant recipients had a higher rate of postoperative complications than daytime recipients [18]. Similarly, it has been shown that night time liver transplant recipients have a 2-fold increased short-term mortality [19]. The possibility to prolong preservation times and thus render lung transplantation into a semi-elective and day time procedure has the potential to improve patient outcomes and as quality of life for transplant professionals, which would profoundly change current practice.

Postpone Implantation for Logistic Reasons

The limited preservation time of grafts can create significant logistic problems for lung transplant centers. In this light, extending the time window of implantation by being able to store grafts at 10°C increases flexibility and offers several advantages: (i) scheduled elective cases can be finished by deliberately moving the implantation to the afternoon; (ii) surgically complex recipients can be transplanted during the day when the team can perform at its best, or the most experienced team is available; (iii) small and medium-sized lung transplant centers may be able to accept concurrent donor offers and safely implant the organs one after another. These logistical advantage has already in fact been successfully described by the Madrid transplant program, which accepted two parallel donors and postponed the most complex of the two cases until daytime, thus avoiding parallel surgery in the operating room overnight [20]. In addition, prolonged organ storage provides the opportunity to optimize a recipient preoperatively. As highly sensitized recipients are increasingly accepted by lung transplant programs, preoperative sensitization protocols including immunoadsorption or plasmapheresis could potentially be performed without time constraints. Finally, higher logistical flexibility can have a direct impact on the quality of life of transplant candidates. Patients on the waiting list who reside in remote areas may have the opportunity to remain in their hometown despite longer transport times without the need to relocate near the transplant center—a major social and economic advantage for them.

Broader Geographic Distribution of Donor Organs

The shortage of donor lungs remains one of the biggest hurdles in clinical lung transplant practice. In Europe, North America and around the world, a large number of optimal donor offers are often declined, simply due to the long transport times involved. An easy-to-use, cost effective and reusable temperature-controlled device that ensures organ storage at 10°C for an extended period of time could fundamentally change our current practice. Based on the scientific evidence presented above, such a device could safely extend the geographic boundaries and facilitate a broader organ sharing.

Another interesting concept that is linked with increased preservation times, is fostering environmentally friendly transportation modes. Currently, the majority of donor lungs are transported by charter airplane flights [21]. Especially in Europe, where travelling distances are comparably short, most organs could be transported by commercial flights, car or train in the near future. This has the potential to significantly improve the carbon footprint of organ procurement and decrease costs [22].

Reduce the Number of "False-Calls" for Recipients

"Dry runs" are common in lung transplantation with rates of up to 40 percent being reported in DCD donors [23]. Currently, recipients are immediately informed when an organ has been allocated to them and they usually have to promptly come to the hospital. "Dry runs" pose an enormous emotional burden to recipients and their families. Such false calls could be completely avoided by prolonged 10°C preservation as patients would only be informed when a lung has been finally accepted for transplantation.

FUTURE DIRECTIONS OF ORGAN PRESERVATION

Donor lung preservation is one of the most studied topics of clinical lung transplantation. Most of recent research aims to either prolong preservation times or improve organ quality. We foresee and increasing role of *ex-vivo* lung perfusion with a constant improvement in perfusion and ventilation strategies. Several attempts have recently been made to optimize perfusion solutions in order to prolong EVLP times, i.e., adding nutrients or maintaining perfusate osmolality and pH [24, 25]. Another interesting concept might be the use of hypothermic *ex-vivo* lung perfusion [26]. HOPE is already routinely used in liver and kidney transplantation [27]. In addition, it has recently also been successfully tested in heart transplantation [28].

The other main perspective of *ex-vivo* lung perfusion is its role as a repair platform where reversible conditions of the donor organ can be treated. Promising data from animal study are already available. EVLP has been successfully used to reduce bacterial load [29], to reverse inflammatory damage related to aspiration [30], and to clear lungs from infections such as HCV [31]. In a subsequent step EVLP could be used to manipulate donor lungs and render them into 'super organs'. Modulating immunogenicity by inducing IL-10 overexpression or cleaving surface antigens are possible approaches.

CONCLUSION

Clinical donor lung preservation will significantly change in the future. Storage of grafts at 10°C will have a considerable impact on

transplant programs around the world by extending acceptable and safe preservation times. This will shift in our clinical practice towards an unprecedented semi-elective transplantation practice with numerous beneficial effects.

AUTHOR CONTRIBUTIONS

KH and AB wrote the manuscript. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

SK serves as Chief Medical Officer of Traferox Technologies, SK and MC receive personal fees from Lung Bioengineering and Traferox Technologies. SK and MC are shareholders of Traferox Technologies. SK and MC fully adhere to policies at University Health Network that ensure academic integrity and management of potential interest.

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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ORIGINAL RESEARCH
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Revised Heart Allocation Policy Improved Waitlist Mortality and Waiting Time With Maintained Outcomes in En-Bloc Heart-Lung Transplant Candidates and Recipients

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The revised United Network for Organ Sharing heart allocation policy was implemented in October 2018. Using a national transplant database, this study evaluated the transplant rate, waitlist mortality, waiting time, and other outcomes of en-bloc heart-lung transplantation recipients. Adult patients registered on the national database for heart-lung transplants before and after the policy update were selected as cohorts. Baseline characteristics, transplant rates, waitlist mortality, waiting times, and other outcomes were compared between the two periods. In total, 370 patients were registered for heart-lung transplants during the pre- and post-periods. There were significantly higher transplant rates, shorter waitlist times, and substantially reduced waitlist mortality in the post-period. Registered patients waitlisted in the post-period had significantly higher utilization of intra-aortic balloon pumps, extracorporeal membrane oxygenation, and overall life support, including ventricular assist devices. Transplant recipients had significantly longer ischemic times, increased transport distances, and shorter waiting times before transplantation in the post-policy period. Transplant recipients held similar short-term survival before and after the policy change (log-rank test, $p = 0.4357$). Therefore, the revised policy significantly improved access to en-bloc heart-lung allografts compared with the prior policy, with better waitlist outcomes and similar post-transplant outcomes.

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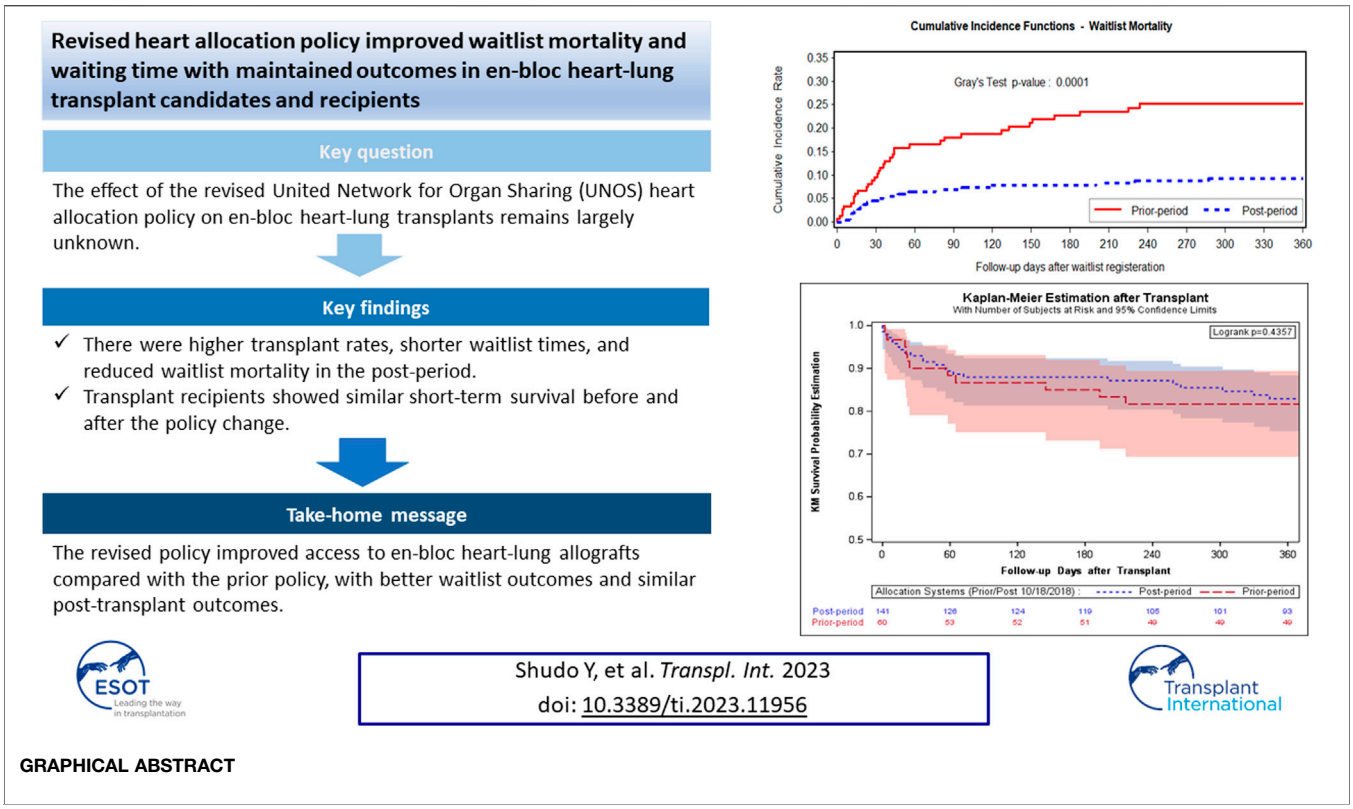
Keywords: heart-lung transplant, heart allocation policy, waitlist mortality, post-transplant outcomes, waitlist outcomes

INTRODUCTION

En-bloc heart-lung transplantation (HLTx) is well-established as an effective and definitive treatment for patients with advanced cardiopulmonary failure. Since the first successful operation performed in 1981 [1], >3,200 patients have undergone HLTx worldwide [2, 3].

Furthermore, to optimize the utilization of scarce donor hearts, the United Network for Organ Sharing (UNOS) revised the heart allocation policy in the United States, which took effect on

Abbreviations: ECMO, extracorporeal membrane oxygenation; HLTx, heart-lung transplantation; IABP, intra-aortic balloon pump; UNOS, United Network for Organ Sharing; VAD, ventricular assist device.



GRAPHICAL ABSTRACT

18 October 2018 [4]. Briefly, the new policy stratifies recipient candidates into six statuses and prioritizes transplantation of patients requiring temporary mechanical circulatory support, such as extracorporeal membrane oxygenation (ECMO) or an intra-aortic balloon pump (IABP) [4]. Previous studies have also assessed changes in post-transplantation outcomes associated with the allocation policy change. However, most of these studies examined isolated heart transplantation, and the effect of this change on multi-organ transplants remains largely unknown.

This study aimed to evaluate the transplantation rate, waitlist mortality, waiting time, and other outcomes of HLTx candidates and recipients using a national transplantation database.

MATERIALS AND METHODS

This study was based on National Organ Procurement and Transplantation Network STAR database (UNOS) released in January 2023. First-time HLTx registrants aged >18 years were selected from the UNOS database. Two periods were defined to compare the demographic characteristics and outcomes between the previous (pre-period) and new (post-period) allocation systems. Each period was 3.5 years, and the time of year was matched in both periods. The pre-period cohort was defined as patients who registered for HLTx between 18 October 2014, and 17 April 2018; similarly, the post-period was between 18 October 2018, and 17 April 2022. Thus, all patients who were listed within a designed period, but still waiting in waitlist by the end of this period, or died/transplanted/delisted after this period were treated as "censored" in these time-to-event-analyses.

The primary outcomes were waitlist mortality, defined as death from waitlist registration, and overall transplant mortality, defined as death from transplantation. Other waitlist outcomes, such as transplant rate and transplanted patients' hospitalization outcomes, such as graft failure episodes, were assessed and compared between periods.

Continuous variables were described as means ± standard deviation or as medians with interquartile ranges (IQR) (25th and 75th percentiles) as appropriate. The continuous variables were compared using the Student's t-test for mean differences and the Wilcoxon rank-sum test for median differences. Categorical variables were compared using the χ^2 test or Fisher's exact test. Cumulative Incidence Functions (CIF) curves showed tendencies of waitlist mortalities in two periods of time. Gray's cumulative risk test was used to test CIF curves when considering transplanted events and delisted events as competing risks. Kaplan-Meier (KM) survival curves were created to visually depict the overall survival of transplanted groups, and the log-rank test was used to test KM curves of two periods. The Cox proportional hazards models were used to estimate the unadjusted and adjusted hazard ratios of periods on three possible events (transplanted, dead while waiting and delisted) when patients were waiting on the waitlist. When one interested event was estimated, the other two were treated as competing risks. Adjusted hazard ratios were obtained after adjustments of multiple demographic and clinical factors, which included patient

TABLE 1 | Demographic data of the waitlist cohort.

42 months Waitlist cohort N = 370		Pre-period (10/18/2014– 4/17/2018) *n* = 152	Post-period (10/18/2018– 4/17/2022) *n* = 218	*p*-value
Age (y)	Mean ± SD	44.0 ± 12.7	45.7 ± 12.4	0.194
	Median [IQR]	45.5 [33.5, 55]	47 [35, 56]	0.172
Gender	Female, n (%)	69 (45.4%)	105 (48.2%)	0.599
	Male, n (%)	83 (54.6%)	113 (51.8%)	
Race	White, n (%)	114 (75.0%)	147 (67.4%)	0.118
	Black, n (%)	26 (17.1%)	57 (26.2%)	
	Others, n (%)	12 (7.9%)	14 (6.4%)	
Blood Type	A, n (%)	37 (24.3%)	73 (33.5%)	0.105
	B, n (%)	25 (16.5%)	42 (19.3%)	
	O, n (%)	84 (55.3%)	99 (45.4%)	
	AB, n (%)	6 (4.0%)	4 (1.8%)	
BMI (kg/m^2)	Mean ± SD	24.3 ± 4.8	24.8 ± 4.8	0.415
	Median [IQR]	24.0 [20.8, 28.0]	24.2 [21.0, 28.0]	0.538
Conditions at listing				
Prior Cardiac Surgery	n (%)	42 (27.6%)	63 (28.9%)	0.791
ECMO at listing	n (%)	15 (9.9%)	46 (21.1%)	0.004
IABP at listing	n (%)	0 (0.0%)	9 (4.1%)	0.012
Ventilator at listing	n (%)	12 (7.9%)	19 (8.7%)	0.779
VAD at listing	n (%)	5 (3.3%)	9 (4.1%)	0.677
Other Mechanism Life Support at listing	n (%)	17 (11.2%)	37 (17.0%)	0.121
Life Support at listing (including VAD)	n (%)	47 (30.9%)	97 (44.5%)	0.008
Status at end-of-period	Transplanted, n (%)	60 (39.5%)	141 (64.7%)	<.001
	Died-while-waiting, n (%)	37 (23.4%)	24 (11.0%)	
	Delisted, n (%)	16 (10.5%)	31 (14.2%)	
	Still waiting, n (%)	39 (25.7%)	22 (10.1%)	
Time on the waitlist (days)	Mean ± SD	253.3 ± 373.2	163.9 ± 243.7	0.009
	Median [IQR]	137.5 [29, 310.5]	54.5 [14, 230]	0.002

Categorical variables are expressed as n (%), and continuous variables are expressed as mean ± standard deviation or as median with interquartile range (IQR) (25th and 75th percentiles). BMI, body mass index; IABP, intra-aortic balloon pump; ECMO, extracorporeal membrane oxygenation; VAD, ventricular assist device.

age when registering on the waitlist, gender, race, prior cardiac surgery, ECMO at the listing, IABP at the listing, Ventilator at the listing, VAD at the listing, Life Support at the listing, and Other Mechanism Life Support at the listing. For all statistical analyses, statistical significance was set at a two-sided level of 0.05. All analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). This study was based on Organ Procurement and Transplantation Network data as of 4 September 2020.

RESULTS

The demographic data and characteristics of the waitlist cohort are presented in **Table 1**. There were 152 patients listed who registered for heart-lung transplantation between 18 October 2014, and 17 April 2018, and 218 between 18 October 2018, and 17 April 2022. Of these patients, 60 recipients (39.5%) underwent transplantation pre-period and 141 (64.7%) post-period. There was a significantly higher transplantation rate (141/218 vs. 60/152, $p < 0.001$) in the post-period. There was no significant difference in age ($p = 0.194$), gender ($p = 0.599$); however, race ($p = 0.118$), blood type ($p = .0105$), and BMI ($p = 0.415$) differed significantly between periods. Patients registered for HLTx in the post-period had significantly higher utilization of IABP (4.1% vs. 0%, $p = 0.012$), ECMO (21.1% vs. 9.9%, $p = 0.004$), and overall life support including ventricular assist devices (VAD) (44.5% vs. 30.9%, $p = 0.008$) while waitlisted. Moreover, there was a significantly shorter waitlist time (164 ± 244 days vs. 253 ± 373 days, $p = 0.009$) in the post-period. The higher utilization of IABP, ECMO, and overall life support, including VAD, suggests that the cohort registered for HLTx in the current era included recipients with relatively more severe illnesses. Notably, however, the waitlist mortality in the post-period was significantly reduced (11.0% vs. 23.4%, competing risks Gray's test $p = 0.0001$) (**Figure 1**).

As shown in **Table 2**, recipients in the transplanted cohort were, on average, 1.6 years older in the post-period than those in the pre-period, although not significant (44.4 ± 13.1 years vs. 46.0 ± 12.2 years; $p = 0.405$). It was very similar for donor

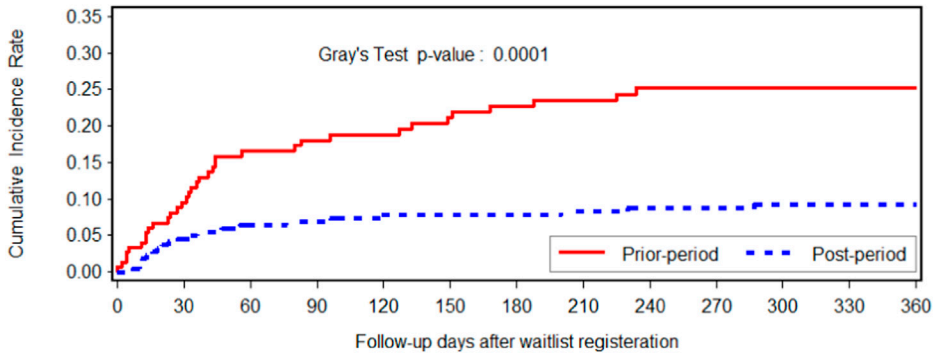


FIGURE 1 | Fine-Gray cumulative incidence function comparing waitlist mortality using transplantation or delisting as competing events. The post-period is in blue, and the pre-period is in red. Fine-Gray *p*-value of .0001 on the cumulative incidence rates of the two groups indicates that waitlist mortality significantly decreased after the allocation change.

age between the two periods (post-period, 32.9 ± 11.5 years vs. pre-period, 32.9 ± 12.7 years; $p = 0.996$). The distribution of transplants between sexes in each period was 53.2% and 58.3% in the post- and pre-periods, respectively ($p = 0.503$), and the proportion of recipient-to-donor sex matches decreased (71.6% vs. 76.7%, in the post- and pre-periods, respectively; $p = 0.461$). No significant difference was observed in recipient blood type ($p = 0.661$) or blood type match between the post- and pre-periods (84.4% and 90.0%, respectively; $p = 0.376$). The mean recipient body mass index was similar between eras (post-period, 24.4 ± 4.9 kg/m^2, and pre-period, 23.7 ± 5.2 kg/m^2; $p = 0.331$).

Transplant recipients receiving HLTx within the post-period tended to have higher utilization of IABP (9.2% vs. 1.7%, $p = 0.069$) and ECMO (31.2% vs. 20.0%, $p = 0.123$) at transplant. However, the difference between periods was not significant. HLTx recipients also had significantly longer ischemic times (Medians 4.0 h vs. 3.5 h, $p = 0.004$) and shorter waiting times before transplantation (median 38 days vs. 117 days, $p = 0.008$) in the post-period following the policy change. Donor organs were transported from significantly farther distances in the post-period than in the pre-period, with the mean distance from the donor hospital to the recipient transplant center being 235.5 ± 201.4 miles in the post-period compared with 129.4 ± 52.9 miles in the pre-period ($p < 0.001$). There was no significant difference in distribution of indications for transplantation ($p = 0.301$). Fewer patients in the post-period had a history of prior cardiac surgery than those in the pre-period (30.0% vs. 38.3%, $p = 0.236$); however, the difference was insignificant. The median length of hospital stay during transplant hospitalization was similar between periods (post-period, 36 days; interquartile ranges (IQR), 20–57 days and pre-period, 33 days; interquartile ranges (IQR), 21–53 days; $p = 0.706$). There were higher risks; however, transplant recipients showed a significantly lower graft failure rate (24.8% vs. 40.0%, $p = 0.031$). Further, these patients tended to have a lower in-hospital mortality (9.9% vs. 11.7%, $p = 0.802$), thus having similar short-term survival before and after the policy change (log-rank test, $p = 0.4357$) (**Figure 2**).

Conversely, 24 listed patients (11.0%) died waiting for a transplant in the new allocation system, whereas 37 recipients (24.3%) died in the pre-period. The waitlist mortality rate was significantly lower (24/218 vs. 37/152, $p < 0.001$) during the post-period. As shown in **Table 3**, recipients in the waitlist mortality cohort were, on average, 4.7 years older in the post-period than those in the pre-period (44.6 ± 12.3 years vs. 50.3 ± 11.3 years; $p = 0.079$). No significant differences in sex ($p = 0.903$) or body mass index ($p = 0.539$) were observed between the two periods. Recipients who died while waiting for transplantation within the post-period showed a significantly higher utilization of life support (70.8% vs. 35.1%, $p = 0.009$) while waitlisted.

Considering the competing risks of waitlist outcomes and controlling for possible confounding factors, the Cox Proportional Hazards regression models were used to estimate the periods' unadjusted and adjusted hazard ratios (**Table 4**). Notably, the unadjusted and adjusted hazard ratios for transplants within periods and death while waiting for transplants were statistically significant. In particular, the hazard ratios of pre-period vs. post-period of transplants within the periods were 0.511 without covariate adjustments and 0.544 after adjustments of covariates ($p < 0.001$ for both), indicating that the transplant likelihood during pre-period was around half of that during post-period. Conversely, the hazard ratios of pre-period vs. post-period of death while waiting for transplants were 2.609 without covariate adjustments and 2.852 after adjustments ($p < 0.001$ for both), which indicated that the death likelihood while waiting for HLTx during pre-period was over 2.6 times of the death likelihood during post-period. This is strong evidence that the new allocation policy has significantly improved patients' survival and saved lives. Notably, there were no significant differences in delisting within these periods.

TABLE 2 | Demographic data of transplanted cohort.

Transplanted groups in a 42 months Waitlist cohort N = 201		Pre-period (10/18/2014– 4/17/2018) *n* = 60	Post-period (10/18/2018–4/17/2022 *n* = 141	*p*-value
Prior Transplant Demographic Data				
Recipient age (y)	Mean ± SD	44.4 ± 13.1	46.0 ± 12.2	0.405
	Median [IQR]	46 [32.5, 55.5]	47 [38, 56]	0.468
Donor age (y)	Mean ± SD	32.9 ± 12.7	32.9 ± 11.5	0.996
	Median [IQR]	32 [22, 43.5]	33 [24, 41]	0.899
Gender	Female, n (%)	25 (41.7%)	66 (46.8%)	0.503
	Male, n (%)	35 (58.3%)	75 (53.2%)	
Gender Match (recipient to donor)	n (%)	46 (76.7%)	101 (71.6%)	0.461
Race	White, n (%)	47 (78.3%)	98 (69.5%)	0.173
	Black, n (%)	8 (13.3%)	35 (24.8%)	
	Others, n (%)	5 (8.3%)	8 (5.7%)	
Recipient Blood Type	A, n (%)	20 (33.3%)	59 (41.8%)	0.661
	B, n (%)	14 (23.3%)	26 (18.4%)	
	O, n (%)	24 (40.0%)	53 (37.6%)	
	AB, n (%)	2 (3.3%)	3 (2.1%)	
Blood Type Match (recipient to the donor)	n (%)	54 (90.0%)	119 (84.4%)	0.376
Recipient BMI (kg/m^2)	Mean ± SD	23.7 ± 5.2	24.4 ± 4.9	0.331
	Median [IQR]	21.9 [20.0, 27.2]	24.1 [20.9, 27.5]	0.165
Indication for Transplant	Congenital heart disease, n (%)	16 (26.7%)	23 (16.3%)	0.301
	Pulmonary Hypertension, n (%)	16 (26.7%)	51 (36.2%)	
	Pulmonary fibrosis, n (%)	12 (20.0%)	32 (22.7%)	
	Other, n (%)	16 (26.7%)	35 (24.8%)	
Prior Cardiac Surgery	n (%)	23 (38.3%)	42 (30.0%)	0.236
Prior Lung Surgery	n (%)	1 (1.7%)	2 (1.4%)	0.994
Operative Data				
Ischemic Time (hrs)	Mean ± SD	3.6 ± 0.9	3.9 ± 0.9	0.006
	Median [IQR]	3.5 [3.1, 4.1]	4.0 [3.4, 4.5]	0.004
Distance, donor hospital to transplant center (miles)	Mean ± SD	129.4 ± 52.9	235.5 ± 201.4	<.001
	Median [IQR]	53 [12.5, 214]	207 [66, 380]	<.001
Time on the waitlist (days)	Mean ± SD	135.0 ± 140.5	96.0 ± 135.9	0.067
	Median [IQR]	117 [23.5, 191.5]	38 [9, 121]	0.008
Preoperative Life Support				
ECMO at listing	n (%)	6 (10.0%)	33 (23.4%)	0.032
ECMO at transplant	n (%)	12 (20.0%)	44 (31.2%)	0.123
IABP at listing	n (%)	0 (0.0%)	8 (5.7%)	0.108
IABP at transplant	n (%)	1 (1.7%)	13 (9.2%)	0.069
Ventilator at listing	n (%)	4 (6.7%)	14 (9.9%)	0.594
Ventilator at transplant	n (%)	7 (11.7%)	13 (9.2%)	0.612
VAD at listing	n (%)	1 (1.7%)	4 (2.8%)	0.981
VAD at transplant	n (%)	2 (3.3%)	4 (2.8%)	0.997
Other Mechanism Life Support at listing	n (%)	11 (18.3%)	28 (19.9%)	0.803
Other Mechanism Life Support at transplant	n (%)	16 (26.7%)	25 (17.7%)	0.151
Life Support at listing (including VAD)	n (%)	22 (36.7%)	68 (48.2%)	0.132
Life Support Pre-transplant (including VAD)	n (%)	30 (50.0%)	84 (59.6%)	0.211
Post Transplant Outcomes				
Length of stay (days)	Mean ± SD	52.7 ± 57.4	50.7 ± 54.0	0.815
	Median [IQR]	33 [21, 53]	36 [20, 57]	0.706
Stoke	n (%)	6 (10.0%)	8 (5.7%)	0.363
Dialysis	n (%)	20 (33.3%)	37 (26.2%)	0.307
PPM	n (%)	2 (3.3%)	2 (1.4%)	0.231
Airway	n (%)	0 (0.0%)	5 (3.6%)	0.469
Graft Failure	n (%)	24 (40.0%)	35 (24.8%)	0.031
In-hospital mortality	n (%)	7 (11.7%)	14 (9.9%)	0.802

Categorical variables are expressed as n (%), and continuous variables are expressed as mean ± standard deviation or as median with interquartile range (IQR) (25th and 75th percentiles). BMI, body mass index; IABP, intra-aortic balloon pump; ECMO, extracorporeal membrane oxygenation; VAD, ventricular assist device; PPM, permanent pacemaker.

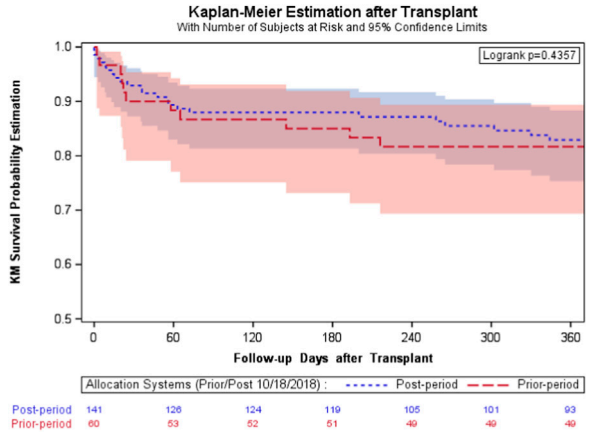


FIGURE 2 | The survival curves after transplantation of the transplanted subgroup. Overall survival Kaplan-Meier estimates stratified between prior (red) and post (blue) policy change: a similar short-term survival before and after policy change (log-rank test, p = .4357).

DISCUSSION

This comprehensive study investigated the impact of the revised UNOS heart allocation policy on transplant rate, waitlist mortality, waiting time, and other outcomes of adult primary HLTx recipients using the UNOS STAR database. We stratified the cohort by disjoint categories of patients registered for HLTx in the allocation system during the previous period, before the policy update (10/2015–04/2018), as well as during the period post-policy update (10/2018–04/2021).

The UNOS updated its heart allocation policy in the United States in October 2018 [4]. Notably, more categories were introduced to better stratify the urgency for recipients of heart transplants, from three categories (status 1A, 1B, and 2) to six categories (status 1–6). These changes were fundamentally implemented to decrease mortality rates for recipients on the waiting list, while providing an opportunity for others to receive organs.

Regarding the allocation of heart and lung combinations, when heart-lung transplantation candidates are registered on the heart, lung, and heart-lung waiting lists, the second organ is allocated to the heart-lung transplantation candidate from the same donor. In practice, donor organs are allocated by running a list of hearts for each recipient. In reality, if the heart offer comes as a primary offer to the heart-lung transplantation candidate, lungs must be offered from the same donor, even if the heart-lung transplantation candidate's need for those lungs is far less urgent than for others on the lung list.

A potential concern was that the new organ allocation system might lead to disadvantages for heart-lung transplantation recipients, since heart-lung transplantation candidates are generally listed as status 4 or 5 in the new system. However, they were listed as status 1B or 2 in the previous system unless they had higher requirements for ECMO, IABP, or other mechanical life support, prolonging the waiting period [5]. Nevertheless, our data showed that the cohort from the new allocation system was associated with higher transplant rate, reduced waitlist mortality, and shorter waiting time. Based on this analysis, the revised heart allocation policy significantly improves access to en-bloc heart-lung allografts than the prior policy, with better waitlist outcomes.

TABLE 3 | Demographic data of patients who died while waiting for transplant.

Died-while-waiting groups in 42 months Waitlist cohort N = 61		Pre-period (10/18/2014– 4/17/2018) *n* = 37	Post-period (10/18/2018– 4/17/2022) *n* = 24	*p*-value
Age (y)	Mean ± SD	44.6 ± 12.3	50.3 ± 11.3	0.079
	Median [IQR]	47 [37, 55]	53.5 [42, 60]	0.077
Gender	Female, n (%)	16 (43.2%)	10 (41.7%)	0.903
	Male, n (%)	21 (56.8%)	14 (58.3%)	
Race	White, n (%)	30 (81.1%)	16 (66.7%)	0.050
	Black, n (%)	4 (10.8%)	8 (33.3%)	
	Others, n (%)	3 (8.1%)	0 (0.0%)	
BMI (kg/m^2)	Mean ± SD	24.3 ± 4.3	25.0 ± 4.9	0.539
	Median [IQR]	24.0 [22.0, 26.6]	24.2 [21.4, 28.1]	0.854
Conditions at listing				
Prior Cardiac Surgery	n (%)	7 (18.9%)	7 (29.2%)	0.353
ECMO at listing	n (%)	5 (13.5%)	7 (29.2%)	0.189
IABP at listing	n (%)	0 (0.0%)	1 (4.2%)	0.393
Ventilator at listing	n (%)	6 (16.2%)	3 (12.5%)	0.776
VAD at listing	n (%)	2 (5.4%)	4 (16.7%)	0.201
Other Mechanism Life Support at listing	n (%)	3 (8.1%)	6 (25.0%)	0.136
Life Support at listing (including VAD)	n (%)	13 (35.1%)	17 (70.8%)	0.009
Time on the waitlist (days)	Mean ± SD	85.8 ± 117.5	144.5 ± 192.9	0.189
	Median [IQR]	37 [16, 127]	41.5 [17, 215]	0.562

Categorical variables are expressed as n (%), and continuous variables are expressed as mean ± standard deviation or as median with interquartile range (IQR) (25th and 75th percentiles). BMI, body mass index; IABP, intra-aortic balloon pump; ECMO, extracorporeal membrane oxygenation; VAD, ventricular assist device; PPM, permanent pacemaker.

TABLE 4 | Hazard ratios of pre-period vs. post-period from Cox PH models.

Specific Event of Interest	# of Interest Event	# of Competing Events	# of Censored	Hazard Ratio Type	HR of Interest Event: Pre-period vs. Post-period	95% CI of HR	P-value
Died While Waiting							
	61	248	61	Unadjusted	2.609	[1.564, 4.351]	<.001
				Adjusted[a]	2.852	[1.670, 4.868]	<.001
Transplanted Within Periods							
	201	108	61	Unadjusted	0.511	[0.381, 0.686]	<.001
				Adjusted[a]	0.544	[0.401, 0.736]	<.001
Delisted Within Periods							
	47	262	61	Unadjusted	0.794	[0.436, 1.445]	0.451
				Adjusted[a]	0.789	[0.413, 1.507]	0.473

HR, Hazard ratio; CI, confidence interval.

[a]*Results adjusted in Cox proportional hazards model by baseline characteristics—patient age when registering on the waitlist, gender, race, prior cardiac surgery, ECMO at the listing, IABP at the listing, ventilation at the listing, VAD at the listing, Life Support at the listing, and other mechanical life support at the listing.*

Our data also showed that recipients who underwent transplantation during the new allocation system included baseline demographics indicating more severe illness, as evidenced by higher utilization of IABP, ECMO, and overall life support, including VAD at transplantation. One may argue that maintaining patients on ECMO in the preoperative phase has been reported as a high-risk resource [3], yet it seems that it has become commonplace in many of our institutions. This study found that >30% of heart-lung transplant recipients were on ECMO at the time of transplant. Nevertheless, the equivalent graft survival was demonstrated by short-term mortality in our study. This result is supported by our institution's previous report, which focused on reasonable outcomes among adult transplant recipients who underwent HLTx bridged from ECMO [6].

In addition to the transplanted recipients' demographics that have been mentioned earlier, the characteristics of patients registered for transplantation who died while waiting were equally important in this study. Our data showed that >70% of patients in the waitlist mortality cohort were on life support, including VAD. This could likely be explained by insufficient access to organs for HLTx candidates with severe illness, and this issue should be addressed in future studies.

Finally, we appear to have made good progress in the pre-transplant phase, with decreased waitlist mortality and faster time to transplant for patients requiring heart-lung transplants. Conversely, post-transplant outcomes seem to have plateaued across the eras. This issue may be partly resolved with the newly developed innovative organ preservation and transport system, which may positively impact long-term survival in this complex patient population [7].

Limitations of the Database

This study has limitations consistent with those of retrospective analyses and the use of a national multicenter database. The UNOS database has some considerable uncollected data for crucial factors during specific periods; however, the UNOS/OPTN registry provided a large sample size to assess the influence of the revised UNOS heart allocation policy on the transplant rate, waitlist mortality, waiting time, and other outcomes of adult HLTx recipients. However, specific recipient characteristics may also contribute to recipient mortality; several have not been included in our analysis. A potential selection bias may have existed wherein physicians believe that obesity is a prohibitive risk factor for HLTx. In addition, only donors whose organs were accepted for transplantation were included. The selection of a suitable donor is a complicated process. Clinicians must consider multiple factors, evaluating recipient urgency against donor characteristics, ischemic time, recipient sensitization, and donor/recipient size mismatches. Therefore, additional characteristics may be responsible for post-transplant graft failure, and these factors were not considered in this analysis.

Conclusion

The revised UNOS policy was associated with higher transplant rates, reduced waitlist mortality, shorter waiting times, and similar post-transplant short-term survival rates. Based on this analysis, the revised heart allocation policy significantly improved access to en-bloc heart-lung allografts than the prior policy, with better waitlist outcomes and similar post-transplant outcomes.

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

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conceptualization, YS; methodology, YS and HH; formal analysis, HH; investigation, YS and HH; resources, YS and YW; data curation, HH and SE; writing—original draft preparation, YS and HH; writing—review and editing, SE; supervision, YW; project administration, YS and HH; funding acquisition, YS and YW. All authors contributed to the article and approved the submitted version.

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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