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Relative Change in Donor-Derived Cell-free DNA is Superior to Absolute Values for Diagnosis of Acute Lung Allograft Dysfunction

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Background. Donor-derived cell-free DNA (dd-cfDNA%) is a biomarker of early acute lung allograft dysfunction (ALAD), with a value of ≥1.0% indicating injury. Whether dd-cfDNA% is a useful biomarker in patients >2 y posttransplant is unknown. Our group previously demonstrated that median dd-cfDNA% in lung recipients ≥2 y posttransplant without ALAD was 0.45%. In that cohort, biologic variability of dd-cfDNA% was estimated by a reference change value (RCV) of 73%, suggesting that change exceeding 73% may be pathologic. In this study, we aimed to determine whether dd-cfDNA% variability or absolute thresholds are optimal for detecting ALAD. **Methods.** We prospectively measured plasma dd-cfDNA% every 3 to 4 mo in patients ≥2 y post–lung transplant. ALAD was defined as infection, acute cellular rejection, possible antibody-mediated rejection, or change in forced expiratory volume in 1 s >10%, and was adjudicated retrospectively. We analyzed area under the curve for RCV and absolute dd-cfDNA% and reported performance of RCV ≥73% versus absolute value >1% for discriminating ALAD. **Results.** Seventy-one patients had ≥2 baseline measurements of dd-cfDNA%; 30 developed ALAD. RCV of dd-cfDNA% at ALAD had a greater area under the receiver operator characteristic curve than absolute dd-cfDNA% values (0.87 versus 0.69, $P = 0.018$). Test characteristics of RCV >73% for ALAD diagnosis were sensitivity 87%, specificity 78%, positive predictive value 74%, and negative predictive value 89%. In contrast, dd-cfDNA% ≥1% had sensitivity 50%, specificity 78%, positive predictive value 63%, and negative predictive value 68%. **Conclusions.** Relative change in dd-cfDNA% has improved test characteristics for diagnosing ALAD compared with absolute values.

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An elevated plasma donor-derived cell-free DNA (dd-cfDNA) to recipient-derived cell-free DNA ratio (dd-cfDNA%) is an effective biomarker for diagnosing acute lung allograft dysfunction (ALAD) in lung transplant recipients.[1] Apoptotic and necrotic cells release intracellular contents, including small fragments of DNA ~150 base pairs in length, into the bloodstream, where they have a short half-life of <1 d. DNA originating from donor cells can be differentiated from DNA released from recipient cells by assaying for differences across multiple single nucleotide polymorphism loci. Therefore, surveillance of peripheral blood dd-cfDNA% can provide a real-time assessment of lung allograft injury.[2] Dd-cfDNA% increases in the setting of acute cellular rejection, antibody-mediated rejection (AMR), and infection in several different cohorts of lung transplant recipients, all within the first 1 to 2 y posttransplant.[3-6] Recently, our group defined baseline dd-cfDNA% levels in a prospective, noninterventional cohort of 51 lung allograft recipients who did not have acute or chronic allograft dysfunction ≥2 y posttransplant.[7] The median dd-cfDNA% in this stable cohort was 0.45 (interquartile range, 0.26–0.69), intraindividual variation (CV_I) was 26%, and interindividual variation (CV_G) was 47%, resulting in a reference change value (RCV) of 73%. This work is an important foundational step toward the potential use dd-cfDNA% as a biomarker for detection of chronic lung allograft dysfunction (CLAD).

Currently, absolute dd-cfDNA% thresholds of 0.85% to 1% have been used to delineate lung allograft injury.[3-6] However, use of absolute measurements may result in high rates of both false positive and false negative tests, depending on normal baseline dd-cfDNA% values. Population-based reference values based on absolute thresholds may have limited use in situations in which within-participant variability (CV_I) of a test is significantly less than the between-participant variability (CV_G).[8] In such a setting, RCV, which provides an estimate of normal biologic and analytic variability for a test, may be a better adjudicator of normal versus abnormal biology. As such, we hypothesize that use of RCV to define ALAD may improve the diagnostic utility of dd-cfDNA% as a biomarker for ALAD in lung allograft recipients. We tested this hypothesis by comparing test performance characteristics for dd-cfDNA%.

MATERIALS AND METHODS

Study Cohort

This observational study cohort included 71 lung transplant recipients from Vanderbilt University Medical Center that were routinely followed in an ambulatory clinic every 3 to 4 mo between January 1, 2021, and October 1, 22 (Institutional Review Board #200233). Patient demographics, inclusion criteria, and exclusion criteria were previously reported.[7] Briefly, adult single or bilateral lung allograft recipients with stable lung function were included in the analysis if there were ≥2 baseline dd-cfDNA% measurements, each >1 mo apart. Immunosuppression and other management details have previously been reported.[7]

Measurement of dd-cfDNA%

Enrolled patients underwent assay of plasma dd-cfDNA% at each routine ambulatory visit using AlloSure Lung kits (CareDx, Inc.- Brisbane, CA). Briefly, peripheral blood was drawn into 2 to 10 mL Streck containers, sealed according to package directions, and delivered to a central laboratory for processing. Numeric results for recipients of single lung transplants were adjusted by doubling the values.[9] The baseline dd-cfDNA% for each patient was calculated as the mean of the 2 lowest dd-cfDNA% values measured in samples obtained when patients did not have symptoms concerning for ALAD, had no acute abnormalities on chest imaging, and when forced expiratory volume in 1 s was ≥90% of the patient's best posttransplant baseline. Patients were followed prospectively. If patients developed ALAD, dd-cfDNA% measured at the time of the ALAD episode was noted. Relative change in dd-cfDNA% at the time of ALAD was calculated as (dd-cfDNA% value at the time of ALAD minus baseline dd-cfDNA%)/(baseline dd-cfDNA%). We then categorized whether relative change exceeded the previously established RCV of 73% or whether absolute dd-cfDNA% at the time of ALAD exceeded a threshold of 1%.[7] To assess test characteristics in patients *without* ALAD, the greatest routinely measured dd-cfDNA% value available was referenced to the average baseline value.

Assessment of Allograft Dysfunction

Patients were monitored for at least 9 mo after initial study enrollment. When patients had ALAD, defined as symptoms of illness or a decline in spirometry (forced expiratory volume in 1 s <90% prior baseline value), they underwent an evaluation to identify the cause of ALAD, dictated by the clinical team. Evaluation testing included assessment for viral, bacterial, and fungal infections (using cultures, polymerase chain reaction, or serum biomarkers), new abnormalities on lung imaging, de novo donor-specific antibodies, or assessment for gastroesophageal reflux. If this evaluation was unrevealing, there was a low threshold to obtain a bronchoscopy with airway inspection, bronchoalveolar lavage, and transbronchial biopsies. The clinical team was blinded to dd-cfDNA% data. Infections (probable or more definitive), ACR, and AMR were defined using International Society for Heart and Lung Transplantation consensus criteria.[10-12]

Statistics

Demographics and clinical characteristics were compared between patients with and without ALAD using Pearson's chi-square testing or Fisher exact test (for categorical variables) or Mann-Whitney testing (for continuous variables) using Stata/BE version 17.0 (College Station, TX). Receiver operating characteristic curves were created and areas under the curve were compared using a paired-sample analysis (SPSS version 28; IBM, Armonk, NY).

RESULTS

A total of 277 patients were to participate in the study; 130 patients responded and were screened. Ninety-seven patients were deemed eligible and 93 patients were ultimately enrolled over a 6-mo period. Of the 93 enrolled patients, 71 had ≥2 dd-cfDNA% measurements obtained ≥3-mo apart in the absence of any signs or symptoms of allograft dysfunction. Twenty-three patients (32%) had at least 1 missing or delayed dd-cfDNA% sample over the course of the study; in 5 instances, the patient did not have routine follow-up, in 6 cases, clinic was purposely deferred because of precautions related to the severe acute respiratory syndrome coronavirus 2 pandemic, and in the remainder, a sample was either accidentally not collected or lost in transit to the central processing facility.

Episodes of ALAD occurred in 30 patients (42%) during the follow-up period; see Table 1 for a list of causes of ALAD in this cohort. Baseline demographics of patients are listed in Table 2, comparing patients who developed ALAD with those

TABLE 1.
Causes of ALAD

Causes	N = 30
Community-acquired respiratory virus	10 (33%)
SARS-CoV-2	6 (20%)
Respiratory syncytial virus	2 (7%)
Adenovirus	1 (3%)
Viral syndrome (not identified)	1 (3%)
Unknown after clinical evaluation	6 (20%)
AMR	5 (17%)
Acute bronchitis	2 (7%)
Pneumonia, organism unknown	1 (3%)
Cytomegalovirus viremia	2 (7%)
Malignancy	1 (3%)
Tobacco smoking	1 (3%)
Acute fibrinous organizing pneumonia	1 (3%)
Gastroesophageal reflux disease	1 (3%)

AMR, antibody-mediated rejection; ALAD, acute lung allograft dysfunction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

who did not. There were no significant baseline differences in traits between patients developing ALAD and patients who did not.

Firstly, the ability of RCV and absolute dd-cfDNA% to diagnose ALAD was compared by calculating the area under the receiver operating characteristic curve for ALAD diagnosis (Figure 1A). RCV had an area under the curve of 0.87, whereas absolute dd-cfDNA% had an area under the curve of 0.69. Comparison of the paired area differences in these curves showed that RCV had superior diagnostic ability compared with absolute dd-cfDNA% ($P = 0.018$).

Then, to determine whether use of RCV was superior to the current practice of diagnosis of ALAD when absolute dd-cfDNA% is ≥1, we compared test characteristics for an RCV of dd-cfDNA% >73% from baseline with an absolute threshold of >1% (Figure 1B). Thirty-five patients (49%) had RCV >73% and 24 patients (34%) had absolute dd-cfDNA% >1. A change in dd-cfDNA% >73% from baseline occurred in 26 of 30 patients with ALAD (87%) and 9 of 41 patients without ALAD (22%). Values of dd-cfDNA% exceeding 1% occurred in 15 patients with ALAD (50%) and in 9 patients without ALAD (22%). Hence, the sensitivity of dd-cfDNA% RCV >73% to detect ALAD was 87%, and the specificity was 78%. The positive predictive value of RCV >73% for ALAD was 74%, and the negative predictive value was 89%. An absolute threshold of dd-cfDNA of 1% had a sensitivity to diagnose ALAD of 50% and a specificity of 78%. The positive predictive value of a threshold of 1% for ALAD was 63%, and the negative predictive value was 68%. Of the 30 patients with ALAD, 3 (10%) were not detected using RCV >73%, and 14 (47%) were not detected using absolute value >1%.

TABLE 2.

Baseline demographics

	All patients (N = 71)	ALAD (N = 30)	No ALAD (N = 41)	*P*
Age (y)	56 (46–62)	56 (42–63)	57 (47–61)	0.81
Female sex	29 (41)	9 (30)	20 (49)	0.09
Bilateral transplant	61 (86)	27 (90)	34 (83)	0.51
White race	60 (86)	25 (86)	35 (85)	1.0
Body mass index	25.2 (20.4–28.7)	26.3 (20.4–28.9)	24.8 (20.4–27.4)	0.29
Lung allocation score	39.22 (34.50–51.17)	39.35 (35.05–45.56)	38.05 (34.50–59.70)	0.84
Total ischemic time (h)	5.5 (4.8–6.1)	5.6 (4.9–6.0)	5.4 (4.8–6.2)	0.82
CMV mismatch (D^+/R^-)	16 (23)	7 (24)	9 (22)	1.0
Lung disease diagnosis				0.47
Obstructive	22 (31)	7 (24)	15 (37)	
Pulmonary vascular	2 (3)	0	2 (5)	
Cystic fibrosis	7 (10)	2 (7)	5 (12)	
Interstitial	40 (56)	21 (70)	19 (46)	
Primary graft dysfunction at 72 h	10 (14)	6 (21)	4 (10)	0.29
Acute cellular rejection	47 (67)	17 (59)	30 (73)	0.30
De novo donor-specific antibody	13 (19)	5 (17)	8 (20)	1.0
Gastroesophageal reflux	40 (57)	18 (62)	22 (54)	0.62
Average baseline dd-cfDNA%	0.37 (0.22–0.63)	0.36 (0.20–0.73)	0.38 (0.23–0.53)	0.72
dd-cfDNA% at ALAD event[a]				
Relative change dd-cfDNA%	73% (35–146)	146% (117–347)	59% (32–80)	<0.01
Absolute dd-cfDNA%	0.63 (0.43–1.29)	1.01 (0.58–1.40)	0.58 (0.41–0.88)	<0.01

Data presented as median (IQR) or number (percentage), as appropriate.

[a]If there was no ALAD event, the greatest dd-cfDNA% measured under non-ALAD conditions was used.

ALAD, acute lung allograft dysfunction; CMV, cytomegalovirus; dd-cfDNA, donor-derived cell-free DNA; IQR, interquartile range.

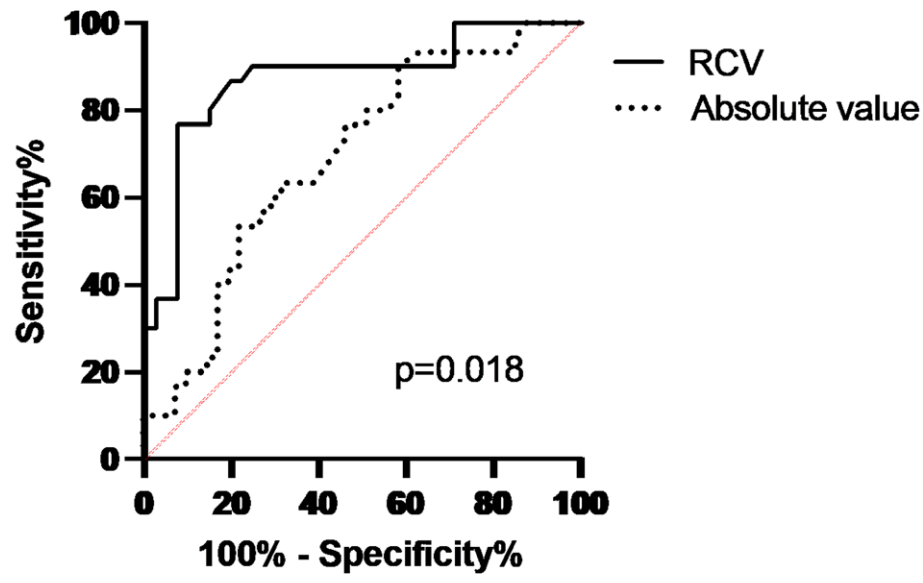
DISCUSSION

In this prospective cohort of lung allograft recipients beyond 2 y posttransplant who had serial monitoring of dd-cfDNA% in the ambulatory setting, the relative change value of dd-cfDNA% is associated with improved diagnostic accuracy for ALAD compared with absolute values.

The use of absolute thresholds of dd-cfDNA% may underestimate the significance of fold change in reflecting the presence and degree of allograft injury.[13,14] For example, a patient with baseline dd-cfDNA values of 0.40% that experiences an increase to 0.80% is likely biologically similar to a patient with baseline values of 0.80% that rises to 1.6%, yet only the latter would be billed as having an ALAD event using an absolute threshold only.[15,16] We show that the sensitivity, specificity, and positive and negative predictive values for predicting ALAD are greater using RCV compared with absolute values of dd-cfDNA%. Other groups have suggested that RCV be incorporated into algorithms that use dd-cfDNA% as a tool to evaluate allograft health. In a cohort of lung allograft recipients early posttransplant in which dd-cfDNA% was used to monitor allograft function instead of transbronchial biopsies, the RCV was 70%,[17] whereas an RCV of 61% was observed in a cohort of stable healthy kidney transplant recipients.[18] In a cohort of kidney transplant patients, 82% of patients with rejection experienced an increase in dd-cfDNA% exceeding RCV.[19] Our study builds on these data by directly comparing the diagnostic utility of RCV and absolute values in the same cohort of patients.

Dd-cfDNA% has become an important biomarker to assess allograft lung health, with plasma levels ≥1% being highly correlative with acute cellular rejection, AMR, and infection, during the early posttransplant period. In fact, because of limitations in performing routine surveillance bronchoscopy during the severe acute respiratory syndrome coronavirus 2 pandemic, dd-cfDNA% temporarily supplanted bronchoscopy as a methodology for lung health surveillance at a number of transplant centers.[20] In this setting, a threshold of >1% was associated with a sensitivity of 74%, specificity of 88%, postive predictive value of 43%, and negative predictive value of 97%. The potential utility for a peripheral biomarker of lung allograft function may be magnified for patients beyond the initial y posttransplant, when intensive monitoring with bronchoscopy wanes, but risk for CLAD increases as patients accumulate multiple events that are CLAD risk factors. Use of serial dd-cfDNA% monitoring may facilitate early detection of graft dysfunction during this vulnerable period, allowing for timely augmentation of immunosuppression or other therapies to stabilize lung function.[21] However, because of the narrow intraindividual variability ($CV_I = 26\%$) and wide interindividual variability ($CV_G = 47\%$) of dd-cfDNA% assessed that we identified in patients >2 y posttransplant, RCV may

A



B

Test characteristic for ALAD diagnosis	Relative change value >73%	Absolute value >1%
Sensitivity	87%	50%
Specificity	78%	78%
Positive predictive value	74%	63%
Negative predictive value	89%	68%

Based on data from 30 patients with acute lung allograft dysfunction and 41 patients without acute lung allograft dysfunction.

FIGURE 1. Relative change value of dd-cfDNA% improves diagnosis of ALAD compared with absolute values. A, ROC curves illustrating the diagnostic abilities of each threshold were generated, and area under the curves were compared using a paired-sample analysis (SPSS version 28). The diagnostic ability of RCV was significantly greater than for an absolute threshold of 1% ($P = 0.018$). B, Use of relative change value of dd-cfDNA% >73% of baseline was compared with an absolute threshold of 1% for identification of ALAD in a cohort of lung allograft recipients >2 y posttransplant (n = 71). ALAD, acute lung allograft dysfunction; dd-cfDNA, donor-derived cell-free DNA; RCV, reference change value; ROC, receiver operator characteristic.

more accurately discriminate between allograft health and injury, a finding supported by data from our current study.

There are several strengths to this study. This is the first study of dd-cfDNA% to directly compare RCV and absolute dd-cfDNA% values for the adjudication of acute injury events in a cohort of lung transplant recipients. As diagnostic test characteristics can be cohort-specific, it is advantageous that the comparison between RCV and a 1% threshold was performed in the same cohort in which the test variables were defined.[22] Finally, this is one of the largest cohorts of lung transplant recipients that are >2 y posttransplant in which dd-cfDNA %variability parameters were studied. Our findings will need to be further validated in additional cohorts to ensure broad generalizability across the lung transplant population. Additionally, as dd-cfDNA% was assessed during routine clinical visits rather than with a defined time interval, it is difficult to know whether some changes in dd-cfDNA% were missed if clinic visits were deferred. Whether test characteristics of dd-cfDNA% using different commercial assays are similar remains unknown, although the genetic discrimination technology would not be expected to affect biological variation within individual patients. Currently, dd-cfDNA% assessment does not distinguish between infectious or immune causes of injury; it would be interesting to test whether different injury types are associated with characteristic levels of change in dd-cfDNA% from baseline.

In summary, we demonstrate that change in dd-cfDNA% is a more accurate indicator of ALAD than an absolute threshold in a cohort of lung transplant recipients >2 y posttransplant. This finding is clinically relevant as it improves the diagnostic accuracy of dd-cfDNA% as a plasma biomarker of lung injury.

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