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BRIEF COMMUNICATION

Comparison of donor-derived cell-free DNA between single versus double lung transplant recipients

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Plasma donor-derived cell-free DNA (dd-cfDNA) is a sensitive biomarker for the diagnosis of acute rejection in lung transplant recipients; however, differences in dd-cfDNA levels between single and double lung transplant remains unknown. We performed an observational analysis that included 221 patients from two prospective cohort studies who had serial measurements of plasma dd-cfDNA at the time of bronchoscopy and pulmonary function testing, and compared dd-cfDNA between single and double lung transplant recipients across a range of disease states. Levels of dd-cfDNA were lower for single vs. double lung transplant in stable controls (median [IQR]: 0.15% [0.07, 0.44] vs. 0.46% [0.23, 0.74], $p < .01$) and acute rejection (1.06% [0.75, 2.32] vs. 1.78% [1.18, 5.73], $p = .05$). Doubling dd-cfDNA for single lung transplant to account for differences in lung mass eliminated this difference. The area under the receiver operating curve (AUC) for the detection of acute rejection was 0.89 and 0.86 for single and double lung transplant, respectively. The optimal dd-cfDNA threshold for the detection of acute rejection was 0.54% in single lung and 1.1% in double lung transplant. In conclusion, accounting for differences in dd-cfDNA in single versus double lung transplant is key for the interpretation of dd-cfDNA testing in research and clinical settings.

Abbreviations: ACR, acute cellular rejection; ALAD, acute lung allograft dysfunction; AMR, antibody mediated rejection; AR, acute rejection; BAL, bronchoalveolar lavage; CLAD, chronic lung allograft dysfunction; dd-cfDNA, donor-derived cell-free DNA; DSA, donor specific antibody; FEV1, forced expiratory volume in 1 second; PFT, pulmonary function test; TBBx, transbronchial biopsy.

KEYWORDS
clinical research/practice, lung transplantation/pulmonology, donors and donation: donor-derived infections, rejection, rejection: acute

1 | INTRODUCTION

When cells undergo apoptosis or necrosis, they release fragments of DNA into the bloodstream known as cell-free DNA (cfDNA). Given that solid organ transplantation creates a unique scenario in which there is genomic admixture between donor and recipient, it is possible to genetically differentiate between cfDNA originating from the recipient versus the donor-derived cell-free DNA (dd-cfDNA). As such, plasma dd-cfDNA has emerged as a novel molecular biomarker of allograft injury after solid organ transplantation, including lung transplant.[1] Prior studies have demonstrated that levels of plasma dd-cfDNA increase in the setting of acute rejection and infection after lung transplantation and have evaluated the performance characteristics of dd-cfDNA to detect these entities.[2-5]

Considerable differences in donor lung mass between single and double lung transplant recipients may influence the interpretation of dd-cfDNA levels, both at baseline and in the setting of acute allograft injury. Previous studies, however, have handled dd-cfDNA levels in single and double lung transplant recipients in different ways. While some studies utilize a "correction" for dd-cfDNA levels in single lung transplant patients by doubling the value,[2,3,6] other studies did not adjust the values to account for differences in lung mass.[4,5,7] This heterogeneity of investigational approaches may contribute to difficulties in interpreting results of prior and ongoing research of dd-cfDNA in lung transplantation. Further insight into how levels of dd-cfDNA differ between single and double lung transplant recipients is therefore critical for the adequate interpretation of assay results. In this study, we aimed to determine whether the levels of dd-cfDNA are higher in double vs. single lung transplant recipients, by (1) comparing levels of dd-cfDNA in single and double lung transplant recipients and (2) comparing the performance characteristics of dd-cfDNA for the detection of allograft injury between single and double lung transplant recipients.

2 | MATERIALS AND METHODS

2.1 | Study design and participants

We conducted an observational analysis of subjects enrolled in two prospective cohort studies. The first study, Genome Transplant Dynamics (GTD) (NCT01985412), was conducted between December 1, 2010 and December 31, 2012 at the Stanford University Hospital. The second study, Genome Research Alliance for Transplantation (GRAfT) (NCT0243070), is currently ongoing and began enrollment in 2015 at three centers (the Inova Fairfax Hospital, Johns Hopkins Hospital, and University of Maryland Medical Center). Both studies were designed to evaluate the utility of dd-cfDNA to monitor for acute rejection and included subjects >18 years of age awaiting lung transplantation. All patients underwent routine post-transplant monitoring with regular clinic visits, pulmonary function testing (PFT), surveillance bronchoscopy with bronchoalveolar lavage (BAL) and transbronchial biopsy (TBBx), and donor specific antibody testing (DSA). Patients also received serial plasma sampling for dd-cfDNA on Days 1, 3, 7, 14, 21 post-transplant, and at the time of all surveillance and for-cause bronchoscopies. Patients who died within 30 days of transplantation were excluded from the study. This study was approved by the Institutional Review Board at each participating center and the National Heart, Lung, and Blood Institute.

2.2 | Clinical endpoints

The primary clinical endpoint of this study was acute rejection, a composite outcome of acute cellular rejection (ACR) grade A2 or higher, ACR grade 1 with allograft dysfunction (>10% decline in forced expiratory volume in 1 second (FEV1)), and clinical antibody mediated rejection (AMR). All endpoints were adjudicated by centralized multidisciplinary adjudication committees blinded to %dd-cfDNA data as previously described.[2,6,8] Endpoints were adjudicated using center level data to remain consistent with usual care practices. ACR was defined as histopathologic evidence of ACR on biopsy and graded by pathologists at each center according to International Society for Heart and Lung Transplant (ISHLT) guidelines.[9] Antibody mediated rejection was defined according to ISHLT guidelines for the diagnosis of possible, probable and definite clinical AMR.[10] Subclinical AMR (AMR not associated with allograft dysfunction) was not included. Allograft dysfunction was categorized according to the degree of spirometric decline in FEV1 as "no" (<10%), mild (10 to <15%), or severe (>15%). Pathogens were defined as positive microbiology on bronchoalveolar lavage and were further categorized as being associated with allograft dysfunction or not. The endpoint of acute lung allograft dysfunction (ALAD) was defined as a composite of ACR, AMR, and the presence of a pathogen accompanied by allograft dysfunction.[7] Endpoints were paired with dd-cfDNA levels drawn on the same day that the diagnostic studies (TBBx, BAL, PFT) were performed (and immediately prior to the TBBx). Controls were defined as those without the evidence of ACR, AMR, pathogens, or allograft dysfunction.

2.3 | Measurement of dd-cfDNA

Measurement of dd-cfDNA was performed using an automated shotgun sequencing method as previously described.[8,11] First, donor and recipient whole blood was obtained before transplant to

extract genomic DNA. Genotyping was performed using Illumina whole-genome array at 2.5 million positions (HumanOmni 2.5-8v1.2). Donor and recipient genotype information was compared to identify informative SNPs, which are SNPs to which the recipient is homozygous and different from the donor. After transplantation, whole blood was collected from recipients and spun to obtain plasma. Cell-free DNA was isolated from recipient plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen) and used to prepare DNA libraries (Mondrain Ovation SP Ultralow Library System). DNA libraries were sequenced using the Illumina HiSeq-2500 at 12.7 million reads (range 10.0–21.5 million reads). The sequence reads were filtered and trimmed to remove duplicates and low-quality reads, allowing for the remaining sequence reads to be aligned with the human genome build hg19 (www.ucsc.edu). These mapped reads were then surveyed to assign them as recipient and donor cfDNA using informative SNPs identified from genotyping. The dd-cfDNA was then calculated as a percentage (%) of the number of donor reads to number of donor plus recipient reads. Values for single lung transplant were either "corrected" by doubling the dd-cfDNA value to account for lung mass or "uncorrected" (not doubled).

2.4 | Statistical analysis

Continuous variables were summarized using mean (standard deviation (SD)) or median (interquartile range (IQR)), and categorical variables were summarized using counts (%). Nonlinear regression using an exponential, two-phase decay model was used to model post-transplant dd-cfDNA decay kinetics. We used a generalized estimating equation (GEE) approach to compare dd-cfDNA levels between different groups, while accounting for the correlation among repeated dd-cfDNA measurements in the same subject.[12] dd-cfDNA was log-transformed as $\log_2(x + 0.01)$ to reduce the skewness for the GEE analysis. Median values of dd-cfDNA are presented for clarity. We conducted receiver-operating characteristic (ROC) analysis by treating the dd-cfDNA level as the predictor, and acute rejection as the binary outcome. All analyses were performed using R software version 4.0.2 (Copyright 2020 The R Foundation for Statistical Computing) and GraphPad Prism 9.2.0. The R package "geepack" was used for GEE analyses.[13] Area under the curve was calculated by using the R package "pROC."[14] *p*-values were 2-sided with significance indicated by a value ≤ .05.

3 | RESULTS

3.1 | Cohort description and study endpoints

Of the 225 patients enrolled in the GRAfT and GTD studies at the time of our analysis, 4 patients died within 30 days post-transplant and were excluded, leaving 221 patients in the study, 135 from GRAfT and 86 from GTD (Figure 1). The average age (SD) was 49.7 (17) years, the average LAS score (SD) was 48.1 (18.4), the most common indication for transplant was interstitial lung disease (48%), and 71% of patients underwent bilateral lung transplant (Table 1). Over the median (IQR) 41.3 (25.0, 52.9) months of follow-up, 948 biopsies were performed for histopathology (654 in GRAfT and 294 in GTD). In order to account for post-transplant logarithmic dd-cfDNA decay kinetics, histopathology performed

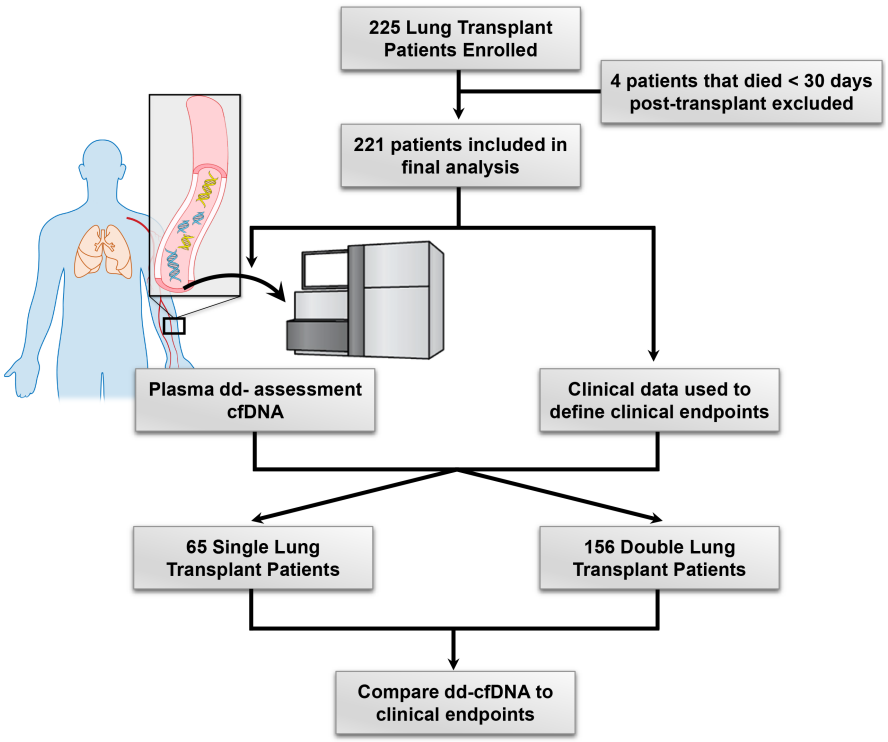

FIGURE 1 Flowchart representing the study design. A total of 221 patients were included in the final analysis. dd-cfDNA, donor-derived cell-free DNA [Color figure can be viewed at wileyonlinelibrary.com]

<45 days post-transplant and biopsy specimens that lacked allograft tissue were eliminated, leaving 714 samples. There were 115 episodes of acute rejection over the course of the study, including 36 episodes of ACR (≥A2 or A1 with allograft dysfunction) and 79 episodes of clinical AMR. A pathogen was isolated in 265 BAL samples, of which 67 were associated with allograft dysfunction. 222 samples were classified as controls with no evidence of ACR, AMR, pathogens, or allograft dysfunction.

3.2 | Post-transplant trends in dd-cfDNA

Overall, there were 2201 dd-cfDNA levels measured over the course of the study (≈9 per patient). The median (IQR) levels of dd-cfDNA on day 1 post-transplant were similar in double lung vs. single lung transplant (23.00% [17.98, 30.67] vs. 21.66% [17.10, 32.54], $p = .57$). Levels then decayed logarithmically for both single and double lung transplant with an initial half-life of 1.5 days in single and 1.2 days in double lung transplant, followed by a slower decay with a half-life of 20.4 days for single and 10.2 days for double. For stable controls, median dd-cfDNA levels reached 0.38% (0.17, 0.80) in single lung transplant and 0.85% (0.34, 1.67) in double lung transplant by 45 days after transplant. At 3 months post-transplant, single lung transplant reached levels of 0.18% (0.10, 0.45) and double lung transplants reached levels of 0.48% (0.26, 0.96) (Figure 2).

TABLE 1 Patient demographics

Recipient age (years [SD])	49.7 (17)
Lung allocation score (mean [SD])	48.1 (18.4)
Male recipient (%)	53%
Double lung transplant (%)	71%
Indication	
COPD	21%
Cystic fibrosis	19%
Interstitial lung disease	48%
Pulmonary arterial hypertension	2%
Other	10%
Race	
White	83%
Black	11%
Asian	2%
Other	4%

3.3 | Levels of dd-cfDNA in single vs. double lung transplant

For both ACR and AMR, single and double lung transplant patients exhibited higher levels of dd-cfDNA than controls. Considering stable controls, with no infection or rejection, single lung transplant patients exhibited lower levels of dd-cfDNA than double lung transplant patients (Median [IQR]: 0.15% [0.07, 0.44] vs. 0.46% [0.23, 0.74], $p < .01$). Left sided single lung transplants had lower dd-cfDNA levels than right sided single lung transplant patients (0.09% [0.06, 0.17] vs. 0.26% [0.12, 0.58], $p = .04$). For episodes of acute rejection, single lung transplant patients exhibited lower levels of dd-cfDNA than double lung transplant patients (1.06% [0.75, 2.32] vs. 1.78% [1.18, 5.73], $p = .05$). Levels of dd-cfDNA were higher in single lung transplant patients with acute rejection than in controls (1.06% [0.75, 2.32] vs. 0.37% (0.16, 0.83), $p < .01$). Double lung transplant patients with acute rejection also demonstrated higher levels of dd-cfDNA vs. controls (1.78% [1.18, 5.73] vs. 0.37% [0.16, 0.83], $p < .01$) (Table 2).

When analyzing dd-cfDNA levels by the type of acute rejection, single lung transplant patients with AMR exhibited lower levels of dd-cfDNA than double lung transplant patients with AMR (1.09 [0.78, 3.25] vs. 2.66[1.55, 6.94], $p = .04$); however, dd-cfDNA values were not significantly different between single lung vs. double lung transplant patients with ACR ($p = .85$), although the sample size for single lung transplant patients with ACR was small ($n = 8$). Notably, single lung transplant patients had higher mean

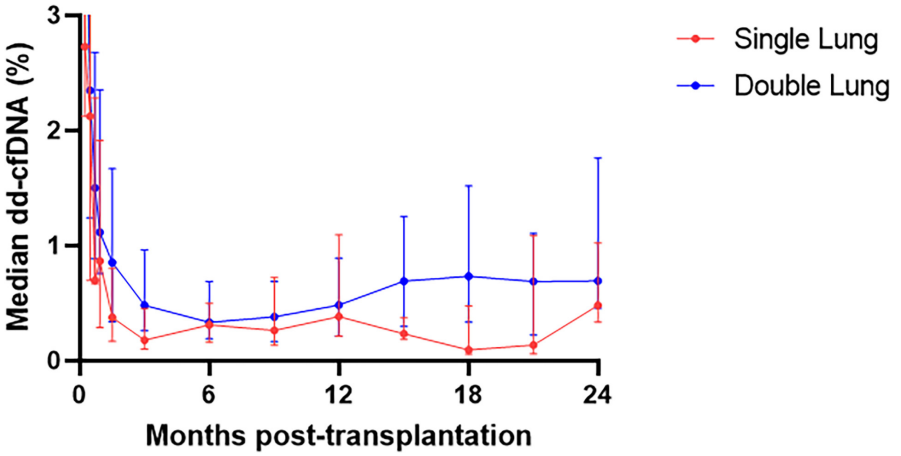

FIGURE 2 Median dd-cfDNA vs. time post-transplantation for both single and double lung transplant patients over the first 24 months post-transplant. dd-cfDNA, donor-derived cell-free DNA [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Comparison of dd-cfDNA levels in single vs. double lung transplant patients

Clinical endpoint	Single lung transplant median (IQR) dd-cfDNA (%)	Double lung transplant median (IQR) dd-cfDNA (%)	*p* value*
Controls	0.15% (0.07, 0.44)	0.46% (0.23, 0.74)	$p < .01$
Acute rejection	1.06% (0.75, 2.32)	1.78% (1.18, 5.73)	$p = .05$
ACR	0.87% (0.72, 1.32)	1.18% (0.52, 1.52)	$p = .85$
AMR	1.09% (0.78, 3.25)	2.66% (1.55, 6.94)	$p = .04$
Pathogens	0.46% (0.14, 1.21)	0.73% (0.30, 1.86)	$p = .26$
Pathogens with allograft dysfunction	0.64% (0.41, 1.64)	1.64% (0.66, 4.05)	$p = .08$
ALAD	0.91% (0.61, 2.12)	1.69% (0.95, 4.47)	$p = .04$

Abbreviations: ACR: acute cellular rejection; ALAD: acute lung allograft dysfunction; AMR: antibody mediated rejection; dd-cfDNA: donor-derived cell-free DNA.

*p-values comparing dd-cfDNA levels between single vs. double lung transplant patients across a range of pathologies using generalized estimating equations.

histological grades of ACR than double lung transplant patients (2.35 vs. 1.95, $p = .03$).

In patients with ALAD (composite of ACR, AMR, and pathogen + allograft dysfunction), levels of dd-cfDNA were lower in single vs. double lung transplant (0.91% [0.61, 2.12] vs. 1.69% [0.95, 4.47], $p = .04$). Both single and double lung transplant patients with ALAD exhibited higher levels of dd-cfDNA than controls. However, single lung transplant patients with pathogens did not have significantly lower levels of dd-cfDNA than double lung transplant (0.46% [0.14, 1.21] vs. 0.73% [0.30, 1.86], $p = .26$). Similarly, there was no significant difference between single and double lung transplant patients with pathogens + allograft dysfunction (0.64% [0.41, 1.64] vs. 1.64% [0.66, 4.05], $p = .08$).

3.4 | Doubling single lung dd-cfDNA values to correct for allograft mass

As prior studies have attempted to "correct" single lung transplant dd-cfDNA values by multiplying the value by a factor of 2 in order to account for differences in lung tissue mass, we compared dd-cfDNA values between single vs. double lung transplant recipients in which the single lung values were corrected by doubling the value. Correction by doubling dd-cfDNA for single lung transplant controls resulted in similar dd-cfDNA levels to double lung transplant controls (0.30% [0.01, 0.87] vs. 0.46% [0.23, 0.74], $p = .59$). Likewise, for acute rejection, doubling the levels of dd-cfDNA for single lung transplant resulted in similar levels to double lung transplant (2.11% [1.50, 4.63] vs. 1.78% [1.18, 5.73], $p = .53$). However, while doubling single lung values resulted in similar levels to double lung patients in the setting AMR (2.17% [1.55, 6.5] vs. 2.66% [1.55, 6.93], $p < .01$), doubling the single lung transplant patient values for patients with ACR resulted in significantly higher levels than double lung transplant patients (1.74% [1.44, 2.29] vs. 1.18% [0.52, 1.52], $p = .03$). There was no difference in dd-cfDNA values between corrected single vs. double lung transplant patients with ALAD, pathogens, and pathogen + allograft dysfunction.

TABLE 3 Performance characteristics of dd-cfDNA for the detection of endpoints

Clinical endpoint	Single lung transplant AUC (95% CI)	Double lung transplant AUC (95% CI)
Acute rejection	0.89 (0.82–0.97)	0.86 (0.81–0.90)
ACR	0.87 (0.77–0.97)	0.73 (0.64–0.83)
AMR	0.91 (0.84–0.98)	0.91 (0.87–0.95)
ALAD	0.84 (0.75–0.93)	0.83 (0.78–0.88)

3.5 | Performance characteristics of dd-cfDNA for detecting allograft injury in single vs. double lung transplant

The AUC of dd-cfDNA for detecting acute rejection was similar between single and double lung transplant patients, 0.89 (95% CI: 0.82, 0.97) and 0.86 (95% CI: 0.81, 0.90), respectively (Figure 3). The optimal threshold value for the detection of acute rejection in single lung transplant patients was 0.54%, with a sensitivity of 92% and specificity of 80%. For double lung transplant patients, the optimal threshold value was 1.1%, with a sensitivity of 78% and specificity of 83%. Thus, *corrected* single lung transplant and double lung transplant patients both demonstrated an optimal threshold value of 1.1% for the detection of acute rejection. The performance characteristics of dd-cfDNA for the detection of additional clinical endpoints are presented in Table 3.

4 | DISCUSSION

In this study, we found that levels of dd-cfDNA are higher in double vs. single lung transplant patients across a range of clinical endpoints including stable controls, acute rejection, AMR, and ALAD. Doubling dd-cfDNA levels in single lung transplant patients, in order to account for differences in lung mass, largely eliminated the differences in values between single and double lung transplant. Furthermore,

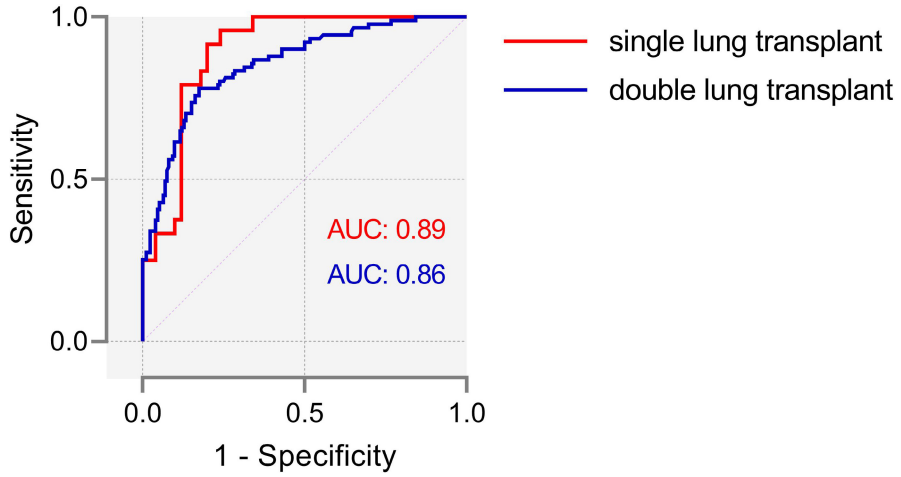

FIGURE 3 Performance characteristics of dd-cfDNA to detect acute rejection for both single and double lung transplant recipients. dd-cfDNA, donor-derived cell-free DNA; DL, double lung; SL, single lung [Color figure can be viewed at wileyonlinelibrary.com]

the performance characteristics of dd-cfDNA to detect acute rejection were similar between single and double lung transplant patients with a similar threshold value ~1% for both double lung and "corrected" single lung transplant values, supporting the practice of providing a correction for dd-cfDNA in single lung transplant by doubling the value. These results provide valuable insight into the appropriate interpretation of dd-cfDNA levels in single and double lung transplant patients.

In one of the first studies evaluating the association of dd-cfDNA with episodes of acute rejection, De Vlaminck et al. noted that the measured cell turnover rate was approximately two-fold higher in double vs. single lung transplant recipients, providing the basis for accounting for differences in tissue mass in single vs. double lung transplant by multiplying the dd-cfDNA level for single lung transplant patients by a factor of 2.[3] Subsequent studies have utilized this method with varying frequency, with some studies doubling dd-cfDNA levels in single lung transplant[2,6,8] and other studies electing not to provide this correction.[4,5,7] However, our findings confirm that differences in dd-cfDNA levels between double and single lung transplant patients require further consideration in the interpretation of values for both clinical and investigational purposes.

These considerations are relevant to the establishment of threshold values for the detection of various forms of allograft failure. In our cohort, the optimal threshold value for the detection of acute rejection was 1.1% in double lung transplant but 0.54% in single lung transplant. If this threshold value of 1.1% was applied to a combined population of both double and single lung transplant patients, the sensitivity and specificity of dd-cfDNA to detect acute rejection in the single lung transplant patients would now be only 50% and 88%, respectively—vastly different than double lung transplant patients only (78% sensitivity and 83% specificity). This implies that either different threshold values should exist for single vs. double lung transplant patients or that dd-cfDNA levels in single vs. double lung transplant should be corrected to account for differences in tissue mass. Furthermore, these findings have considerable implications for performance of future research and the interpretation of pre-existing research that may not have accounted for inherent differences in dd-cfDNA levels between single vs. double lung transplant patients. As demonstrated above, studies evaluating the performance characteristics and the establishment of threshold values for the detection of various clinical pathologies may be inaccurate if they did not perform adjustment for single vs. double lung transplant. These same considerations may apply to future studies evaluating dd-cfDNA in the diagnosis of other conditions such as chronic lung allograft dysfunction or changes in dd-cfDNA in response to treatment of acute rejection or infection.

In contrast to dd-cfDNA levels in single vs. double lung transplant patient controls, acute rejection, AMR, and ALAD, it is notable that there were no such differences in levels of dd-cfDNA between single and double lung transplant patients with pathogens and ACR. In fact, corrected single lung transplants with ACR had higher levels of dd-cfDNA than double lung transplant with ACR. This may be due to the small sample size of single lung patients with ACR and pathogen + allograft dysfunction; also, single lung patients in our cohort had more severe grades of ACR than double lung transplant patients.

To the best of our knowledge, this is the first study providing a comprehensive evaluation of differences in dd-cfDNA levels in single vs. double lung transplant patients. However, this study does have limitations. The lack of additional radiographic and clinical data precluded a more comprehensive assessment of allograft infection, rather than the simple presence of pathogens on microbiology. However, our combined outcome of pathogen plus allograft dysfunction may provide a better approximation of clinically significant allograft infection. While the overall sample size in our analysis was relatively robust, the sample size for single lung transplant patients alone was more modest. This analysis is also limited by the lack of a validation cohort. Future studies should aim to validate our results, particularly utilizing a larger sample size of single lung transplants. Finally, there may be variability between centers in the identification and grading of histopathology for acute rejection.

In summary, levels of dd-cfDNA are higher in double vs. single lung transplant patients in the stable state and during various types of allograft injury, but demonstrate similar performance characteristics. Accounting for differences in dd-cfDNA in single vs. double lung transplant patients is paramount to proper interpretation, and doubling of dd-cfDNA levels in single lung transplant patients to

account for differences in lung tissue mass appears to be an effective strategy.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

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