Impact of Pseudomonas Aeruginosa Infections and Donor-Specific Antibody Development on Outcomes post-Lung Transplantation: Evaluation and Insights into the Development of the Hungarian Lung Transplantation Program

PhD thesis

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1. Introduction

Lung transplantation (LuTx) is a widely used and accepted modality of care for patients with end-stage pulmonary disease, which presumes complex professional teamwork before and after the operation. Among other solid organ transplantations, LuTx is especially challenging due to various factors. The intricate structure of the lung anatomy and the perioperative use of extracorporeal membrane oxygenator (ECMO) and other highly invasive modalities make this procedure both surgically and therapeutically high-risk. The elevated incidence of acute and chronic rejection requires vigorous immunosuppressive regimens; however, unlike other solid organs, the lungs are in constant, breath-to-breath connection with the environment and are inherently exposed to pathogens, making adequate immunosuppression difficult to achieve. Improved patient selection, better pre- and post-operative care, and meticulous education are the fundamentals of long-term management strategies that contribute to the increasing success of a lung transplant program.

Recipient survival rates have improved over the years, but long-term success is often complicated by chronic rejection, infection, and other post-operative challenges. The most recent

data published by the International Society for Heart and Lung Transplantation (ISHLT) in 2023 focuses on mortality and retransplantation rates among LuTx recipients. For adult recipients, the median survival was approximately 6.2 years. The 1-year survival rate was around 85%. Approximately 54% of recipients survived five years post-transplant. Despite its potential, LuTx remains a high-risk procedure, associated with relatively high rates of mortality and morbidity compared to other solid organ transplants.

The number of patients waiting for LuTx exceeds markedly the number of available donors. Despite significant progress made in recent decades, several challenges persist. The main obstacles today are the shortage of donor organs, proper selection of candidates, early complications like primary graft dysfunction (PGD), the increased potential for infections, and the risk of chronic lung allograft syndrome (CLAD). Although LuTx has become increasingly successful as a surgical procedure, significant work remains to improve outcomes, address the donor shortage, and individualise immunosuppressive regimens. Additionally, advancements in less-invasive surgical approaches are being pursued to reduce surgical trauma and enhance recovery times.

2. Objectives

In the first part of the present study, we reassessed our real-world data (*RWD study*) to evaluate waitlist management, donation activity, and recipient morbidity and mortality. Comparing our results with other centres prompted further investigation into infectious and immunological outcomes. Immunosuppressed recipients often develop severe pulmonary infections, with pathogen-induced tissue damage and impaired healing contributing to CLAD. Pseudomonas aeruginosa (P. aeruginosa) frequently colonises the airways of LuTx recipients and has been linked to the progression of CLAD. Recent studies have shown that its presence correlates with a higher risk of developing donor-specific antibodies (DSAs).

In the second part (*DSA study*), we examined the link between airway infections and DSA using MFI stratification, correlating results with clinical signs of antibody-mediated rejection (AMR) and immune cell profiles in bronchoalveolar lavage (BAL). We assessed their impact on graft loss and CLAD-free survival. Combining serum DSA and BAL data may aid in predicting outcomes.

3. Methods

The *RWD study* was a retrospective observational cohort analysis evaluating clinical outcomes of lung transplant recipients. All patients evaluated, listed, and/or transplanted in the Hungarian Lung Transplantation Program during its first three years (Dec 12, 2015 – Dec 31, 2018) were included. Eligibility for LuTx was determined in accordance with international guidelines. No exclusions were needed for missing key outcome data. RWD were gathered from the Program's existing databases and Eurotransplant (ET) databases, then systematically reviewed for quality, consistency, and accuracy. We applied predefined variable definitions, data dictionaries, and SOPs for extraction and analysis, including stratification by clinical outcomes. Follow-up extended up to five years post-transplant or until death.

The *DSA study*, also a retrospective study, included all patients from December 12, 2015, to August 7, 2021, who underwent DSA testing, with follow-up until August 15, 2022 (median: 735 days). Uniform protocols guided patient management were applied. Retransplantation was considered a separate outcome. Primary outcomes included graft loss (death

or retransplantation) and CLAD-free survival. Donors with concordant antigens were avoided if pretransplant DSA MFI exceeded 3000. No standard desensitisation was used, but DSA-positive patients received plasmapheresis and IVIG. HLA antibodies were assessed using LABScreen Single Antigen tools (One Lambda), with DSA positivity defined as an MFI greater than 1000; the antibody with the highest MFI was labelled as immunodominant.

BAL involved 120 mL of 0.9% saline in 40 mL fractions; the fluid was analysed for neutrophil percentage and pathogens, with more than 25% of neutrophils deemed “high”. Infections with P. aeruginosa, Gram-negatives, or fungi were diagnosed using a 10^3 CFU/ml threshold. CLAD and AMR definitions followed the guidelines of the ISHLT. AMR was subclinical (based on DSA, C4d, histology) or clinical (based on FEV1 decline, radiology, exclusion of confounders).

Primary outcomes included short- and long-term survival (3 months to 5 years), airway infections, BAL data, DSA levels, and CLAD-free survival. *Secondary outcomes* included waitlist time, waitlist mortality, and factors influencing morbidity (e.g., LAS, ECMO, MV, ICU stay, transfusion, infection, rejection, bronchial issues, CLAD within 5 years).

Statistical methods employed included descriptive statistics, Kaplan-Meier survival curves, and subgroup comparisons. Cox regression identified survival predictors; logistic regression evaluated morbidity risks. Propensity score matching minimised bias, and pairwise t-tests identified differences. Prism GraphPad 9 and R, version 4.2.1, were used for the data analysis of the DSA study. One- and two-way ANOVA tests were used to assess group differences, while contingency analyses evaluated odds ratios using Chi-square tests. MFI values, AMR status, HLA-DQ specificity, and infections were evaluated independently. Survival analyses used univariate and multivariate Cox models with time-dependent variables (e.g., AMR, presensitization, BAL neutrophils, DSA levels, infections). AMR and DSA were not included together due to collinearity. P-values <0.05 were significant.

Data privacy complies with GDPR. Patient identifiers were removed, and subjects were anonymised. Due to the retrospective design, informed consent was waived. The DSA study was conducted in accordance with the Declaration of Helsinki and was approved by Hungary's Medical Research Council (ETTTUKEB, BM/15225-1/2023).

4. Results

Referrals to the Lung Transplantation Committee (LTC) increased steadily: 69 in 2015, 75 in 2016, 85 in 2017, and 92 in 2018. Median waitlist time was 55.5 days, with 12 deaths (15% mortality). During the RWD study period, 62 patients underwent LuTx in Hungary, all included in the analysis. The mean Lung Allocation Score (LAS) was 37.91 (SD 12.46); four recipients had high LAS (>50), qualifying for ET-prioritised allocation. Of the 62 surgeries, 61 were bilateral and one single-lung. A clamshell incision was used in 60 cases and anterolateral thoracotomy in two. Central VA-ECMO supported 59 patients, with two additional peripheral VA-ECMO cases in recipients with PPH. The mean ECMO time was 203.8 minutes (SD 36.4); three patients required prolonged ECMO (3-4 days). On average, 4.9 units (SD 2.27) of filtered, irradiated RBCs were transfused. Cold Ischemia Time (CIT) averaged 320.46 minutes (SD 40.69) for the first lung and 401.7 minutes (SD 42.15) for the second.

PGD incidence was low, with no severe cases or renal replacement therapy. PRES occurred in seven patients, and pancytopenia in two. Mean ICU stay was 24.6 days (SD 18.18), and MV support averaged 11.02 days (SD 13.10).

Tracheostomy was performed in 23.6% of cases (median day, 8; IQR, 5.5). Impaired clamshell healing was the most common late surgical complication. P. aeruginosa infection occurred in 13 patients (21%), BAL neutrophilia in 20 (32.25%), and high DSA in 15 (24.2%)—mainly class II DQ—all associated with clinical AMR. Bronchial strictures requiring bronchoscopy occurred in four patients (6.45%). CLAD was developed in 24 recipients (38.7%) within five years. No intraoperative deaths occurred. Mortality was 14.5% at 3 months, 22.5% at 6 months, and 27.4% at 1 year. One-year, three-year, and five-year survival rates were 73%, 55%, and 48%, respectively.

RWD subgroup analysis compared three cohorts: Cohort 1 (n = 17, <12-month survival), Cohort 2 (n = 24, CLAD), and Cohort 3 (n = 12, >12-month survival without CLAD). High DSA and clinical AMR were more frequent in Cohort 1 (36.4%) compared to Cohort 2 (14.3%), and absent in Cohort 3. P. aeruginosa was most common in Cohort 2 (20.8%), and BAL neutrophilia was also highest in this group (50%), suggesting associations with CLAD but not early mortality.

ANOVA showed significant differences (p = 0.0) in MV and ICU duration across cohorts. Cohort 1 had the longest MV and ICU stays; Cohort 3 had the shortest MV (3.5 days) but a

somewhat longer ICU stay (20.36 days), possibly due to early-era protocols. Tracheostomy was most frequent in Cohort 1 (47.1%), indicating poorer outcomes.

In the *DSA study,* 87 of 116 recipients were analysed (29 were excluded for not undergoing DSA testing). Among them, 36% were DSA-positive. Class I DSAs occurred in 19%, class II in 32%, and both in 49%. HLA-DQ DSAs were most common and showed the highest MFI (mean 8.527; $p < 0.0001$), typically within three months postoperatively.

Clinical AMR was associated with poorer graft survival (HR, 7.95; CI, 3.67–17.23; $p < 0.001$) and CLAD-free survival (HR, 16.22; CI, 3.02–87.22; $p = 0.001$). Subclinical AMR showed no significant differences vs. no AMR. Clinical AMR increased CLAD risk (OR 7.8; p=0.009) and had higher MFI (median 6.823) vs. subclinical AMR (3.377; p<0.001). HLA-DQ DSAs had the highest MFI (median 11.321; p<0.0001). Clinical AMR was an independent predictor of reduced graft survival (HR 7.98; $p < 0.001$) and CLAD-free survival (HR 34.79; $p = 0.001$).

Stratification by MFI (DSA-negative, DSA low: 1,000–8,000; DSA high: >8,000) revealed significantly worse graft and CLAD-free survival in the high DSA group (HR 5.77–6.64 for graft; HR 6.47–10.82 for CLAD; all p<0.02).

No significant differences were found between low and negative groups. The DSA-high group had a higher CLAD risk (OR 8.6; p=0.006), although not more severe CLAD. Most high MFI DSAs were class II (86%) and HLA-DQ (76%), which was not explained by broad HLA mismatch. DSA-high was confirmed as an independent predictor for reduced graft survival (HR 7.37; $p < 0.001$) and CLAD-free survival (HR 22.04; $p = 0.001$).

P. aeruginosa was present in 40.5% of BALs in DSA-positive vs. 13% in DSA-negative patients (OR 4.5; p=0.0042). In MFI stratified groups: 15.2% in DSA-negative, 30.8% in low, and 53.3% in high (OR 3.75–6.67; $p<0.05$). Other pathogens showed no significant link. Clinical AMR was independent of infection, as 82% of cases did not involve P. aeruginosa. Infections did not impact graft or CLAD-free survival.

BAL neutrophils were elevated in DSA-high patients (26.3%) compared to low (7.9%) and negative (8.04%) patients ($p < 0.001$), with peak ratios observed during DSA-high periods. High neutrophil counts predicted poorer graft survival (HR 3.45; $p < 0.001$), a finding confirmed in multivariate models (HR ~2.8; $p < 0.02$), with no association with concurrent infections ($p = 0.562$).

5. Conclusions

The number of patients registered on the LuTx waiting list has increased steadily over the first three years. Waiting time on the list and wait list mortality data aligned with the ET average. Comparing our numbers with the ET data shows that our adjusted waiting list load pmp is only 20.3% of the ET average. The preference for bilateral transplants is based on the evidence of better long-term outcomes. This was made possible by the sufficient donation activity in Hungary. Compared to international data, the low incidence of PGD is most likely due to the consistent use of intra-operative ECMO, which provides protective reperfusion conditions for the first implanted lung. None of our patients required kidney replacement therapy due to careful fluid balance management during the early postoperative period, intending to maintain a negative fluid balance to protect the grafts from fluid overload. Bronchial complication rates were also lower than reported, but PRES incidence was higher. Post-transplant ICU and MV support times were significantly longer than reported in international papers. Based on the ISHLT reports, our recipients' early survival rate (1-year survival) was lower than the international average.

In subgroup RWD analysis, shorter survival (<12 months) was associated with longer MV duration and ICU stay than in the CLAD and long-term survival cohorts. High DSA and AMR were more prevalent in the short-survival cohort than in the CLAD or long-term survival groups. The high neutrophil ratio in BAL was most frequent in the CLAD group, possibly indicating a link to chronic rejection. P. aeruginosa infection was most common in the CLAD group rather than those with poor survival outcomes.

In our current study, we examined these outcomes concerning the de novo DSA response, stratified by MFI levels. Also, we analysed the influence of P. aeruginosa infection on the humoral response. We identified high MFI DSAs and clinical AMR as independent prognostic factors for graft loss and poor CLAD-free survival. Furthermore, P. aeruginosa infection correlated with DSA development, and BAL neutrophilia served as a readily measurable indicator of poor allograft prognosis.

In our cohort, recipients with clinical AMR exhibited a strong correlation with graft loss and reduced CLAD-free time. Through multivariate Cox regression models, we identified clinical AMR as an independent risk factor for both outcomes; however, subclinical AMR did not exhibit the same

association. DSAs associated with clinical AMR had higher MFI values and primarily exhibited HLA-DQ specificity, which was identified as a significant risk factor for AMR and graft damage.

By establishing a cutoff based on clinical data, we demonstrated that high MFI DSAs have a significant impact on graft survival. MFI stratification is a relevant and accessible tool for evaluating future graft damage. We found that high levels of MFI DSAs reduce CLAD-free survival, while no similar effect was observed in the low DSA group.

Our cohort identified a higher risk for CLAD, which can be attributed to our MFI stratification method. Most of the >8000 MFI DSAs we studied were class II and HLA-DQ specific, showing consistent traits with previously reported studies, where class II DSAs were identified as risk factors for BOS, and 76% of the HLA-DQ-specific DSAs were linked to CLAD.

We suggest that the underlying mechanisms are related to the inflammatory environment within the lungs, where class II HLA expression may increase, as demonstrated by the finding that inflammatory cytokines (IFN-γ, TNF-α, IL-1β) elevate HLA class II expression on endothelial cells.

Our study has limitations, as it is a single-centre analysis with a limited number of participants. The study's retrospective nature may introduce confounding variables, and our clinical approach inevitably leaves underlying mechanisms hypothetical. MFI is a semi-quantitative measure of DSA levels, and the absence of standardised diagnostic protocols may lead to variations in DSA cutoff results across different centres. (101) Additionally, serum DSA levels do not accurately reflect the number of antibodies deposited in the lungs, which may result in misleadingly low MFI values. Still, several questions remain unanswered and warrant further investigation in future studies. It is currently unknown whether DSAs play a significant role in the initiation or progression of CLAD, which could have implications for the timing of desensitisation therapy. Our findings regarding CLAD grade support the idea that DSAs may be more critical in the initiation phase. Moreover, investigating whether P. aeruginosa's role in enhancing the humoral response is causal or merely a bystander effect requires further exploration.

Launching and running a LuTx program is a complex mission that requires coordinated teamwork and high expertise from specialists across various fields. Despite our program's gradual, multi-phase implementation, we continually face

challenges that must be addressed. Currently, the most significant challenge is the large deficit on the waiting list. The treatment process requires a considerable amount of time and resources. As the number of recipients increases and the waiting list deficit shows signs of declining, we must also focus on improving donation processes. Theoretically, if we increase donation activity and lung utilisation to the average levels of ET, we could gain about 17 to 20 additional organs per year. This would result in a potential 50% increase in the number of explanted organs.

Focusing on surgical techniques and transitioning to less invasive procedures, such as bilateral thoracotomy or minimally invasive techniques, will also promote a fast-track surgical mentality in LuTx. Proper donor and recipient selection and improved physical and mental preparation for recipients will likely result in shorter MV times and ICU stays, fewer infections, and potentially reduced immunological complications, ultimately leading to better survival rates.

DSAs typically emerge shortly after LuTx, while consequential graft loss or CLAD follows in relative delay. The interval between DSA detection and clinical outcome may be sufficient to implement therapeutic interventions. However, because all desensitisation protocols have side effects and

significantly increase the risk of infections, monitoring MFI and BAL neutrophilia may serve as prognostic factors. This approach could guide clinicians in determining when aggressive intervention is warranted.

Looking ahead, we aim to increase the number of transplants, as international benchmarks suggest that Hungary, with a population of 9.8 million, would require at least 30 - 40 LuTxs annually.

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Cumulative impact factor: ***25.929***

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