Assessment of dd-cfDNA Levels in Clinically Stable Lung Allograft Recipients Beyond the Initial 2 y Posttransplant

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Background. Donor-derived cell-free DNA (dd-cfDNA) is a useful biomarker for the diagnosis of acute allograft injury within the first 1 to 2 y after lung transplant, but its utility for diagnosing chronic lung allograft dysfunction (CLAD) has not yet been studied. Understanding baseline dd-cfDNA kinetics beyond the initial 2 y posttransplant is a necessary first step in determining the utility of dd-cfDNA as a CLAD biomarker. We seek to establish baseline dd-cfDNA% levels in clinically stable lung allograft recipients who are >2 y posttransplant. Methods. We performed a prospective, single-center, observational study to identify plasma dd-cfDNA levels in clinically stable lung allograft recipients >2 y posttransplant. Results. Fifty-one subjects were enrolled and ≥3 baseline dd-cfDNA measurements were acquired during a median of 252 d. The median baseline percent dd-cfDNA level in our cohort was 0.45% (interquartile range [IQR], 0.26–0.69). There were statistically significant differences in dd-cfDNA based on posttransplant duration (≤5 y posttransplant median 0.41% [IQR, 0.21–0.84] versus >5 y posttransplant median 0.50% [IQR, 0.33–0.76]; P < 0.02). However, the clinical significance of this small change in dd-cfDNA is uncertain because this magnitude of change is within the biologic test variation of 73%. Conclusions. This study is the first to define levels of dd-cfDNA in clinically stable patients who are >2 y post-lung transplant. These findings lay the groundwork for the study of dd-cfDNA as a possible biomarker for CLAD.

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INTRODUCTION

Long-term survival after lung transplantation is limited, with a median survival of 6.7 y.1 The leading cause of mortality and allograft failure beyond the initial year posttransplant is chronic lung allograft dysfunction (CLAD).2 CLAD is progressive, irreversible fibrosis of the lung parenchyma with differences in prognosis based on the phenotype of bronchiolitis obliterans or restrictive allograft syndrome.3 CLAD develops because of an amalgam of acute insults over time; risk factors for CLAD include acute cellular rejection (ACR), antibody-mediated rejection, primary graft dysfunction, gastroesophageal reflux disease, and infections with Staphylococcus species, Pseudomonas, cytomegalovirus (CMV), or community-acquired respiratory viruses.4 By 5 y posttransplant, >50% of lung transplant recipients will develop CLAD.4

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Although anti-inflammatory therapies can delay CLAD onset or stabilize lung function once CLAD is diagnosed, there are no known cures. As such, prevention and early diagnosis are paramount. Recent work has identified donor-derived, cell-free DNA (dd-cfDNA) as a novel biomarker with increased utility for CLAD prediction within the first 3 mo posttransplant.

Circulating cell-free DNA are short fragments of double-stranded DNA 50 to 200 base pairs in length, released by apoptotic and necrotic cells. During times of allograft injury, dd-cfDNA levels increase, which upon cessation of injury levels return to baseline. Given differences in single-nucleotide polymorphisms across the genome between donor and recipient, the percent of DNA originating from the allograft can be determined without knowledge of either donor or recipient genotype. Levels of dd-cfDNA correlate with ACR in lung transplant recipients, with a sensitivity of 77%, specificity of 84%, positive predictive value of 60%, and negative predictive value of 90% at a threshold of 1%. dd-cfDNA is also a biomarker of antibody-mediated rejection.

Given the diagnostic utility of dd-cfDNA for acute lung allograft dysfunction (ALAD), we postulated that dd-cfDNA might identify CLAD in patients who are >2 y posttransplant. However, little is known about dd-cfDNA kinetics in patients beyond the initial 2 y posttransplant. Therefore, an important initial step is to understand the relationship between time and baseline levels of dd-cfDNA in clinically stable patients at time points more distant from transplantation. Given that findings that altered senescence, telomere length, and other aging mechanisms may contribute to CLAD, it is possible that levels of dd-cfDNA in clinically stable lung allograft recipients might change with time. We performed a single-center, prospective, observational study in clinically stable lung allograft recipients ≥2 y posttransplant, measuring plasma dd-cfDNA% at routine ambulatory clinic appointments to define the trajectory of dd-cfDNA release beyond 2 y after lung transplantation. We included subjects with stable lung function, with an absence of symptoms of acute respiratory illness, and with ≥3 dd-cfDNA measurements separated by at least 1 mo. The primary outcome was median dd-cfDNA% for 2 distinct subcohorts based on time posttransplant (≤5 y, >5 y). Secondary outcomes included intra- and interindividual coefficients of variation (CV1 and robust CV2, respectively), index of individuality (II), and reference change value (RCV). We hypothesized that baseline dd-cfDNA% in patients >2 y posttransplant would increase over time.

**PATIENTS AND METHODS**

**Study Cohort**

We performed a prospective, single-center, observational study at the Vanderbilt University Medical Center (VUMC) between January 1, 2021, and June 1, 2022 (VUMC Institutional Review Board #200233). Adult (aged >18 y) recipients of either single or bilateral transplants transplanted before January 1, 2019, and followed for routine care at VUMC who had stable lung function (absence of CLAD) compared with peak baseline average levels (FEV1 >90%, forced vital capacity >90%, and forced expiratory flow between 25% and 75% of exhaled breath >75% peak baseline average, defined as the best 2 values measured >3 wk apart) were eligible for inclusion. After obtaining written informed consent, patients were enrolled in subgroups based on time posttransplant to ensure inclusion of patients over a wide range of times since transplant to allow for possible CLAD development; we planned to have an equal number of patients ≤5 and >5 y posttransplant. Patients were invited to participate via email at the start of the study and every 3 mo thereafter. We excluded subjects who were recipients of allogeneic stem cell transplants, had prior solid organ transplants (including prior lung transplant), could not participate in spirometry, had a diagnosis of a nondermatologic malignancy posttransplant, received organs from syngeneic donors, or were pregnant during the study period. At any point during the study, dd-cfDNA samples were excluded if patients had symptoms concerning for ALAD (such as acute onset of cough, fever, dyspnea), acute infiltrates on chest imaging, or a reduction in FEV1 >10% from baseline. Samples from patients with prior episodes of ALAD were included if there was resolution of symptoms and return of spirometry to pre-ALAD values and as long as >30 d had passed after the ALAD episode. Patients were only included in the analysis if there were ≥3 dd-cfDNA measurements, each >1 mo apart. We used Research Electronic Data Capture (REDCap) for data collection and management.

**Management of Patients Posttransplant**

All patients received induction immunosuppression with basiliximab. Standard maintenance immunosuppression included a calcineurin inhibitor (tacrolimus preferred, goal trough 10–14 ng/mL within the first year posttransplant, followed by goal 8–12 ng/mL thereafter), an antiproliferative agent (mycophenolate mofetil 1000 mg twice daily preferred), and prednisone (tapered from 20 to 5 mg/d during the first 3 mo posttransplant). The antiproliferative was reduced or held if a patient had persistent or recurrent infection, malignancy, or cytopenias. Standard infection prophylaxis included trimethoprim–sulfamethoxazole, valganciclovir (for 6–12 mo posttransplant depending on donor/recipient CMV serostatus), and an antifungal (posaconazole preferred through 2 mo posttransplant). Patients were routinely followed in ambulatory clinic every 3 to 4 mo after the initial year posttransplant. Routine follow-up included laboratory analysis (complete metabolic panel, complete blood count with differential, CMV polymerase chain reaction, calcineurin inhibitor trough level, spirometry, and 2-view chest radiography).

**Measurement of dd-cfDNA**

Patients enrolled in the study underwent quantification of plasma dd-cfDNA at each routine ambulatory visit using AlloSure Lung kits (CareDx, Inc, Brisbane, CA). Briefly, this test uses next-generation sequencing to distinguish between dd-cfDNA and recipient-derived circulating cell-free DNA fragments in peripheral blood based on differences in single-nucleotide polymorphisms across 405 single-nucleotide polymorphisms. Peripheral venous blood was collected in 2 to 10 mL Streck containers, sealed according to package directions, and delivered to CareDx for processing, next-generation sequencing performance, and data analysis per the manufacturer’s proprietary methodology. Numeric results were reported to VUMC for each patient expressed as the percent of cell-free DNA that is donor-derived (dd-cfDNA%). For recipients of single lung transplants, dd-cfDNA% levels were adjusted by doubling the values. In studies of patients within 1 y of lung transplant, a value of >1% is interpreted as indicative of allograft injury.
**Assessment of Biologic Variation**

We assessed intraindividual CVp, CVg (robust), II, and RCVs for the entire cohort. CVp was calculated by dividing the standard deviation by the mean of the samples for each subject. Robust CVg was calculated by obtaining the median value of the median absolute deviation (median-Xi) and dividing it by the median.22 II was the ratio of CV/CVg. RCV was calculated using the formula $RCV = \frac{1}{2} \times 1.96 \times (CVA^2 + CVg^2^{0.5})$. Analytic coefficient of variation (CVa) for the AlloSure test has been previously established as 2.7%.23

**Statistics**

Categorical values were compared using the Pearson chi-square test or Fisher exact test, where appropriate. Continuous values were compared using the Mann-Whitney U test. Correlations between continuous variables were performed using Spearman’s coefficient. Statistical calculations were performed on Stata/BE, version 17.0 (College Station, TX).

**RESULTS**

**Demographics of Study Cohort**

Among the cohort of 310 living lung transplant recipients followed at VUMC, 211 were >2 y posttransplant at the time of study enrollment. Patients in this eligible pool were invited to participate via email, of which 125 patients expressed interest. Ultimately, 93 patients were enrolled in the study (Figure 1). Twenty-nine patients did not meet enrollment criteria because of impaired lung function, 2 patients did not provide consent, and 1 patient dropped out before blood sample collection. Patients included in the analysis were a median of 1762 d posttransplant (interquartile range [IQR], 1104–2563); the subcohort of patients transplanted ≤5 y were a median of 1149 d posttransplant (IQR, 969–1492), whereas those transplanted >5 y were a median of 2796 d posttransplant (IQR, 2191–3405). Fifty-one patients had ≥3 viable samples of dd-cfDNA within the study period and were included in the final analysis. Baseline demographics of subjects included in the analysis are listed in Table 1 and are divided into groups based on time posttransplant (≤5 y [N=29], >5 y [N=22]). The cohort that was ≤5 y posttransplant had statistically fewer episodes of ACR (50% versus 82%; $P=0.04$).

**dd-cfDNA Levels in Patients >2 y Posttransplant**

The mean number of samples per patient was 3.5. The median time between the first and last sample collected was 252 d (IQR, 212–328). There was no significant difference in median collection time between patients ≤5 y and those >5 y posttransplant (238 d [IQR, 205–307] versus 270 d [IQR, 233–324]; $P=0.08$). The median dd-cfDNA% value overall was 0.45% (IQR, 0.26%–0.68%; Figure 2). The 95th percentile value was 1.54%, and the 97.5th percentile was 1.90%. There was a statistically significant difference in median dd-cfDNA% between patients ≤5 y posttransplant versus those >5 y (≤5 y group median 0.41% [IQR, 0.21%–0.64%], >5 y group median 0.50% [IQR, 0.33%–0.76%]; $P=0.02$). There was no significant relationship between dd-cfDNA% and recipient sex, FEV1, previous history of ACR, or recipient age (Figure 3).

**Biological Variation of dd-cfDNA in Lung Allograft Recipients >2 y Posttransplant**

We determined the biological variability of plasma dd-cfDNA in this population (Figure 4). The coefficient of intraindividual variability (CVp) for the entire cohort was 26% (IQR, 17%–43%). Interindividual coefficient of variability (CVg) was estimated using the robust coefficient of variability and was 47%. II was 56%. RCV was 73%. These data demonstrate that the detected changes in dd-cfDNA% in patients ≤5 y posttransplant and those >5 y posttransplant fall within the biological variation of repeated testing.

**DISCUSSION**

In a prospective, single-center, observational cohort study, we demonstrate that the median dd-cfDNA% in clinically stable lung allograft recipients >2 y posttransplant is 0.45%. Although there is a statistically significant change in median dd-cfDNA% based on time posttransplant, these values are within the normal biologic variation for this test. Our study defines baseline levels of dd-cfDNA% in patients >2 y post–lung transplant. Levels in this time frame are similar to the baseline values of dd-cfDNA in patients within the first 2 y after lung transplantation (<0.5%).7,10,24 We initially hypothesized that dd-cfDNA may increase over time because of allograft aging processes related to cellular senescence, telomere biology, and other mechanisms. Although there is a statistically significant increase in dd-cfDNA% in the subcohort of patients >3 y posttransplant, the clinical significance of this small change is unclear because these levels are still within the variability of the test in this population. One possibility is that these patients have a greater propensity for subclinical CLAD, independent of normal aging processes, because of higher rates of previously resolved ACR. Assessing levels in a much larger cohort over time may elucidate whether this incremental increase in dd-cfDNA% reflects longitudinal allograft cellular turnover.

Keller et al11 demonstrated that normal biological variation within 2 y of transplant is 70%, up to the 95th percentile of 1.0. Our data show a similar amount of intraindividual variation in patients beyond 2 y posttransplant. We found that

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**FIGURE 1.** Study recruitment and enrollment. Flowchart describing participant screening and enrollment for this study. dd-cfDNA, donor-derived, cell-free DNA; VUMC, Vanderbilt University Medical Center.
interindividual variation is numerically greater in our cohort of patients >2 y posttransplant than reported values. Interestingly, several patients had dd-cfDNA >1.50%, despite having normal baseline spirometry and being asymptomatic. Although these values may be falsely positive, an alternative explanation is that dd-cfDNA may detect subclinical allograft injury in this population. We did assess dd-cfDNA versus relative FEV1 (Figure 3D) to determine whether there was a direct correlation between dd-cfDNA and subclinical chronic injury states (“pre-CLAD”); there did not seem to be any relationship, although such an analysis is limited by our inclusion criteria that selected for patients without CLAD. Moreover, this trial was designed as a noninterventional study, so we did not direct management based on results of dd-cfDNA. Future studies should test the feasibility of using dd-cfDNA as a tool to detect subclinical injury by incorporating a diagnostic schema that includes bronchoscopy with transbronchial biopsies, screening for de novo donor-specific antibodies, and assessments for gastroesophageal reflux disease or tobacco use. Notably, it is possible that variability in recipient-derived cfDNA could also impact the proportion of total cfDNA that is donor-derived. For example, nonpulmonary infection or extreme exercise could reduce dd-cfDNA% by increased recipient-derived cfDNA release. In kidney transplant patients, there are some data suggesting that recipient cfDNA increases over time; this dilution results in a decrease of dd-cfDNA percentage.25

Our study has several strengths. We focused specifically on defining the characteristics of plasma dd-cfDNA over time.
after transplant in seemingly healthy lung transplant recipients. Our study cohort is relatively large compared with other studies of dd-cfDNA in lung transplant recipients. Analysis of serial samples in each patient increases the likelihood that dd-cfDNA in our cohort truly represents clinical stability. The main limitations of our study are its single-center design and the lack of additional objective measurements of potential subclinical graft injury. We included samples from 3 patients with previous ALAD with a documented resolution of symptoms and recovery of changes in lung function. These patients may not only contribute to the variability of dd-cfDNA beyond 2 y after transplant but also reflect a real-world patient population suggesting that dd-cfDNA levels at distant times after transplant may have clinical utility. Finally, our findings are based on the use of the AlloSure test kit and may not apply to other platforms for assaying dd-cfDNA, especially regarding biologic variation. However, dd-cfDNA% thresholds that define acute lung allograft injury have been comparable across different propriety platforms. 

In conclusion, this study defines baseline levels of dd-cfDNA in clinically stable patients who are >2 y post–lung transplant. We show that median values of 0.45% with biologic variation of 73% up to 1.54 are the baseline in this population. These findings lay the groundwork for dd-cfDNA for future studies to test if dd-cfDNA is a powerful tool for early, noninvasive detection of CLAD.
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