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## ***Pseudomonas aeruginosa* and acute rejection independently increase the risk of donor-specific antibodies after lung transplantation**

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### **Abstract**

Factors contributing to donor-specific HLA antibody (DSA) development after lung transplantation have not been systematically evaluated. We hypothesized that the isolation of *Pseudomonas aeruginosa* in respiratory specimens would increase the risk of DSA development. Our objective was to determine the risk of DSA development associated with the isolation of *Pseudomonas aeruginosa* after lung transplantation. We conducted a single-center retrospective cohort study of primary lung transplant recipients and examined risk factors for DSA development using Cox regression models. Of 460 recipients, 205 (45%) developed DSA; the majority developed Class II DSA (n = 175, 85%), and 145 of 205 (71%) developed DSA to HLA-DQ alleles. Univariate time-dependent analyses revealed that isolation of *Pseudomonas* from respiratory specimens, acute cellular rejection and lymphocytic bronchiolitis are associated with an increased risk of DSA development. In multivariable analyses, *Pseudomonas* isolation, acute cellular rejection, and lymphocytic bronchiolitis remained independent risk factors for DSA development. Additionally, there was a direct association between the number of positive *Pseudomonas* cultures and the risk of DSA development. Our findings suggest that proinflammatory events including acute cellular rejection, lymphocytic bronchiolitis, and

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#### DISCLOSURE

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#### DESCRIPTION OF SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

#### DATA SHARING POLICY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

*Pseudomonas* isolation after transplantation are associated with an increased risk of DSA development.

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## 1. INTRODUCTION

Lung transplantation is the ultimate treatment for patients with advanced lung disease. However, long-term outcomes remain disappointing, and the median survival after transplantation is 6.5 years<sup>1</sup>. Chronic lung allograft dysfunction (CLAD) is the leading cause of death beyond the first year after transplantation and is attributed to immune-mediated injury<sup>1-4</sup>. The development of donor specific antibodies (DSA) to mismatched human leukocyte antigens (HLA) has been increasingly recognized as an independent risk factor for CLAD and death<sup>5-11</sup>. Furthermore, multiple studies have reported a high incidence of DSA after lung transplantation<sup>10,11</sup>. Indeed, in a prospective multicenter observational study, 36% of patients developed DSA within 4 months of transplantation<sup>12</sup>. These data underscore the role of humoral immune responses in lung allograft rejection. Previous studies have identified risk factors for the development of DSA including pre-transplant allosensitization, re-transplantation, the lung allocation score (LAS) and primary graft dysfunction (PGD)<sup>12-15</sup>. The associations of LAS and PGD with DSA suggest that pro-inflammatory events early after transplantation increase the risk of DSA development. However, these studies have not examined the role of infection as a risk factor for the development of DSA, yet infections are common complications after lung transplantation and have been linked to the development of CLAD<sup>16-18</sup>. Emerging data support the paradigm that infections may augment or trigger alloimmune responses that promote rejection<sup>19</sup>. Although various mechanisms have been proposed to explain this association, most of which involve analyzing T cell mediated immune responses, a role for humoral immune activation, especially DSA, has not been defined. *Pseudomonas aeruginosa* is known to damage the lung epithelium, and can facilitate proliferation of T and B cell subsets.<sup>20</sup> Hence, we hypothesized that the isolation of *Pseudomonas aeruginosa* in respiratory specimens after lung transplantation increases the risk of DSA development independent of other risk factors including the underlying diagnosis. To that end, we sought to identify risk factors for the development of DSA. Some of the results from this manuscript have previously been reported in an abstract form<sup>21</sup>.

## 2. MATERIALS AND METHODS

### 2.1. Study Design, Settings and Participants

We performed a retrospective single-center cohort study and included all 477 primary lung transplant recipients at Barnes-Jewish Hospital between January 1, 2008 and December 31, 2015, with follow-up through December 31, 2018. We excluded multi-organ transplant recipients (n = 2), those who underwent desensitization before transplantation (n = 4), those who had pre-transplant DSA (n = 1), those who developed HLA antibodies but donor specificity could not be established (n = 4), and those who died or underwent a retransplant prior to first DSA screen (n = 5) (Figure 1). The Washington University School of Medicine Institutional Review Board for Human Studies approved the study protocol (ID # 201811073).

## 2.2. Clinical Management

As part of our routine clinical protocol, most patients were treated with anti-thymocyte globulin or basiliximab for induction immunosuppression. All recipients were treated with three-drug maintenance immunosuppression. In the immediate post-transplant period, all patients were treated with broad-spectrum antibacterial antibiotics that were tailored according to recipient and donor cultures for at least 14 days. DSA screens were performed at 10 days, 1, 2, 3, 6, and 12 months after transplantation and every 3 months thereafter. Additional DSA screens were performed during episodes of allograft dysfunction. Similarly, bronchoscopy with bronchoalveolar lavage (BAL) and transbronchial lung biopsies was performed at 1, 2, 3, 6, and 12 months after transplantation and to evaluate episodes of allograft dysfunction. Bronchial washings and BAL fluid were sent for routine bacterial cultures and additional microbiological studies. All positive bacterial cultures were treated with appropriate oral or intravenous antibiotics for at least 14 days. Additional details regarding our program's clinical management protocols are provided in the online data supplement.

## 2.3. Variables

HLA antibodies were detected using the LABScreen™ Single Antigen assay, and DSA was considered positive if the mean fluorescence intensity (MFI) was  $\geq 2000$ . In cases where the donor was not high-resolution typed and DSA could not be definitively identified, the patients were excluded from the study (Figure 1). The time from transplantation to the first detection of DSA was defined as **time to DSA positivity**. **DSA clearance** was defined as all DSA tests being negative after an initial positive result, and the time between the first positive and the negative result after which all other results remained negative was defined as the **time to DSA clearance**. A positive bacterial culture from a bronchial wash or BAL was defined as **bacterial isolation**<sup>22-26</sup>. We did not attempt to distinguish between infection and colonization. In other words, we considered *Pseudomonas aeruginosa* isolation if *Pseudomonas aeruginosa* was detected in the bronchial wash, BAL or sputum, and the first time it was detected post-transplantation was considered as the **time to *Pseudomonas* isolation**. However, based on the *a priori* defined objectives of our study, we did not distinguish whether bacterial isolation was on a surveillance bronchoscopy or during a hospital admission for deterioration of respiratory status or lung function. One of the reasons to do this was that we wanted to identify the risk of developing DSA in the event certain bacteria were isolated from the lungs; and our clinical interpretation of the risk would be unlikely to change in either setting. Rather, we focused on the cumulative number of isolates, and whether they were *de novo* or persistent (i.e., present prior to transplantation in the recipient). PGD and acute cellular rejection were defined based on the International Society of Heart and Lung Transplantation (ISHLT) criteria<sup>27,28</sup>. CLAD was defined as a persistent decline in FEV1  $\geq 80\%$  for at least 3 weeks without a specific cause<sup>29</sup>. **CLAD-free survival** was defined as the earliest occurrence of either CLAD or death, whichever came first<sup>2</sup>.

Given that colonization with *Pseudomonas aeruginosa* (as well as other infections) and the occurrence of acute rejection and lymphocytic bronchiolitis could occur simultaneously with the development of donor-specific antibodies (DSA), we treated these risk factors as time-

dependent covariates in a Cox proportional hazards model. Thus, our analyses accounted for the time of occurrence of the explanatory variables when determining the risk conferred by these variables on the development of DSA. In such analyses (say, for the development of DSA), the “time to outcome” was identified as occurring when the **first** DSA was detected, and the risk factor was treated in a time-dependent manner by selecting the **first** time after transplant that *Pseudomonas* was isolated in the bronchial wash, bronchoalveolar lavage or sputum. Therefore, in all cases in these analyses, the explanatory variables occurred before the outcome<sup>30,31</sup>.

#### 2.4. Statistical Analysis

Differences in patient demographics were evaluated using the Pearson Chi-Square test when comparing categorical variables and the Mann-Whitney test for continuous variables. Results were reported as median (interquartile range) unless otherwise specified. Survival analysis included Kaplan-Meier and Cox proportional hazard models using events after transplantation as time-dependent covariates after conducting regression diagnostics. Specifically, acute rejection, lymphocytic bronchiolitis and bacterial isolation were treated as time-dependent covariates when evaluating risk factors for the development of DSA and DSA clearance. To minimize confounding, we defined the covariates for each of the three multivariable models *a priori* based on review of the literature<sup>12,13,32</sup>, and the focus of the study:

**Model 1:** *Pseudomonas aeruginosa* isolation post-transplantation as a time-dependent covariate, CPRA, PGD grade 3 at any point, LAS,

**Model 2:** Acute cellular rejection (Grade A2) as a time-dependent covariate, CPRA, PGD grade 3 at any point, LAS,

**Model 3:** Lymphocytic bronchiolitis (Grade B1R) as a time-dependent covariate, CPRA, PGD grade 3 at any point, LAS.

As a result, certain variables (for example, recipient age) were not included even if the p value was < 0.1 on univariate analysis<sup>33</sup>. We included only one time-dependent variable in each multivariable model to avoid overinflation of risk; as a result, we present three multivariable models<sup>34</sup>. Given the interaction between PGD and cardiopulmonary bypass (CPB), the results were presented before and after adjusting for CPB. Because of the risk of DQ-DSA on adverse outcomes, we performed a subgroup analysis to identify risk factors for developing DQ-DSA<sup>8,10,15,35</sup>. Similar multivariable models were evaluated as described above, for the development of DQ-DSA. An interval-censored survival analysis was also performed to handle the timing of screening when calculating the time of bacterial isolation and time of DSA-positivity. Statistical analysis was performed using SPSS 26.0 (SPSS Inc., Chicago, IL) and p values <0.05 were considered statistically significant.

We confirmed the proportional hazards assumption for all time-independent variables in the Cox proportional hazards models, using the log-negative-log method. We performed regression diagnostics for the multivariable models by identifying outliers using the DFBETA test, ran the time-dependent Cox proportional hazards models and plotted the

residuals against the study ID (Supplementary Figure 1). Finally, we excluded nonlinearity for all variables in the Cox proportional hazards models, using partial residual plots.

### 3. RESULTS

#### 3.1. Demographic characteristics of the cohort

The 460 patients in our cohort were followed up for a mean of  $4.82 \pm 2.86$  years (range: 0.03 – 10.99 years). We had 4,500 DSA results, with each patient having a mean of  $9.74 \pm 4.55$  DSA tests. We had 3,110 culture results for bacterial isolates, with each patient having a mean of  $6.73 \pm 3.13$  isolates. We had 2,549 transbronchial biopsy results, with each patient having a mean of  $5.52 \pm 2.34$  biopsies. Among the 460 patients included in this cohort, 205 (45%) developed DSA during the study period (Figure 2). Those who developed DSA were more likely to be younger, have a higher CPRA prior to transplantation, and have required cardiopulmonary bypass during surgery (Table 1 and 2). Of note, there was no difference in the distribution of recipient gender or underlying diagnosis between the 2 groups. Additional baseline characteristics of the 2 groups are shown in Table 1. DSA-free survival was 4.26 years (Figure 2, 95% CI: 3.83 – 4.69 years). Among the 205 who developed DSA, 175 (85%) developed DSA to Class II HLA, and 145 (71%) developed DSA to HLA-DQ alleles (Figure 3). *Pseudomonas aeruginosa* was the most frequently isolated bacterial organism from bronchoscopy specimens after transplantation; among the 460 recipients, 147 (32%) had at least one positive culture for *Pseudomonas aeruginosa*. 87 of the 147 recipients (59%) who had *Pseudomonas aeruginosa* isolation after transplantation did not have *Pseudomonas aeruginosa* isolation before transplantation. While the DSA appeared before *Pseudomonas* isolation in 10.7% of our cohort, these cases were excluded from the time-dependent Cox proportional hazards analyses and did not contribute to the risk conferred by *Pseudomonas* isolation on DSA development<sup>30,31</sup>. Gram-negative bacilli other than *Pseudomonas aeruginosa* were isolated at least once in 182 (40%) recipients, and gram-positive cocci were isolated in 103 (22%) recipients. *Candida* and *Aspergillus* species were cultured more frequently; 358 (78%) recipients had at least one positive culture for *Candida* species, and 158 (34%) had at least one positive culture for *Aspergillus* species. Among the 147 who had at least one positive culture for *Pseudomonas aeruginosa* after transplantation, 70 (47%) had cystic fibrosis, 38 (26%) had interstitial lung disease, 23 (16%) had COPD, and 16 (11%) had other underlying diagnoses (Table S1). Patients with cystic fibrosis in whom *Pseudomonas* was isolated comprised of 15% (70/460) of the total cohort (Table S1). Among these 70 patients, 38 (54%) had DSA and 32 (46%) did not.

#### 3.2. Risk factors for DSA development

Among the variables that we selected for inclusion into multivariable models *a priori*, *Pseudomonas aeruginosa* isolation (HR = 1.68; 95%CI: 1.15 – 2.45,  $p = 0.007$ ), acute rejection grade A2 (HR = 1.97; 95%CI: 1.39 – 2.78,  $p < 0.001$ ), and lymphocytic bronchiolitis grade B1R (HR = 1.69; 95%CI: 1.18 – 2.41,  $p = 0.004$ ) were associated with a significantly increased risk of DSA development in univariate analyses (Table 2). Additionally, *Pseudomonas aeruginosa* isolation was associated with an increased risk of DSA development (HR 1.9; 95%CI: 1.13 – 3.19,  $p = 0.015$ ) even after excluding cases ( $n=80$ ) in whom *Pseudomonas aeruginosa* was isolated pretransplant. The cumulative acute

rejection A score and the cumulative lymphocytic bronchiolitis B score after transplantation were also associated with an increased risk of DSA development (Table 2). PGD grade 3 at any time point within the first 72 hours was not a significant risk factor (HR = 1.28; 95%CI: 0.94 – 1.72, p = 0.114). In a subgroup analysis of recipients in whom transfusion data were available (n=168), the number of packed red blood cells received at the time of transplantation did not confer an increased risk of DSA development (HR = 1.01, 95%CI: 0.95 – 1.08, p = 0.720). However, there was no association between the LAS at the time of transplantation and DSA development (HR = 1.00; 95%CI: 0.99 – 1.01, p = 0.607). *Post-hoc* analysis of baseline characteristics that were different between those who developed DSA and those who did not revealed that the use of cardiopulmonary bypass was associated with an increased risk of DSA development (HR = 1.46; 95%CI: 1.11 – 1.92, p = 0.008), while recipient age was inversely associated with the risk of DSA development (HR = 0.99; 95%CI: 0.98 – 1.00, p = 0.010, Table 2). CMV viremia or CMV isolation in bronchoscopy specimens, the isolation of *Pseudomonas aeruginosa* in pre-transplant respiratory specimens, the isolation of gram-positive bacteria post-transplant, the isolation of *Aspergillus* post-transplant and the isolation of CARV post-transplant were not associated with an increased risk of DSA development (Table 2).

In multivariable models, each of the time-dependent covariates (1) *Pseudomonas aeruginosa* isolation (2) acute rejection grade A2, and (3) lymphocytic bronchiolitis grade B1R, was associated with a significantly increased risk of DSA development (Table 3 and S2). CPRA remained a significant and independent risk factor in the 3 models. PGD grade 3 at any time point within the first 72 hours was not a significant risk factor on multivariable models. This association also held true as a part of a sensitivity analysis when considering the excluded patients with “possible DSA” (Table S3) and on an interval-censored survival analysis (Table S4). The magnitude of the risk remained independently associated with DSA development when stratified on cardiopulmonary bypass (Table 3). When restricted to the development of DSA to HLA-DQ, the three time-dependent covariates (1) *Pseudomonas aeruginosa* isolation, (2) acute rejection grade A2, and (3) lymphocytic bronchiolitis grade B1R were also independently associated with an increased risk in both univariate and multivariable analyses (Table 4 and Table 5). In contrast, CPRA was not associated with an increased risk of DSA development to HLA-DQ (Table 4).

In addition, there was a direct relationship between the number of positive cultures for *Pseudomonas aeruginosa* and the risk of DSA development in both univariate (HR = 1.08 for every +”n” positive culture, 95%CI: 1.04 – 1.13, <p=0.001, Table 2) and multivariable analyses when adjusted for CPRA, LAS and PGD (HR = 1.08 for every +”n” positive culture, 95%CI: 1.04 – 1.13, p=<0.001). The isolation of other Gram-negative bacteria, or other organisms (i.e., Gram-positive bacteria, *Aspergillus*, CMV, CARV) was not associated with an increased risk of DSA development (Table 2 and 4). After accounting for CPRA, LAS and PGD in the multivariable analysis, the magnitude of risk for developing DSA to HLA-DQ remained high in recipients who had *Pseudomonas aeruginosa* isolation after transplantation (Table 5).



### 3.3. *Pseudomonas aeruginosa* isolation and long-term outcomes

Among the 460 recipients, 306 (67%) developed CLAD or died during the study period. The development of DSA was associated with worse CLAD-free survival (HR = 1.80, 95% CI: 1.43 – 2.25,  $p < 0.001$ , Table 6). Among those who developed DSA ( $n=205$ ), clearance of DSA was associated with better CLAD-free survival (HR = 0.39, 95% CI: 0.28 – 0.55,  $p < 0.001$ , Table 6). In other words, there was a 71% reduction in the risk of CLAD or allograft failure in those who cleared DSA compared to those who did not clear DSA. Given that isolation of *Pseudomonas aeruginosa* has been associated with worse long-term outcomes after transplantation, we examined its association with CLAD-free survival. Isolation of Gram-positive and Gram-negative bacteria, including *Pseudomonas*, was associated with worse CLAD-free survival on univariate analysis (Table 6). The cumulative number of positive cultures for *Pseudomonas aeruginosa* after transplantation was also associated with worse CLAD-free survival (HR = 1.06 for every +”n” positive culture, 95% CI: 1.03 – 1.10,  $p < 0.001$ ), but the isolation of *Pseudomonas aeruginosa* before transplantation was not. We also found that lymphocytic bronchiolitis was associated with worse CLAD-free survival on univariate and multivariable analysis (HR = 1.29, 95% CI 1.02 – 1.62,  $p = 0.035$ , Table 6 and 7). On multivariable analysis of *a priori* defined variables, *Pseudomonas aeruginosa* isolation after transplantation remained a risk factor for CLAD-free survival after adjusting for PGD Grade 3 at any time point and LAS (HR = 1.42, 95% CI: 1.11 – 1.81,  $p=0.005$ , Table 7) as well as after adding the cumulative acute rejection A score post-transplantation (Table S5); and in a separate model after adding the cumulative lymphocytic bronchiolitis B score post-transplantation (Table S5).

## 4. DISCUSSION

The development of DSA portends poor outcomes after lung transplantation<sup>5-10</sup>. In this study, we show that there are distinct risk factors associated with the development of DSA. Specifically, we confirm that acute rejection is associated with an increased risk of DSA, as previously reported, and identify a similar risk attributable to lymphocytic bronchiolitis<sup>12</sup>. Moreover, we now identify *Pseudomonas aeruginosa* isolation from the respiratory tract as an independent risk factor for the development of DSA, which has not been previously reported. Furthermore, we identify a significant association between *Pseudomonas aeruginosa* isolation and the development of DSA to mismatched DQ alleles, which is independently associated with an increased risk of CLAD<sup>10,35</sup>. These findings suggest that innate immune responses to *Pseudomonas aeruginosa* can activate humoral alloimmune responses after lung transplantation. The association of *Pseudomonas aeruginosa* isolation and DSA was independent of the underlying diagnosis. In addition, our findings corroborate previous studies by demonstrating that the development of DSA is a strong risk factor for CLAD and death<sup>7-10</sup>, whereas DSA clearance mitigates this risk. Additionally, we report that *Pseudomonas aeruginosa* isolation is associated with worse CLAD-free survival, as is the cumulative number of positive cultures for *Pseudomonas aeruginosa*. Our multivariable analysis also identified *Aspergillus* isolation, and the development of DSA as risk factors for worse CLAD-free survival on multivariable analysis, which is consistent with reports of them being risk factors for deleterious long-term outcomes after lung transplantation<sup>8,22,23</sup>. Our findings are consistent with those of the UCLA group who had previously reported that

the likelihood of transition from transplant to chronic rejection and death is increased by the interactions between *Pseudomonas aeruginosa*, *Aspergillus* and ELR+ CXC chemokines derived from lung epithelial cells that can act via its receptor CXCR2 (IL-8 receptor  $\beta$ ).<sup>22</sup> Interestingly, CARV did not appear to be a risk factor for the development of DSA in our analyses. Our findings are consistent with the findings of the CTOTC-03 study, which although done in pediatric lung transplant recipients, also did not find a relationship between CARV infection and the development of alloimmune or autoimmune humoral or cellular responses<sup>36</sup>.

Previous studies have identified risk factors for the development of DSA, but these have not examined the role of microbial isolation from the respiratory tract although infection is a common pro-inflammatory complication after lung transplantation. Indeed, the focus of the study identifying PGD as a risk factor for DSA was to evaluate the role of pro-inflammatory cytokines, with the underlying hypothesis that PGD-induced inflammation augments alloimmunity, thereby predisposing to CLAD<sup>13</sup>. Similarly, the HALT (HLA Antibodies after Lung Transplantation) study identified acute rejection and the lung allocation score as independent risk factors for DSA development; however, the primary focus of the study was to define the incidence of DSA and their characteristics early after transplantation in a prospective multi-center fashion<sup>12</sup>. Our results complement these studies in identifying PGD and acute rejection as risk factors for DSA and expand the identified pro-inflammatory complications associated with DSA to *Pseudomonas aeruginosa* isolation. Taken together, these findings support the paradigm that pro-inflammatory events after transplantation increase the risk of DSA development, which has been proposed in both pre-clinical and clinical studies<sup>18,22,37,38</sup>.

Over half of those who had *Pseudomonas aeruginosa* isolation after transplantation were culture-negative for *Pseudomonas aeruginosa* before transplantation. Moreover, the association between post-transplant *Pseudomonas aeruginosa* and DSA persisted even after excluding those who were culture-positive for *Pseudomonas aeruginosa* before transplantation. This suggests that the acquisition of *Pseudomonas aeruginosa* after transplantation promotes the development of donor-specific antibodies. Our data indicate that this relationship is unique to *Pseudomonas aeruginosa*, as we did not detect an association between other bacteria, fungi or viruses and DSA in our cohort. Several reports have demonstrated that *Pseudomonas aeruginosa* stimulates potent innate immune responses, including airway neutrophilia<sup>39-41</sup>. Our group has also shown that *Pseudomonas aeruginosa* infection in a mouse model of lung transplantation promotes neutrophilia through a G-CSF-dependent mechanism that prevents established tolerance<sup>39</sup>. Notably, we also observed that *Pseudomonas aeruginosa* infection induced allograft-infiltrating neutrophils to upregulate the B7 molecules CD80 and CD86, which in turn could drive alloantigen-specific T cell responses through providing B7:CD28 co-stimulation in trans.<sup>42</sup> Although in that study we did not measure DSA, it is interesting to speculate that *Pseudomonas aeruginosa*-mediated neutrophil B7 upregulation could bolster CD28-mediated CD4<sup>+</sup> T cell help to B cells to promote DSA generation in a similar manner. Ultimately, the inflammatory environment induced by *Pseudomonas aeruginosa* may promote the clonal expansion of alloreactive T-cells and the polyclonal activation of B-cells<sup>13,43,44</sup>, and upregulation of HLA molecules on airway epithelial cells<sup>45,46</sup> to amplify the humoral alloimmune response.



*Pseudomonas aeruginosa* is also capable of facilitating affinity maturation and promoting isotype switching and somatic mutations in alloreactive B-cells, especially if these antigens are already exposed on the allograft<sup>47,48</sup>. Moreover, *Pseudomonas aeruginosa* isolation from the respiratory tract or the resultant clinical treatment with antibiotics may modulate the gut microbiome and result in both protective and deleterious humoral responses<sup>49,50</sup>. Thus, altering the microbiome in itself may modulate the severity of allograft injury<sup>51,52</sup>. Together these results establish a precedent to investigate mechanistically how *Pseudomonas aeruginosa* may promote alloimmune responses, and how these responses can be mitigated to improve long-term outcomes after lung transplantation.

There is a possibility that the abovementioned crosstalk between the innate and adaptive responses contribute to the association between *Pseudomonas* and CLAD, specifically through DSA driving antibody-mediated rejection (AMR). To that effect, we have previously reported in an abstract that *Pseudomonas* isolation may be a trigger for AMR.<sup>53</sup> Since DSA is an integral component of the clinical definition of AMR and is thought to be the cause of AMR, one would expect a clear association between *Pseudomonas*, DSA, AMR and CLAD. However, most patients who have DSA do not develop AMR, although they are at a higher risk for CLAD.<sup>54</sup> A mechanistic link to explain how *Pseudomonas* may trigger antibody-mediated rejection through DSA is currently being actively investigated by our research group.

There are limitations that are inherent to this study's design that should be considered when interpreting our data. First, this was a single-center retrospective study in patients transplanted between 2008–2015. Although the majority of patients were treated with a similar clinical protocol after transplantation, there may be patient-specific deviations that might impact alloimmune responses that have not yet been identified as modulating DSA development. Second, we did not differentiate colonization from infection when considering *Pseudomonas aeruginosa* isolation. We believe that this distinction would be fraught with complications in a retrospective analysis, particularly because it is often difficult to make this distinction prospectively in clinical practice. For this reason, we elected to use the term "isolation" to avoid implying that an invasive infection was present. Nevertheless, this does not detract from our findings that isolating *Pseudomonas aeruginosa* in respiratory specimens after transplantation is associated with the development of DSA. Third, we cannot exclude the possibility that some patients had DSA below the threshold that our laboratory has chosen (MFI = 2000), which cross that threshold after the isolation of *Pseudomonas aeruginosa*. However, the prevalence of DSA in our cohort was consistent with or even higher than other centers that have used a similar or lower cutoff.<sup>8,10,35</sup> Despite this, the interpretation that *Pseudomonas aeruginosa* increases the risk of DSA development needs to be tested in *in vivo* model systems with a lower threshold for reporting DSA. Fourth, as we used a Cox proportional hazards with time-dependent covariates, we did not consider waxing and waning status when determining how *Pseudomonas* (or acute rejection) affect the risk of developing DSA, as the "time to risk factor" and/or "time to event" was the **first** reported time to detection of the risk factor or event. As a result, we did not report on how the clearance of *Pseudomonas* alters the risk for DSA development or CLAD-free survival. Rather, we reported the number of *Pseudomonas* isolates from the same individual,

and found this to be a significant risk factor for both DSA and CLAD-free survival. One of the reasons for approaching the analysis in this manner is to avoid introducing bias into the analysis. For example, our surveillance bronchoscopies are only done at 1, 2, 3, 6 and 12 months post-transplant (thus, within the first year) and only clinically indicated thereafter. Hence, there are more data points in the first 6 months, compared to thereafter. As a result, although the patient may clear *Pseudomonas* in month 13, that may not be documented. Nevertheless, we aimed to handle this issue by reporting number of positive *Pseudomonas* cultures and the risk that it confers. Similarly, when evaluating CLAD-free survival, we considered “time to DSA clearance” as time-dependent variable. This would allow us to account for when the DSA was **first** identified as being cleared; however, we did not include it in the analysis if it recurred after initial clearance. Fifth, our data suggest a strong association between *Pseudomonas* isolation and the development of DSA, but this does not imply causality. It creates a precedence for subsequent mechanistic studies to determine how *Pseudomonas* contributes to DSA development.

Through this manuscript, we propose that pro-inflammatory events after lung transplantation, such as acute rejection and *Pseudomonas aeruginosa* isolation are associated with an increased risk of DSA development, independent of the underlying diagnosis. These findings suggest that the isolation of *Pseudomonas aeruginosa* should be followed by close monitoring for the development of DSA. Additionally, our findings would need to be validated in an independent cohort and warrant *in vivo* verification. Results from such logical extension of our findings may then form the basis for clinical investigations focused on ameliorating the risk of alloimmunity associated with *Pseudomonas aeruginosa* isolation. Additionally, because DSA development is a critical component of antibody-mediated rejection (AMR), our work lays the foundation for studies evaluating whether *Pseudomonas aeruginosa* isolation is associated with AMR.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## ABBREVIATIONS

<b>AMR</b>	antibody-mediated rejection
<b>BAL</b>	bronchoalveolar lavage
<b>CARV</b>	community-acquired respiratory viruses

<b>CI</b>	confidence interval
<b>CLAD</b>	chronic lung allograft dysfunction
<b>CMV</b>	cytomegalovirus
<b>CPB</b>	cardiopulmonary bypass
<b>CPRA</b>	calculated panel reactive antibody
<b>DSA</b>	donor specific antibodies
<b>G-CSF</b>	granulocyte-colony stimulating factor
<b>HALT</b>	HLA Antibodies after Lung Transplantation (study)
<b>HLA</b>	human leukocyte antigen
<b>HR</b>	hazard ratio
<b>ISHLT</b>	International Society of Heart and Lung Transplantation
<b>IQR</b>	interquartile range
<b>LAS</b>	Lung Allocation Score
<b>MFI</b>	mean fluorescent intensity
<b>PGD</b>	primary graft dysfunction

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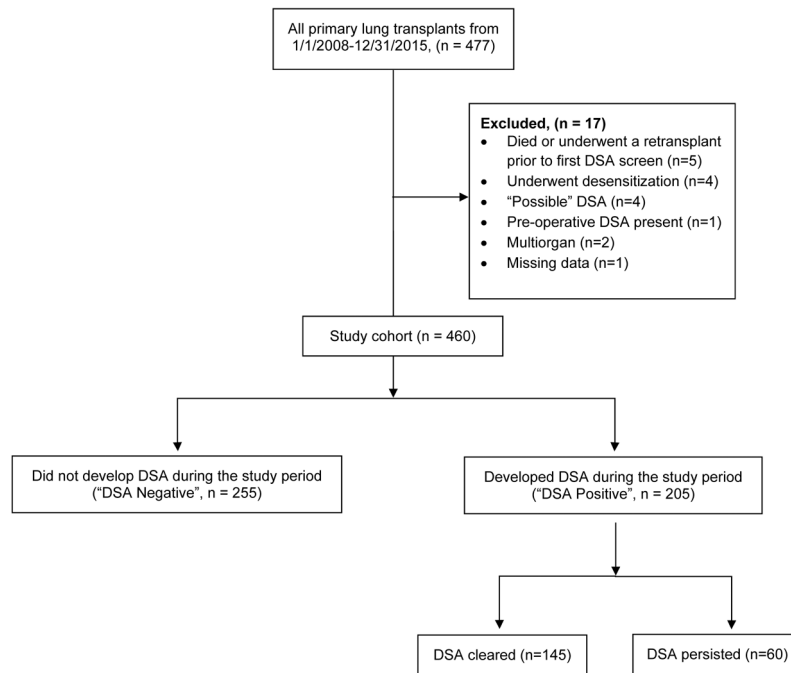
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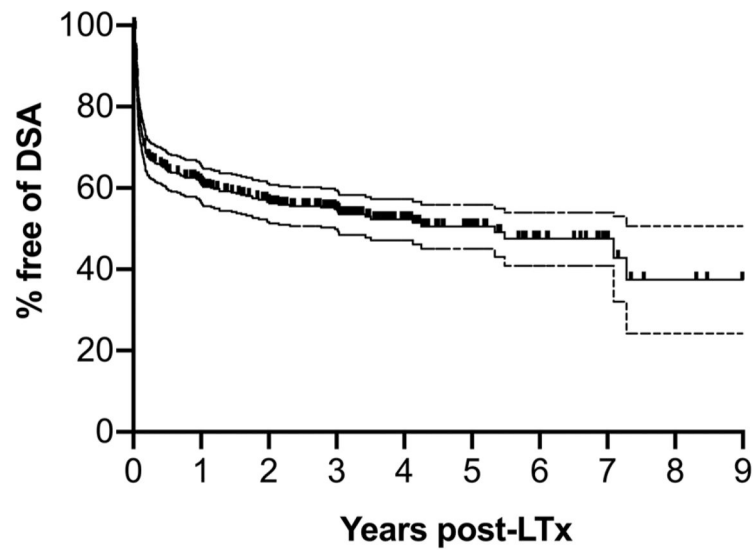
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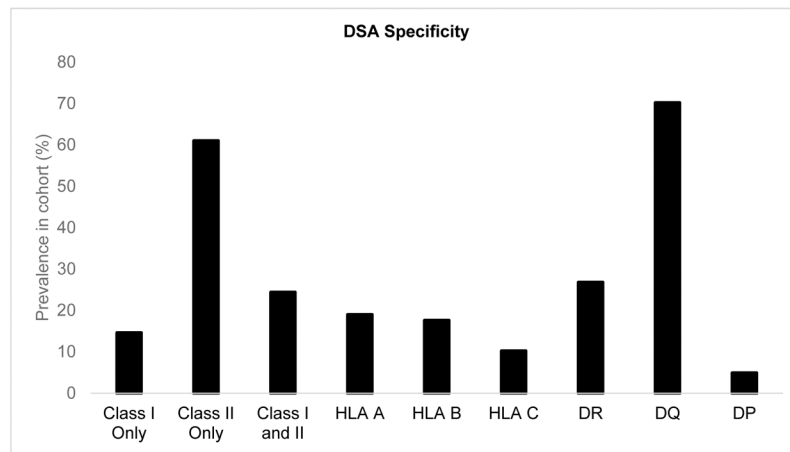
**Figure 1: Flow diagram: study screening and eligibility.**

Derivation of the study cohort. Flowchart of lung transplant recipients and application of eligibility criteria that resulted in the final study cohort stratified by whether they developed donor-specific antibodies (DSA), and whether they cleared it.



Year	0	1	2	3	4	5	6	7	8	9
<b>At-risk</b>	460	242	184	136	71	42	20	10	4	1
<b>Events</b>	0	173	189	194	199	201	203	203	205	205

**Figure 2: Freedom from donor-specific antibodies (DSA) after lung transplantation (LTx).** Kaplan-Meier survival methods estimated the proportion of recipients in whom DSA had not occurred at follow-up after lung transplantation (LTx). The numbers below the x-axis time points represent those at risk. Cohorts consisted of primary LTx reported from January 1, 2008 and December 31, 2015, with follow-up through December 31, 2018. Analyses censored for end of study follow-up and loss to follow-up.



**Figure 3: Characteristics of donor-specific antibodies (DSA).**

This graph represents the specificity of donor-specific antibodies (DSA) present in those recipients transplanted between January 1, 2008 and December 31, 2015, with follow-up through December 31, 2018 (n=205), represented as a proportion (out of 100%). Each recipient may have more than one DSA and thus, may have contributed to more than one bar.

**Table 1:**

## Demographics

Variable	DSA negative (n=255)	DSA positive (n=205)
Female recipient, n (%)	104 (40.8)	85 (41.5)
Recipient age at first transplant	59 (16.8)	55 (22.3)
Diagnosis, n (%)		
Interstitial lung disease/pulmonary fibrosis	115 (45.1)	87 (42.4)
COPD	65 (25.5)	44 (21.5)
Cystic fibrosis (CF)/non-CF bronchiectasis	40 (15.7)	44 (21.5)
Pulmonary hypertension	4 (1.6)	3 (1.5)
Other	31 (12.2)	27 (13.2)
Lung allocation score at transplant	40.5 (16)	42.4 (24)
GERD	113 (44.3)	77 (37.6)
Pre-transplant cPRA	10.5 (21.3)	15.2 (24.7)
Total HLA mismatch number	8 (2)	8 (2)
Transplant type, n (%)		
Single	6 (2.4)	7 (3.4)
Bilateral	249 (97.6)	198 (96.6)
Intraoperative CPB, yes n (%)	113 (44.3)	111 (54.1)
Ischemic time, min	267.5 (97)	277.5 (110)
CMV mismatch (yes), n (%)	73 (28.9)	59 (28.9)
Allosensitized, n (%)	91 (35.7)	72 (35.1)
Pre-operative ECMO, n (%)	2 (0.8)	2 (1.0)
Post-operative ECMO, n (%)	9 (3.5)	5 (2.4)
Induction immunosuppression		
Basiliximab	207 (81.2)	150 (73.2)
ATGAM	43 (16.9)	44 (21.5)
Other	3 (1.2)	5 (2.4)
None	1 (0.4)	3 (1.5)
Unknown	1 (0.4)	3 (1.5)
PGD grade 3 at any time, n (%)	64 (25.1)	60 (29.3)

Abbreviations: COPD: chronic obstructive pulmonary disease; CF: cystic fibrosis; cPRA: calculated panel reactive antibodies; HLA: human leukocyte antigen; CPB: cardiopulmonary bypass; CMV: cytomegalovirus; ECMO: extracorporeal membrane oxygenation; GERD: gastroesophageal reflux disease; ATGAM: antithymocyte globulin; PGD: primary graft dysfunction. Continuous variables are represented as median (interquartile range) except for cPRA, which is represented as mean (standard deviation) as the median was 0 in both groups.

**Table 2:**

Univariable Cox proportional hazards models of risk factors for posttransplant donor-specific HLA antibody (DSA) development.

Variable	HR	95% CI	p
<b>Recipient age</b>	<b>0.99</b>	<b>0.98-1.00</b>	<b>0.01</b>
Diagnosis, CF	1.03	0.95-1.12	0.43
LAS	1.00	1.00-1.01	0.61
GERD	0.83	0.63-1.11	0.83
<b>Pre-transplant CPRA</b>	<b>1.01</b>	<b>1.00-1.01</b>	<b>0.02</b>
HLA mismatch number	1.06	0.97-1.17	0.19
Transplant type, bilateral	0.81	0.38-1.72	0.58
<b>Intraoperative CPB</b>	<b>1.46</b>	<b>1.11-1.92</b>	<b>0.008</b>
Induction, Basiliximab	0.75	0.55-1.03	0.07
PGD grade 3 at any time	1.28	0.94-1.72	0.114
<b>Acute rejection Grade A2*</b>	<b>1.97</b>	<b>1.39-2.78</b>	<b>&lt;0.001</b>
<b>Lymphocytic bronchiolitis Grade B1R*</b>	<b>1.69</b>	<b>1.18-2.41</b>	<b>0.004</b>
<b>Cumulative A rejection score post-LTx</b>	<b>1.09</b>	<b>1.03-1.15</b>	<b>0.001</b>
<b>Cumulative B rejection score post-LTx</b>	<b>1.15</b>	<b>1.06-1.25</b>	<b>0.001</b>
Gram-positive bacteria isolation post-LTx*	0.99	0.66-1.48	0.96
Gram-negative bacteria isolation post-LTx*	1.23	0.90-1.68	0.20
<b><i>Pseudomonas</i> isolation post-LTx*</b>	<b>1.68</b>	<b>1.15-2.45</b>	<b>0.007</b>
<b>Number of positive <i>Pseudomonas</i> cultures</b>	<b>1.08</b>	<b>1.04-1.13</b>	<b>&lt;0.001</b>
CARV isolation post-LTx*	1.05	0.65-1.69	0.86
<i>Pseudomonas</i> isolation pre-LTx	1.10	0.77-1.55	0.61
<i>Aspergillus</i> isolation post-LTx*	1.34	0.88-2.05	0.17
CMV in blood post-LTx*	0.88	0.63-1.22	0.45
CMV in BAL post-LTx*	0.88	0.60-1.28	0.49

**Abbreviations:** BAL: bronchoalveolar lavage; CARV: community-acquired respiratory viruses; CF: cystic fibrosis; CPRA: calculated panel reactive antibodies; CPB: cardiopulmonary bypass; CMV: cytomegalovirus; GERD: gastroesophageal reflux disease; LTx: lung transplant; PGD: primary graft dysfunction. p value is for Cox proportional hazards model with “time to DSA positivity” as time to event.

\* indicates variables which were evaluated using a time-dependent Cox proportional hazards model.

**Table 3:**

Multivariable Cox proportional hazards models of risk factors for posttransplant donor-specific HLA antibody (DSA) development

Variable	Non-stratified			Stratified for CPB		
	HR	95% CI	p	HR	95% CI	p
<b><i>Pseudomonas</i> isolation post-LTx</b>	<b>1.75</b>	<b>1.18-2.60</b>	<b>0.005</b>	<b>1.76</b>	<b>1.19-2.61</b>	<b>0.005</b>
<b>Pre-transplant CPRA</b>	<b>1.01</b>	<b>1.00-1.01</b>	<b>0.009</b>	<b>1.008</b>	<b>1.00-1.01</b>	<b>0.008</b>
PGD grade 3 at any point	1.25	0.91-1.71	0.176	1.173	0.85-1.62	0.331
LAS	1.00	1.00-1.01	0.397	1.00	0.99-1.01	0.879

Variable	Non-stratified			Stratified for CPB		
	HR	95% CI	p	HR	95% CI	p
<b>Acute rejection Grade A2*</b>	<b>2.06</b>	<b>1.43-2.97</b>	<b>&lt;0.001</b>	<b>2.01</b>	<b>1.4-2.9</b>	<b>&lt;0.001</b>
<b>Pre-transplant CPRA</b>	<b>1.01</b>	<b>1.00-1.01</b>	<b>0.017</b>	<b>1.01</b>	<b>1.00-1.01</b>	<b>0.015</b>
PGD grade 3 at any point	1.27	0.92-1.75	0.141	1.2	0.87-1.65	0.276
LAS	1.00	1.00-1.01	0.430	1.00	0.99-1.01	0.927

Variable	Non-stratified			Stratified for CPB		
	HR	95% CI	p	HR	95% CI	p
<b>Lymphocytic bronchiolitis Grade B1R*</b>	<b>1.72</b>	<b>1.18-2.51</b>	<b>0.005</b>	<b>1.72</b>	<b>1.18-2.51</b>	<b>0.005</b>
<b>Pre-transplant CPRA</b>	<b>1.01</b>	<b>1.00-1.01</b>	<b>0.012</b>	<b>1.01</b>	<b>1.00-1.01</b>	<b>0.01</b>
PGD grade 3 at any point	1.3	0.94-1.78	1.109	1.21	0.88-1.68	0.238
LAS	1.00	1.00-1.01	0.389	1.00	0.99-1.01	0.876

**Abbreviations:** CPRA: calculated panel reactive antibodies; CPB: cardiopulmonary bypass; HR: hazards ratio; CI: confidence interval; LAS: lung allocation score; LTx: lung transplant; PGD: primary graft dysfunction.



**Table 4:**

Univariable Cox proportional hazards models of risk factors for posttransplant donor-specific HLA antibody (DSA) development restricted to DQ-DSA

Variable	HR	95% CI	p
<b>Recipient age</b>	<b>0.98</b>	<b>0.97-0.99</b>	<b>0.001</b>
Diagnosis, CF	1.04	0.94-1.14	0.49
LAS	1.00	0.99-1.01	0.93
Pre-transplant CPRA	1.00	0.99-1.01	0.81
Transplant type, bilateral	0.64	0.28-1.45	0.28
Intraoperative CPB	1.32	0.95-1.83	0.1
<b>Induction, Basiliximab</b>	<b>0.66</b>	<b>0.46-0.94</b>	<b>0.02</b>
PGD grade 3 at any time	1.08	0.74-1.56	0.70
<b>Acute rejection Grade A2*</b>	<b>2.05</b>	<b>1.38-3.05</b>	<b>&lt;0.001</b>
<b>Lymphocytic bronchiolitis Grade B1R*</b>	<b>2.07</b>	<b>1.39-3.08</b>	<b>&lt;0.001</b>
<b>Cumulative A rejection score post-LTx</b>	<b>1.11</b>	<b>1.04-1.18</b>	<b>0.001</b>
<b>Cumulative B rejection score post-LTx</b>	<b>1.17</b>	<b>1.07-1.29</b>	<b>0.001</b>
Gram-positive bacteria isolation post-LTx*	0.99	0.62-1.58	0.95
Gram-negative bacteria isolation post-LTx*	1.41	0.98-2.02	0.06
<b><i>Pseudomonas</i> isolation post-LTx*</b>	<b>2.16</b>	<b>1.43-3.26</b>	<b>&lt;0.001</b>
<b>Number of positive <i>Pseudomonas</i> cultures</b>	<b>1.11</b>	<b>1.06-1.16</b>	<b>&lt;0.001</b>
<i>Pseudomonas</i> isolation pre-LTx	1.17	0.78-1.77	0.44
Aspergillus isolation post-LTx*	1.38	0.84-2.26	0.20
CMV in blood post-LTx*	0.77	0.52-1.14	0.19
CMV in BAL post-LTx*	0.83	0.53-1.30	0.42

**Abbreviations:** BAL: bronchoalveolar lavage; CF: cystic fibrosis; CPRA: calculated panel reactive antibodies; CPB: cardiopulmonary bypass; CMV: cytomegalovirus; LTx: lung transplant; PGD: primary graft dysfunction. p value is for Cox proportional hazards model with "time to DSA positivity" as time to event.

\* indicates variables which were evaluated using a time-dependent Cox proportional hazards model.

**Table 5:**

Multivariable Cox proportional hazards models of risk factors for posttransplant donor-specific HLA antibody (DSA) development restricted to DQ-DSA.

Variable	HR	95% CI	p
<b><i>Pseudomonas</i> isolation post-LTx</b>	<b>2.26</b>	<b>1.46-3.50</b>	<b>&lt;0.001</b>
Pre-transplant CPRA	1.00	1.00-1.01	0.55
PGD grade 3 at any point	1.06	0.71-1.58	0.77
LAS	1.00	0.99-1.01	0.82

Variable	HR	95% CI	p
<b>Acute rejection Grade A2*</b>	<b>2.21</b>	<b>1.45-3.37</b>	<b>&lt;0.001</b>
Pre-transplant CPRA	1.00	0.99-1.01	0.78
PGD grade 3 at any point	1.09	0.73-1.62	0.67
LAS	1.00	0.99-1.01	0.82

Variable	HR	95% CI	p
<b>Lymphocytic bronchiolitis Grade B1R*</b>	<b>2.06</b>	<b>1.35-3.15</b>	<b>0.001</b>
Pre-transplant CPRA	1.00	0.99-1.01	0.68
PGD grade 3 at any point	1.13	0.76-1.69	0.54
LAS	1.00	0.99-1.01	0.79

**Abbreviations:** CPRA: calculated panel reactive antibodies; CPB: cardiopulmonary bypass; LAS: lung allocation score; LTx: lung transplant; PGD: primary graft dysfunction.

**Table 6:**

Univariable Cox proportional hazards models of risk factors for chronic lung allograft dysfunction-free survival (CLAD-free survival).

Variable	HR	95% CI	p
<b>Recipient age</b>	<b>0.99</b>	<b>0.98-1.00</b>	<b>0.004</b>
Diagnosis, CF	1.00	0.94-1.07	0.92
LAS	1.00	1.00-1.01	0.18
cPRA	1.00	0.99-1.00	0.27
Transplant type, bilateral	1.08	0.56-2.10	0.82
Intraoperative CPB	1.16	0.92-1.45	0.21
Induction, Basiliximab	1.10	0.84-1.43	0.50
PGD grade 3 at any time	1.24	0.97-1.59	0.09
A2 rejection or greater *	1.15	0.91-1.45	0.23
<b>Lymphocytic bronchiolitis,<math>\geq</math>B1R*</b>	<b>1.29</b>	<b>1.02-1.63</b>	<b>0.03</b>
Cumulative A rejection score post-LTx	1.04	0.99-1.09	0.15
Cumulative B rejection score post-LTx	1.09	1.00-1.18	0.05
<b>Gram-positive bacteria isolation post-LTx*</b>	<b>1.46</b>	<b>1.14-1.87</b>	<b>0.003</b>
<b>Gram-negative bacteria isolation post-LTx*</b>	<b>1.48</b>	<b>1.18-1.85</b>	<b>0.001</b>
<b><i>Pseudomonas</i> isolation post-LTx*</b>	<b>1.42</b>	<b>1.11-1.81</b>	<b>0.005</b>
<b>Number of positive <i>Pseudomonas</i> cultures</b>	<b>1.06</b>	<b>1.03-1.10</b>	<b>&lt;0.001</b>
<i>Pseudomonas</i> isolation pre-LTx	1.07	0.80-1.43	0.64
<b><i>Aspergillus</i> isolation post-LTx*</b>	<b>1.50</b>	<b>1.19-1.90</b>	<b>0.001</b>
CARV isolation post-LTx*	0.98	0.76-1.27	0.90
CMV in blood post-LTx*	1.27	1.00-1.61	0.05
CMV in BAL post-LTx*	1.07	0.85-1.35	0.55
<b>Development of DSA*</b>	<b>1.80</b>	<b>1.43-2.25</b>	<b>&lt;0.001</b>
DSA Class			0.28
DSA Class II only	1.56	0.90-2.72	0.17
DSA Class II DR	1.36	0.98-1.89	0.07
<b>DSA Class II DQ</b>	<b>1.65</b>	<b>1.31-2.07</b>	<b>&lt;0.001</b>
DSA Class II DP	1.04	0.49-2.21	0.91
<b>DSA developing &lt; 30 days post-LTx</b>	<b>0.69</b>	<b>0.50-0.95</b>	<b>0.02</b>
DSA developing < 90 days post-LTx	0.90	0.64-1.27	0.54
<b><i>Clearance of DSA*</i></b>	<b>0.39</b>	<b>0.28-0.55</b>	<b>&lt;0.001</b>

**Abbreviations:** BAL: bronchoalveolar lavage; CARV: community-acquired respiratory viruses; CF: cystic fibrosis; CPRA: calculated panel reactive antibodies; CPB: cardiopulmonary bypass; CMV: cytomegalovirus; DSA: donor-specific antibodies; LTx: lung transplant; PGD: primary graft dysfunction. p value is for Cox proportional hazards model with “time to CLAD-free survival” as time to event.

\* indicates variables which were evaluated using a time-dependent Cox proportional hazards model. Note that those variables in italics were applicable only to those patients who were DSA positive (n=205).

**Table 7:**

Multivariable Cox proportional hazards models of risk factors for chronic lung allograft dysfunction-free survival (CLAD-free survival).

Variable	HR	95% CI	p
<b><i>Pseudomonas</i> isolation post-LTx</b>	<b>1.42</b>	<b>1.11-1.81</b>	<b>0.005</b>
PGD grade 3 at any point	1.22	0.94-1.57	0.14
LAS	1.00	1.00-1.01	0.41

Variable	HR	95% CI	p
<b>Lymphocytic bronchiolitis,<math>\geq</math>B1R*</b>	<b>1.29</b>	<b>1.02-1.62</b>	<b>0.035</b>
PGD grade 3 at any point	1.20	0.93-1.55	0.171
LAS	1.00	1.00-1.01	0.34

**Abbreviations:** DSA: donor-specific antibodies; LAS: lung allocation score; LTx: lung transplant; PGD: primary graft dysfunction.