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Case Report

Real-world Clinical Experience Implementing Donor-derived Cell-free DNA for Detection of Acute Lung Allograft Dysfunction after Lung Transplantation

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Abstract

Background

Donor-derived cell-free DNA fraction (%dd-cfDNA) is a non-invasive plasma biomarker that can detect acute rejection or infection in lung transplant (LT). We investigated the clinical utility of %dd-cfDNA for detection of acute lung allograft dysfunction (ALAD) in real-world clinical practice.

Methods

This single-center, retrospective study enrolled patients within 3-years of LT. Patient management was informed by %dd-cfDNA results combined with standard of practice (SOP) assessments that included flexible bronchoscopy (FB) procedures. We assigned %dd-cfDNA to clinical-pathological cohorts and assessed performance characteristics for %dd-cfDNA for ALAD- (a composite endpoint of acute rejection (ACR + AMR), infection, and indeterminant lung allograft dysfunction (iLDA)) compared to stable patients. %dd-cfDNA kinetics within 90-days after SARS-Co(V)-2/COVID-19 infection were also analyzed. Further, we determined the biological variability for %dd-cfDNA by reference change value (RCV) for patients with ≥3 stable samples.

Results

A total of 109 LT recipients underwent 276 %dd-cfDNA measurements; 34.7% of these were considered ALAD; including 25.0% ACR, 1.0% AMR, 60.4% infection, and 12.5% iLAD. %dd-cfDNA was elevated in ALAD (median, 25-75% IQR) (1.50%, 0.88-3.86) vs stable cohort (0.585%, 0.18-1.07, $p < 0.0001$) with area under receiver operator characteristic curve (AUC-ROC) of 0.783 (95% CI: 0.728-0.838). %dd-cfDNA was also elevated in ACR (1.440, 0.935-3.085, $p<0.0001$), allograft infection (1.650%, 0.84-4.255, $p<0.0001$), chronic lung allograft dysfunction (CLAD) (2.130%, (1.010-5.910), $p<0.0001$), and post-COVID (1.020%, 0.610-2.280, $p=0.0004$) compared to stable. Longitudinal tests from 18 stable LT patients showed a %dd-cfDNA RCV of 72.7%.

Conclusions

In our real-world experience, %dd-cfDNA detected acute lung allograft dysfunction (ALAD) as defined by SOP assessments inclusive of FB procedures. ALAD is a relevant clinical endpoint that encompasses acute rejection and allograft infection events, which should be considered for implementation in future clinical trial design. Ongoing multi-center studies should be leveraged for additional confirmation of performance characteristics of %dd-cfDNA for ALAD and RCV.

Keywords: *Cell-free DNA, lung transplant rejection, biomarker, surveillance, chronic lung allograft dysfunction.*

Introduction

Lung Transplantation (LT) is the best medical approach for several thousand patients annually in the U.S. suffering from a myriad of end stage cardiopulmonary diseases, despite the looming reality of 10-year post-LT survival of only 28% 1. The principal cause of mortality after the initial LT year is allograft failure due to chronic lung allograft dysfunction (CLAD).2 Since there is no effective treatment for CLAD, clinical attention has focused primarily on risk remediation by surveillance for acute allograft rejection (AR) and infection. Current surveillance flexible bronchoscopy (FB) with transbronchial biopsies (TBBx) and bronchial-alveolar lavage (BAL) performed by most U.S. LT centers are invasive and unable to improve long-term outcomes3-5.

Donor-derived cell-free DNA (%dd-cfDNA) has emerged as a valuable non-invasive plasma biomarker for detection of AR and other causes of tissue injury in the lung allograft.6-12 To date, no consensus guidelines have been established by International Society for Heart and Lung Transplantation (ISHLT) regarding the use of %dd-cfDNA for detecting AR and injury in LT while the European Society of Organ Transplantation Learning Journey (ESOT-TLJ-3.0) Delphi Consensus (2022) concluded only "low quality evidence" exists, reaching consensus of a "weak recommendation".13 This may in part be due to the fact that the aforementioned studies involve analysis of biorepository plasma samples without real-world implementation or clinical experiences associated with %dd-cfDNA.7,10-12 Therefore, we believed it imperative to report our real-world clinical experiences to establish the clinical utility of dd-cfDNA in the context lung allograft health in LT patients.

The aims of our current study were several: (1) to assess clinical utility of %dd-cfDNA test monitoring in real-world clinical experiences along with standard of practice (SOP) for detection of a composite endpoint of acute lung allograft dysfunction (ALAD); (2) to assess potential effects of preceding SARS-Co(V)-2/ COVID-19 allograft infection on %dd-cfDNA kinetics after LT; and (3) to analyze %dd-cfDNA coefficient of variation and the reference change value (RCV) in stable patients after LT. Our study will help corroborate earlier studies demonstrating the clinical utility of %dd-cfDNA in LT patients in a real-world clinical setting.

Materials and Methods

Study Design

This study was approved by the Institutional Review Board at Spectrum Health Medical Center (IRB #2015-262). We prospectively collected standard of practice (SOP) test result data on LT patients at Spectrum Health Lung Transplant Center, at intervals as determined by the individual provider: routine laboratory, pulmonary function tests, TBBx histopathology, BAL microbiology, donor-specific antibodies (DSA) for MHC Class I and II antigens, and plasma %dd-cfDNA. Flexible bronchoscopy (FB) with TBBx and BAL was performed as SOP surveillance at 1-, 3-, 6-, 9-, 12-, and 18-months post-LT, while additional "for cause" procedures were based on clinical indications. Histopathology for TBBx specimens was graded for acute cellular rejection (ACR) in accordance with the revised Working Formulation of International Society of Heart and Lung Transplantation (ISHLT).14 Aliquots of BAL fluid was submitted for SOP microbiologic studies which also included a respiratory viral multiplex PCR test. If FB was performed within 4-weeks of the %dd-cfDNA test, these associated results were utilized for correlation with %dd-cfDNA for assignment to diagnostic cohorts. Office-based pulmonary function tests were performed in accordance with American Thoracic Society (ATS) standards15,16 for the assessment of acute lung allograft dysfunction (ALAD) and clinical diagnosis of chronic lung allograft dysfunction (CLAD).2,17

%dd-cfDNA Measurements

Peripheral blood for the %dd-cfDNA (Prospera™ test) was collected in 10-mL cell-free Streck™ tubes and sent overnight at room temperature to the Natera, Inc. (Austin, TX) Clinical Laboratory Improvement amendments (CLIA)-certified, College of American Pathologists (CAP)-accredited laboratory in San Carlos, CA. After extraction, cell-free DNA (cfDNA) was amplified using a massively multiplexed polymerase chain reaction assay targeting a curated panel of >13 000 single-nucleotide polymorphisms designed to maximize variant frequency across ethnicities.14 For each sample, amplicons were sequenced by next-generation sequencing performed on the Illumina NextSeq500 on rapid run with an average of 14 to 15 million reads per sample and sequencing data was processed to estimate the fraction of dd-cfDNA (expressed as a percentage; %dd-cfDNA) in relation to total cfDNA. The Prospera™ test for single LT patients incorporates a workflow multiplicative correction factor (2x) for resulted %dd-cfDNA.7,18

The Prospera™ test evaluates the risk of rejection by analyzing the levels of dd-cfDNA, specifically

considering a threshold of 1.0%., the sensitivity and specificity of the test are also assessed using a threshold of 0.5% dd-cfDNA. The analytical coefficient of variation (CVa) for %dd-cfDNA (Prospera™) has previously been reported as 1.8%.19

Clinical-Pathological Cohort Assignments

%dd-cfDNA test results were assigned to an associated clinical-pathological cohort by the blinded investigators. The clinical-pathologic cohorts were assigned based on the following definitions: Acute cellular rejection (ACR) encompassed the ISHLT Working Formulation definition (Grades A1-A4) and/or histopathologic "organizing pneumonia" in the absence of an infection and was treated with augmented corticosteroids. Antibody mediated rejection (AMR) was defined as the ISHLT subacute or acute with confidence as "possible/definite". 20 "Indeterminant" lung allograft dysfunction (iLAD) was defined by a clinically indicated FB ("for cause") procedure that was associated with nondiagnostic histopathology results and "negative" BAL microbiology studies. In our experience, such challenging episodes are often treated both with augmented corticosteroids and empiric antibiotic therapies, therefore adjudication to a specific diagnostic cohort is problematic. Allograft Infection (Infxn) was defined according to ISHLT criteria for cardiothoracic organ transplant infection with confidence as "probable / definite" based on presence of BAL microbiology, clinical findings, and directed antimicrobial therapy.21 Chronic Lung Allograft Dysfunction (CLAD) was defined by the presence of ≥ stage1, obstructive or restrictive or mixed phenotype, by ISHLT criteria. 2,17,22

The composite cohort of acute lung allograft dysfunction (ALAD) was adopted from methods of Keller, et al.8 that included either acute rejection (ACR or AMR) or allograft infection (Infxn), however additionally this cohort included iLAD episodes in our analysis. SARS-Co(V)-2 / COVID-19 affliction after LT has been associated potentially with significantly higher 28-day mortality than other SOT recipients and the clinical course can be protracted. 23,24 Therefore, %dd-cfDNA tests obtained within a 90-day window after COVID-19 infection were assigned to a separately designated cohort (post-COVID) for analysis. A subset of the infxn group who were actively infected with COVID-19 (active COVID-19) were also used for analysis. The stable cohort was defined by the absence of AR during preceding 6 weeks and freedom from current AR, Infxn, ALAD, Post-COVID, or CLAD. Excluded %dd-cfDNA tests were those drawn less than 28-days after LT or drawn less than 6-weeks after an established AR event or associated with insufficient clinical data.

Statistical Analysis

The distribution of %dd-cfDNA was expressed as a median with 25% to 75% interquartile range (IQR) for each clinical-pathological diagnostic cohort, with normality assessed by the Kolmogorov-Smirnov test. As lack of normality of %dd-cfDNA was detected in the cohorts, comparisons across cohorts were performed using the nonparametric Mann-Whitney U test ($p<0.05$). Linear regression was utilized where appropriate for analysis of %dd-cfDNA vs time after LT. Area under the receiver operator characteristic curve (AUC-ROC) for differentiating ALAD from stable was determined. Statistical analyses were performed in GraphPad™. Reference Change Value (RCV) was calculated from patients with ≥ 3 samples classified as stable within 3 years of LT. Intra-subject coefficient of variation (CVi) and inter-subject coefficient of variation (CVg) were calculated as robust CV for the required nonparametric analysis whereupon the robust CV = median absolute deviation / median and median absolute deviation = median distance away from median dd-cfDNA%. The index of individuality (II) was determined as the CVi:CVg ratio. The coefficient of variation for the analyte (CVa) for Prospera™ test has been previously reported as 1.8%.19 The implemented equation for determination of RCV = $^{21}/2$ x 1.96 x $(CV^{a2} + CVi2)^{1/2}$.25

Results

General Data Overview

The study was performed on 109 LT patients from a single-LT center (Spectrum/Corewell Health System). between November 2021 and March 2023. Patient demographics and native lung disease for LT are summarized in Table 1a-b. Median patient age was 66.4 years (IQR: 61.8-71.0), 68.0% of the cohort were male, 92.7% were Caucasian, and 83.5% had double LT. Indications for LT were predominantly related to idiopathic interstitial pneumonitis (IIP) and chronic obstructive lung disease (COPD)/emphysema).

The accompanying SOP test result data were retrospectively reviewed to ascribe a blinded, clinical-pathological diagnostic cohort assignment for each %dd-cfDNA test results. Blood samples were collected to assess %dd-cfDNA prospectively. A total of 288 %dd-cfDNA results from 109 patients were assigned to defined clinical-pathological diagnostic cohorts (Figure 1). Twelve %dd-cfDNA tests (12 patients) were excluded from analysis due to either time < 28-days post-LT (N=9) or preceding ACR event within 6-weeks of testing (N=3).

Flexible bronchoscopy (FB) with TBBx + BAL procedures were performed and paired with 214 dd-

cfDNA% testing while the remaining 74 dd-cfDNA% tests were obtained and assigned without associated FB procedures. Overall, 75% of FB with TBBX and BAL procedures were performed for routine surveillance or follow-up of prior ACR diagnosis and 25% were performed "for cause" based on clinical indications. %dd-cfDNA levels by clinical-diagnostic cohorts is shown in table 2.

The %dd-cfDNA values encompassing all clinical-diagnostic cohorts versus time demonstrated statistically increased median %dd-cfDNA over time after LT ($y=0.001124x + 1.211$; $R^2 = 0.0365$; $p=0.0012$). Median time post-LT for ACR (303 days, IQR: 59.5-419.0), AMR (738.5 days, 137-1340), Infxn (349.5 days, 138-545), and iLAD (365 days, 185-673) were not different from the stable cohort (268 days, 169-370.5, p=NS). Time post-LT as anticipated, was longer for CLAD (1008 days, 496-1287) compared to stable ($p<0.0001$).

%dd-cfDNA was Elevated in Cohorts with Acute Rejection, Allograft Infection, and Chronic Lung Allograft Dysfunction compared to Stable Cohort

Compared to stable cohort (0.585%; 0.180-1.068), %dd-cfDNA was significantly elevated for ACR (1.440%, 0.935-3.085, $p<0.0001$), Infxn (1.650%, 0.840-4.255, $p<0.0001$), and CLAD (2.130%, 1.010-5.910, $p<0.0001$) (Figure 2). Dd-cfDNA was not statistically different between ACR group the Infxn cohort ($p=0.287$). Only two episodes of "possible AMR" (9.810%, 6.840-12.790) were observed, based on presence of (+) HLA DSA and graft dysfunction but no associated histopathologic or immunohistochemistry findings; these were excluded from further statistical analyses. CLAD clinical stage at time of %dd-cfDNA analysis, predominantly obstructive phenotype, included: Stage I (N=5), II (N=3), III (N=2), and IV (N=1).

Indeterminant Acute Lung Allograft Dysfunction was not Significantly Different from the Composite Acute Lung Allograft Dysfunction Cohort, while Both were Elevated Compared to a Stable Cohort

As shown in Supplementary Figure 1, the %dd-cfDNA in iLAD cohort (1.390%, 0.738-2.79) was statistically higher compared to the stable cohort (0.585%, 0.180-1.068, $p=0.0036$). The %dd-cfDNA in the composite ALAD cohort which encompassed ACR, AMR, Infxn, and iLAD, was also significantly higher (1.500%, 0.880-3.860) than the stable cohort ($p<0.0001$). By contrast, no difference was observed in %dd-cfDNA between iLAD and the composite ALAD cohort ($p=0.661$).

%dd-cfDNA up to 90-Days after COVID-19 Lung Allograft Infection was Elevated Compared to a Stable Cohort

%dd-cfDNA in post-COVID (1.020%, 0.610-2.280) and Infxn (1.650, 0.480-4.255) cohorts were significantly elevated compared to the stable cohort ($p=0.0004$, $p<0.0001$, respectively) (Figure 3). By contrast although a potential trend was noted, there was no statistical difference for %dd-cfDNA in the post-COVID cohort versus either the total Infxn cohort ($p=0.064$) or with active COVID-19 infection ($p=0.055$). The specific positive microbiologic results are summarized in Supplementary table 1 across bacterial, viral, and fungal domains that represented the Infxn cohort.

%dd-cfDNA Predicted a Robust Composite Primary Endpoint of Acute Lung Allograft Dysfunction Capturing Both Acute Rejection and Allograft Infection Events

The primary endpoint of assessing the performance of %dd-cfDNA to differentiate between ALAD (N=96) (which encompassed acute rejection and allograft infection and indeterminant allograft dysfunction events) and Stable (N=146), yielded an area under the receiver operator characteristic curve (AUC-ROC) of 0.783 (95% C.I.: 0.728-0.838, $p<0.0001$) (Figure 4). Using a %dd-cfDNA threshold of 1.0% yielded a sensitivity of 70.1% (95% C.I.: 61.6-77.4), specificity of 73.3% (65.6-79.8), negative predictive value (NPV) 82.2%, positive predictive value (PPV) of 82.2% (based on a prevalence for ALAD of 34.7%). Implementing a lower 0.5% cut-point yielded a sensitivity of 85.8% (78.7-90.8), specificity of 45.9% (38.0-54.0), NPV of 85.9%, and PPV of 45.7%.

%dd-cfDNA was Elevated for an aggregated Cohort Testing Positive for HLA Donor-Specific Alloantibodies Relative to the Cohort Negative for DSA.

In an exploratory analysis, %dd-cfDNA was found to be elevated in a combined cohort including "positive" for HLA donor-specific alloantibodies (DSA), either major histocompatibility complex (MHC) Class I or Class II or both, (1.280%, 0.660-3.020) compared to the aggregated DSA(-) cohort (0.900%, 0.330-1.580, $p=0.0049$) (Figure 5). The DSA(+) group consisted of 16.4% of the total %dd-cfDNA tests; of these, approximately 36% were MHC Class I, 42% Class II, 6% Class I + II, and 17% were unknown.

Analysis of Intra-patient and Inter-patient Coefficients of Variation and Determination of a Reference Change Value for %dd-cfDNA

The cohort contained 18 patients with ≥ 3 %dd-cfDNA tests who were < 3-years post-LT and had been assigned to the stable cohort. With median of three %dd-cfDNA tests for analysis, the intra-patient robust coefficient of variation (CVi) was 26.2% and inter-patient robust coefficient of variation (CVg) was 73.0% (IQR: 0.17-1.02). The calculated index of individuality (II) was 0.36. Inputting the previously published analytical coefficient of variation (CVa) for the Prospera test of 1.8%, the determined reference change value (RCV) was 72.7%.

Table 1: Demographics

a. Patient Demographics

Characteristics	
Age	66.4 years (Median, IQR: 61.8-71.0)
Sex	F 32% M 68%
Race / Ethnicity	Black / AA 1.8% Asian <1.0% Hispanic / Latino 4.6% Caucasian 92.7%
Type of Procedure	(L) Single LT 10.1% (R) Single LT 6.4% Double LT 83.5%

b. Pre-LT Native Lung Diseases

Diagnosis	**%**
COPD / Emphysema	20.2
Alpha-1-Antitrypsin Deficiency	1.8
Cystic Fibrosis	1.8
Idiopathic Interstitial Pneumonitis	46.8
Pulmonary Fibrosis (Other)	14.7
Systemic Sclerosis-Fibrosis	1.8
Systemic Sclerosis -Pulmonary Hypertension	1.0
COVID-19	3.7
Sarcoidosis	1.0
Re-Transplant	1.8
Secondary Pulmonary Hypertension	1
Bronchiectasis	2.7
Hypersensitivity Pneumonitis	1.8

Table 2: dd-cfDNA% Levels by Diagnostic Cohort

Cohort	**N (Samples)**	**Median Days Post-LT (25-75% IQR)**	**Median dd-cfDNA% (25-75% IQR)**
Stable	146	268 (169-370.5)	0.585 (0.180-1.068)
ACR	24	303 (59.5-419)	1.440 (0.935-3.085)
AMR	2	738.5 (137-1340)	9.810 (6.84-12.79)
Infxn	58	349.5 (138-545)	1.650 (0.840-4.255)
CLAD	11	1008 (496-1287)	2.130 (1.010-5.910)
Post-COVID	23	NA	1.020 (0.610-2.290)
iLAD	12	365 (185-673)	1.390 (0.737-2.79)
ALAD	96	NA	1.500 (0.880-3.860

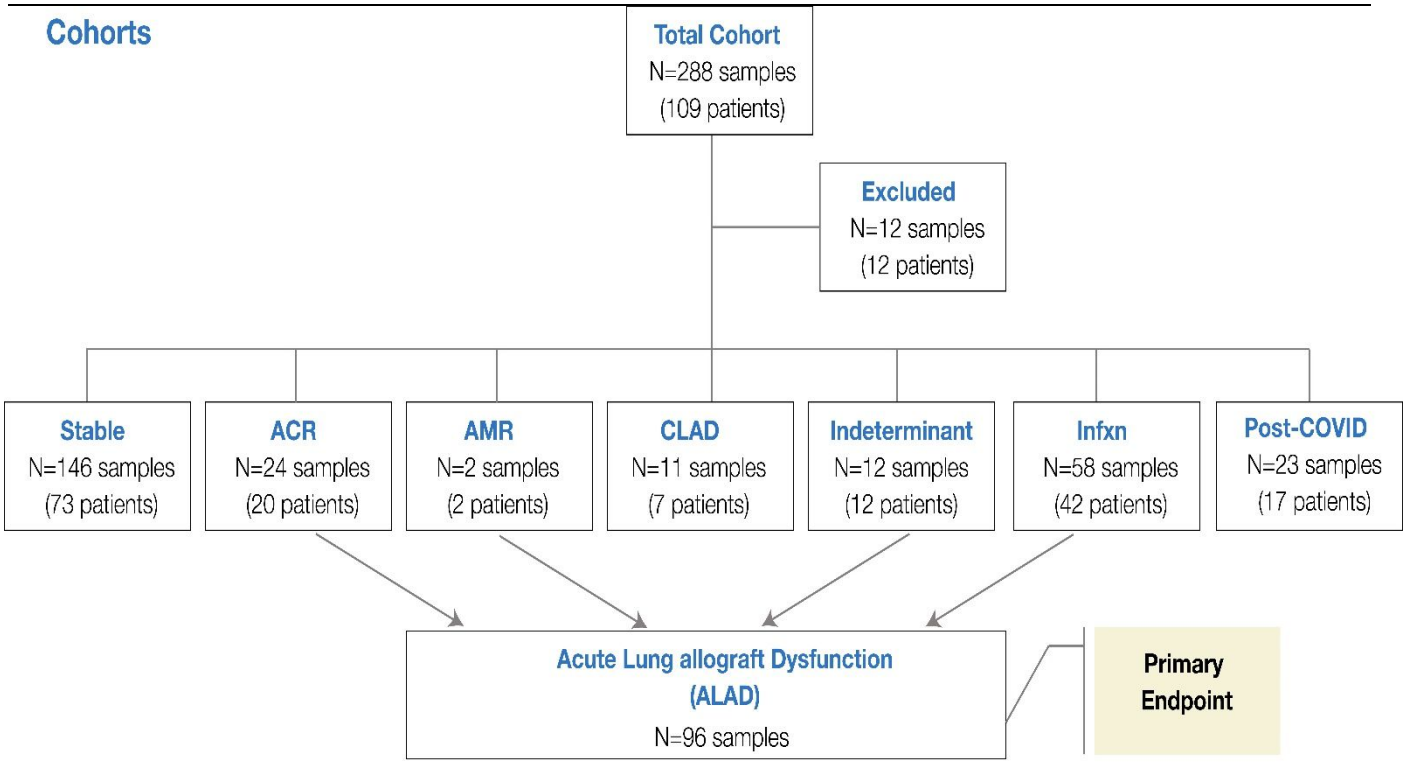


Figure 1: Patients and Samples by Clinical-Pathological Diagnostic Cohort Assignment. [ACR: acute cellular rejection, AMR: antibody-mediated rejection, CLAD: chronic lung allograft dysfunction, iLAD: Indeterminant lung allograft dysfunction, Infxn: allograft infection, post-COVID: %dd-cfDNA obtained within 90-days after SARS-Co(V)-2 / COVID-19 lung infection].

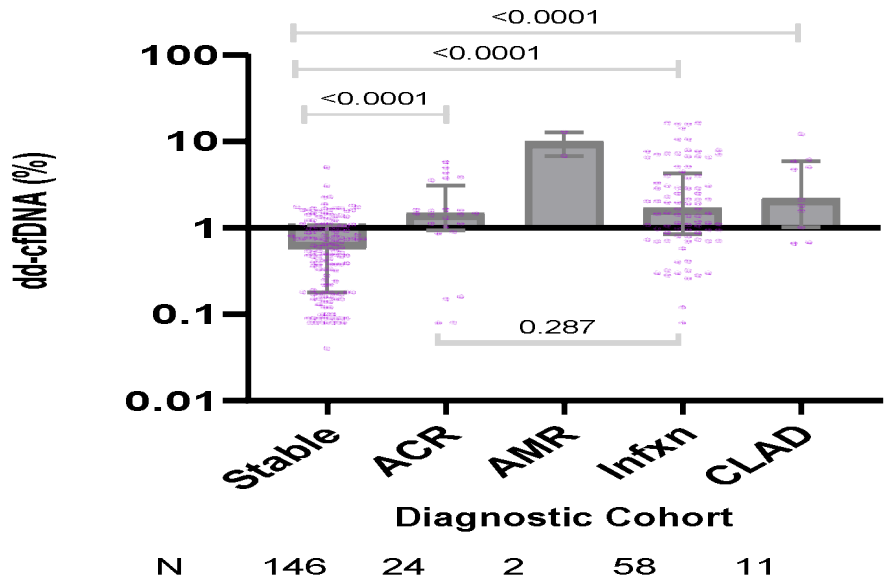


Figure 2: %dd-cfDNA for defined clinical-pathologic diagnostic cohorts (box = median value, brackets = 25-75% interquartile range, dots = individual data points). Median %dd-cfDNA in all cohorts was significantly elevated compared to a stable cohort. No statistical difference observed for ACR versus Infxn cohort. [ACR = acute cellular rejection, AMR = antibody-mediated rejection, Infxn = lung allograft infection, CLAD = chronic lung allograft dysfunction, dd-cfDNA = donor-derived cell-free DNA].

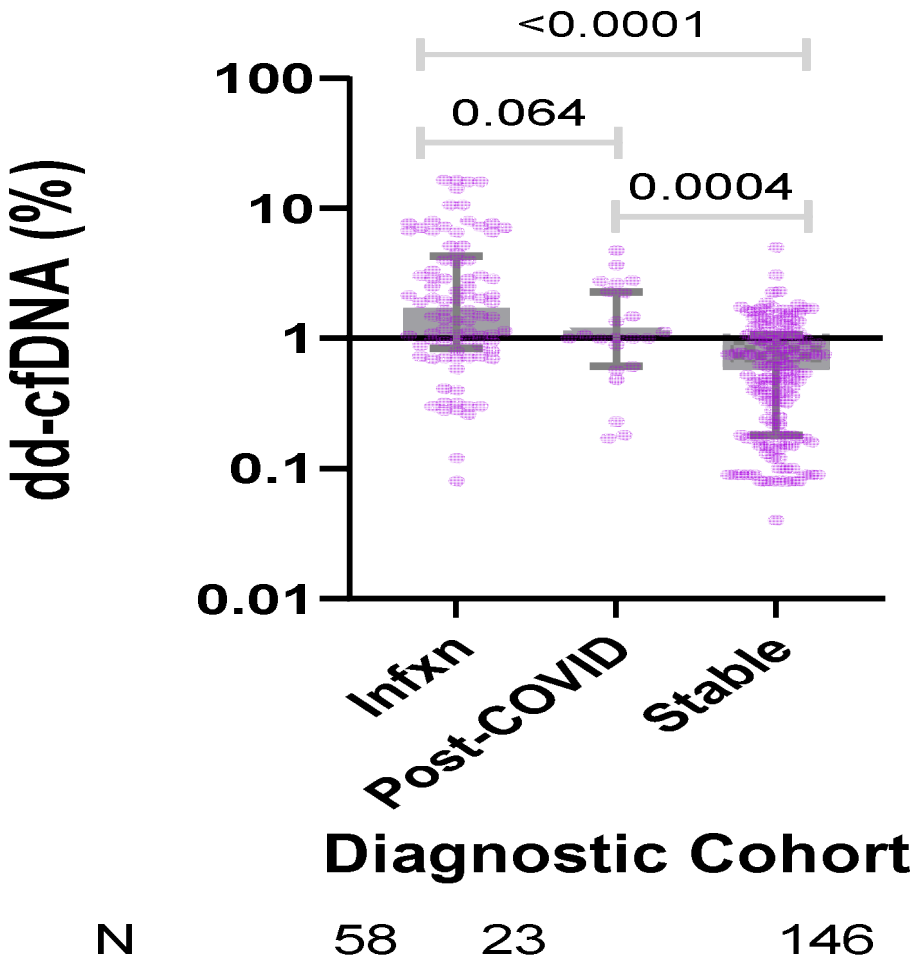
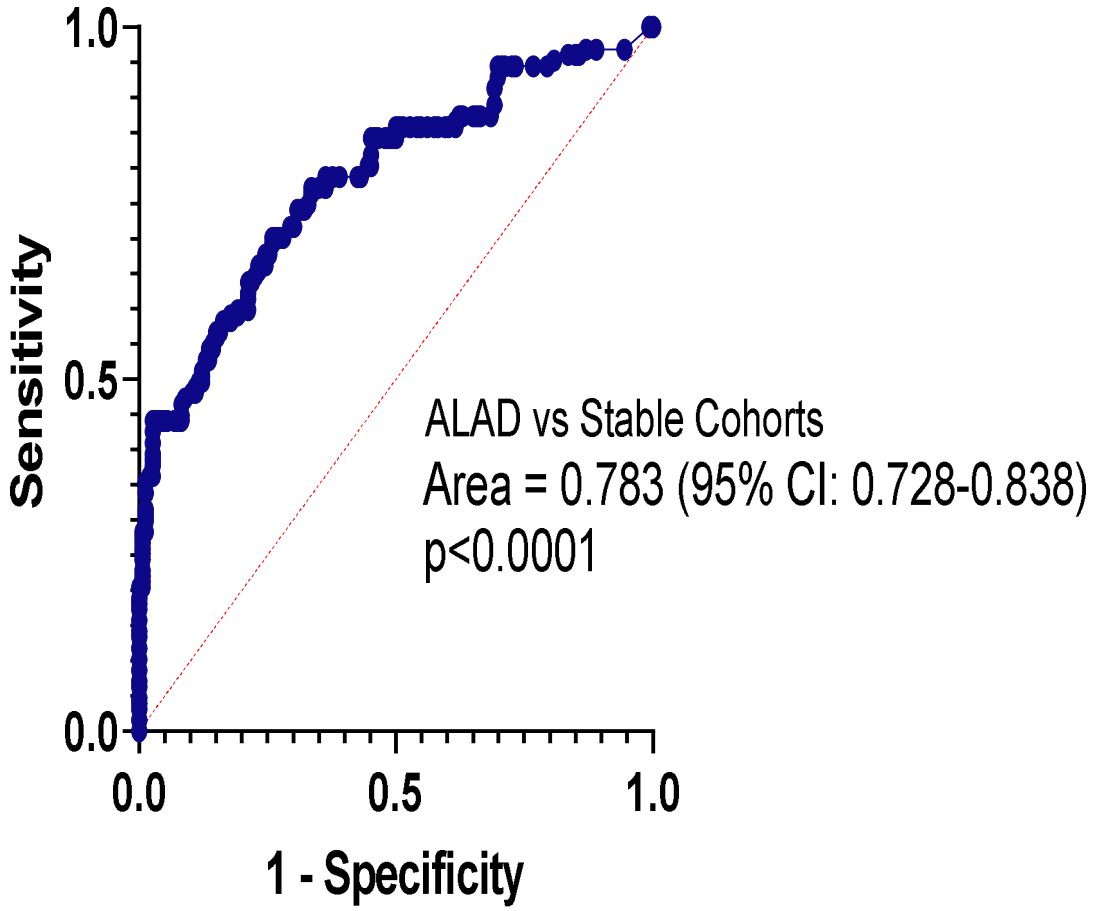


Figure 3: %dd-cfDNA for clinical-pathologic diagnostic cohorts of Infxn, post-COVID, and stable (box = median value, brackets = 25-75% interquartile range, dots = individual data points). Median %dd-cfDNA for post-COVID and Infxn was significantly elevated compared to a stable cohort. No statistical difference observed for post-COVID versus Infxn cohort. [Infxn = lung allograft infection, post-COVID = %dd-cfDNA drawn up to 90-days after SARS-Co(V)-2 / COVID-19 lung allograft infection, dd-cfDNA = donor-

derived cell-free DNA].



Cut-point dd-cfDNA%	**Sensitivity** (95% C.I.)	**Specificity** (95% C.I.)
>0.5%	85.8% (78.7-90.8)	45.9% (38.0-54.0)
≥1.0%	70.1% (61.6-77.4)	73.3% (65.6-79.8)

Figure 4: Receiver operator characteristic (ROC) curve for %dd-cfDNA detection of the composite primary endpoint of ALAD demonstrated an area under the curve (AUC) of 0.783 (p<0.001). ALAD encompassed cohorts of acute rejection, allograft infection, and indeterminant causes of allograft dysfunction. [ALAD = acute lung allograft dysfunction].

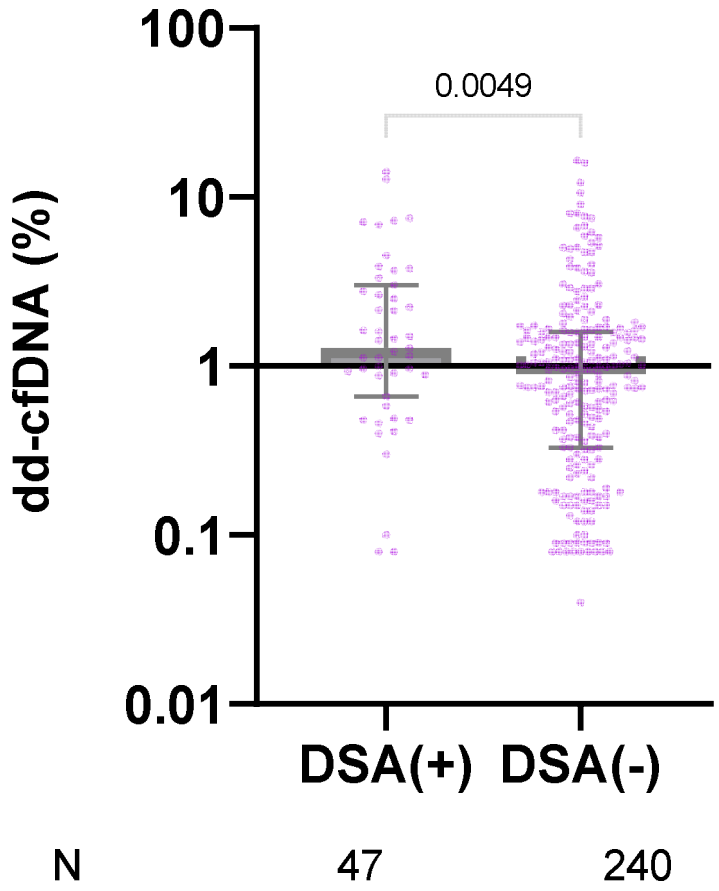


Figure 5: %dd-cfDNA associated with patients aggregated by either MHC Class I or II DSA (+) versus DSA (-) (box = median value, brackets = 25-75% interquartile range, dots = individual data points). The aggregated cohort for DSA (+) had significantly elevated median %dd-cfDNA versus DSA (-) cohort. [MHC = major histocompatibility complex, DSA = donor-specifical alloantibodies, dd-cfDNA = donor-derived cell-free DNA].

Discussion

Lung transplant patients experience significant hurdles to ensuring both their physical and mental health with a sobering reality that median survival is only about 6-years.26,27 Chronic lung allograft dysfunction (CLAD), an enigmatic manifestation of chronic rejection, is the proximate cause of late allograft failure and contributes to more than 40% of mortality after the initial transplant year.27,28 In efforts to thwart development of CLAD, surveillance with FB has been adopted as SOP in a majority of LT centers29. Regardless, invasive surveillance FB procedures have not been demonstrated through randomized-controlled clinical trials, to enhance long term outcomes.3,5 To our knowledge, our current investigation represents the first real-world assessment of clinical utility of %dd-cfDNA combined with SOP after LT, inclusive of surveillance FB procedures.

Our study confirmed the %dd-cfDNA performance findings of prior studies7,10-12 and found %dd-cfDNA was elevated across cohorts associated with various types of lung allograft injury events including AR, allograft infection, and CLAD. Indeed, for both ACR and and Infxn cohorts, the median %dd-cfDNA was elevated approximately 3-fold relative to the stable cohort. Only two episodes of AMR precluded formal analysis yet the %dd-cfDNA was significantly elevated. Noteworthy was our finding that %dd-cfDNA in isolation could not distinguish injury related to ACR from that of infection of the lung allograft.

Adopting the methods of Keller, et al.,8 we sought to analyze performance characteristics for %dd-cfDNA for a composite endpoint of acute lung allograft dysfunction (ALAD). Since %dd-cfDNA alone cannot distinguish AR from infection events, we anticipated that elevation in this biomarker (regardless of etiology) should typically prompt additional clinical evaluation inclusive of possible “for cause” FB, to assess etiology and hence direct management. Further, we considered a cohort of “indeterminant” lung allograft dysfunction (iLAD) as defined by a “for cause” FB procedure but unclear etiology per the results of clinical, histopathological and microbiology data. Intriguing was our finding that median %dd-cfDNA was similar for iLAD compared to the composite ALAD cohort. We speculate that this finding of allograft injury by %dd-cfDNA assessment despite unrevealing FB interrogation, underpin inherent limitations ascribed to standard histopathology that may be clarified significantly by the complementary assessment of tissue “gene expression profiling”, such as with Molecular Diagnostic System (MMDx™)30-32.

We further speculate that the combined injury cohort ALAD should be considered as a valuable primary endpoint for design of future clinical and therapeutic trials, as evidenced by %dd-cfDN ability to identify ALAD with an AUC-ROC of 0.783. An issue requiring further investigation, however, will need to focus on the optimal threshold for %dd-cfDNA during clinical surveillance for ALAD. While lowering threshold from 1.0% to 0.5% results in a substantial increase in sensitivity, from 70% to 86%, the specificity decreased from 73% to 46%, suggestive of a potential for an increase in unnecessary "for cause" FB procedures.

When comparing our findings in relation to ALAD to those of Keller and colleagues, we identified several noteworthy similarities but also some differences.8 Due to the SARS-Co(V)-2 / COVID-19 pandemic risks evident during their investigation, SOP surveillance FB (TBBx and BAL) had been largely suspended and only 'for cause' procedures were performed. The authors analyzed %dd-cfDNA test performance combined with telehealth visits by a retrospective chart review design over a 6-month study duration. Their study included a total of 297 %dd-cfDNA test results from 157 LT patients < 3-years post-LT. In their noninvasive surveillance protocol, %dd-cfDNA was performed approximately monthly for patients < 1-year and every 3-months for years 2 - 3 post-LT. Their implemented threshold for %dd-cfDNA and ALAD surveillance was >1.0% which would then prompt additional SOP clinical assessments. Results in the range of 0.5-1.0% also prompted a repeat test in 2 weeks to assess for any upward trend. The median %dd-cfDNA was 1.7% (25-75% IQR: 0.63-3.1) for the composite endpoint of ALAD versus 0.35% (0.22-0.79; $p<0.001$) for a stable cohort. Since FB surveillance procedures had been omitted, the stable cohort was defined entirely based on clinical criteria and 82% of %dd-cfDNA test results were adjudicated without FB correlation versus only 26% in our investigation. In their analysis, %dd-cfDNA performance for detecting ALAD showed a sensitivity and specificity of 74% and 43%, respectively with an AUC-ROC of 0.82 when considering a clinical definition of stable, as opposed to 76% and 70%, respectively, for the 52 %dd-cfDNA results when SOP surveillance FB had been resumed for cohort assignment. The analysis conducted by Keller, et al., estimated a potential for reduction in surveillance FB by 82.7% of procedures based on %dd-cfDNA monitoring; similarly, our analysis suggested an approximately 50% reduction in invasive FB procedures. The principal findings from their study are therefore quite consonant with our observations and emphasize a valuable role of %dd-cfDNA for surveillance of LT recipients as a clinical tool to assess allograft status.

Our investigation queried the potential influence of prior COVID-19 allograft infection on latter

assessments for %dd-cfDNA and kinetics. Although insufficient longitudinal data precluded our intra-patient analysis of kinetics, this study demonstrated significantly elevated %dd-cfDNA (1.020%) compared to the stable cohort (0.585%, p=0.0004) persisting up to 90-days after infection. Intriguing but yet unclear from our study, is whether pathogenesis of community-acquired respiratory viruses (CARV) with enhanced inflammatory cytokines or chemokines, altered innate immunity, and exosome-mediated events can contribute to ongoing allograft injury (after resolution of the inciting viral infection) and a predisposition to latter CLAD as suggested by prolonged %dd-cfDNA elevation.33-35

We discerned a significant increase in median %dd-cfDNA associated with the presence of HLA donor-specific alloantibodies relative to those without DSA. Although the DSA (+) analysis included of all clinical-pathological cohorts, our finding is congruous with reports which had associated DSA with observed worsened long-term outcomes in solid organ transplant.36-38 Further, Hachem, et al., had reported improvement in obstructive CLAD-free survival when preemptively treated patients experienced successful clearance of HLA DSA after LT.39

Pertinent to protocol development for %dd-cfDNA during longitudinal surveillance, we analyzed our dataset for estimation of a reference change value (RCV) and biological variability for patients with ≥3 tests assigned to the stable cohort and < 3-years post-LT. This metric should be valuable to the interpretation of the clinical significance of a change in %dd-cfDNA when observed during longitudinal assessment. Biological variability requires detailed assessment in particular when considering a T1/2 of only approximately 60-90 minutes for circulating cell-free DNA.40 Although representing single-center data and limited by a relatively small 'N', the RCV of 72.7% from our study is similar to the 70% value reported for LT by Keller, et al.,41 and slightly higher than the 61% as reported for kidney transplant by Bromberg, et al.42 Furthermore, the RCV for %dd-cfDNA in LT is remarkably comparable to that of other blood analytes such as cardiac Troponin-I (72.2%)43 and alanine amino-transferase (72.1%).44 Nevertheless, RCV for %dd-cfDNA in LT should be confirmed in the context of multi-center longitudinal studies and an adequately diverse patient population.

Limitations to our study are several. Firstly, although attempting to combine the %dd-cfDNA test with our SOP assessments, the implementation of the test was at the discretion of the provider with only heterogeneous longitudinal measurements. Secondly, our analysis of %dd-cfDNA kinetics during longitudinal assessment was limited by a small cohort size of only 18 patients. Thirdly, our study had limitations which are inherent to single-center trial design, limited patient diversity, and lack of

randomization. Regardless, this study provides an important framework with potential insights for future LT clinical protocol design and research. We opine that mounting data in LT should provide the additional "high quality" evidence indicated to further support clinical utility of %dd-cfDNA in surveillance for lung health and hence, establish its incorporation into ISHLT and ESOT clinical practice guidelines. To this point, promising multi-center collaborative studies currently include-Lung Allograft Monitoring using Blood Dd-cfDNA (Prospera™) Assessment (LAMBDA 002; NCT05170425), AlloSure™ Lung Assessment and Metagenomics Outcomes Study (ALAMO, NCT05050955) and the TRIFECTA-Lung dd-cfDNA (Prospera™)-MMDx™ Study (NCT05837663).

Author Contributions:

Study design: RG and DR, data analysis: RG, CL, DR, SB, manuscript development: RG, DR, SB.

Authors' Disclosures:

GR reports grants from Pfizer, grants from United Therapeutics, personal fees from Altavant, personal fees from Boerhinger Ingelheim, personal fees from Genentech, outside the submitted work. DR SB are employees of Natera, Inc. with stocks or options to buy stocks in the company. CL declares no conflicts of interest.

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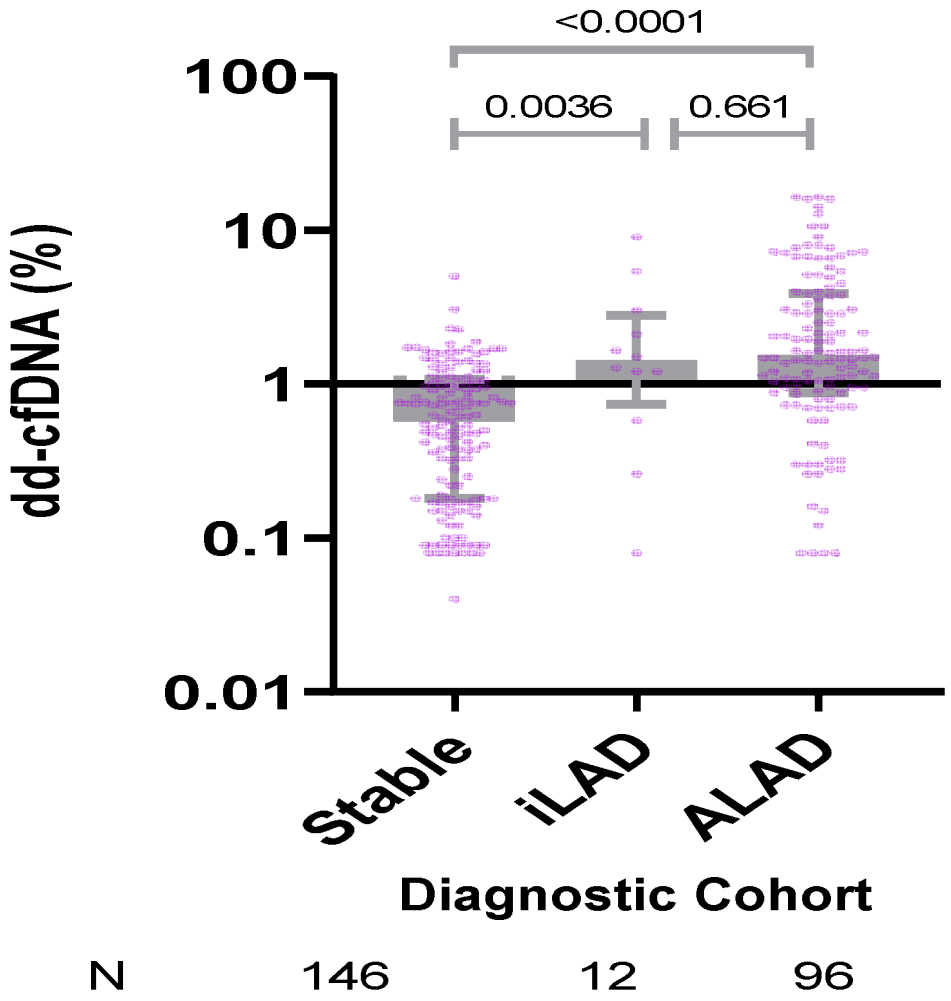
Supplementary material:

Supplementary Figure 1: %dd-cfDNA for clinical-pathologic diagnostic cohorts of stable, iLAD, and ALAD (box = median value, brackets = 25-75% interquartile range, dots = individual data points). Median %dd-cfDNA for ALAD and iLAD was significantly elevated compared to a stable cohort. No statistical difference observed for iLAD versus the composite ALAD cohort. [iLAD = indeterminant lung allograft dysfunction, ALAD = acute lung allograft dysfunction, dd-cfDNA = donor-derived cell-free DNA].


<0.0001
0.0036
0.661
100
10
1
0.1
0.01
dd-cfDNA (%)
Stable
iLAD
ALAD
Diagnostic Cohort
N
146
12
96

Domain	Genus	N
Bacterial		
	Burkholderia spp	1
	Klebsiella spp.	1
	Mycobacteria chelonae	1
	Pseudomonas spp.	9
	Staphylococcus spp.	1
	Streptococcus spp.	1
Viral		
	Adenovirus	2
	Cytomegalovirus (CMV)	6
	Non-COVID Coronavirus	1
	COVID-19	12
	Human Metapneumovirus	2
	Influenza type A	2
	Influenza type B	1
	Parainfluenza	1
	Rhinovirus	5
	Respiratory syncytial virus (RSV)	3
Fungal		
	Aspergillus spp.	8
Unknown Pathogen		6

Supplementary Table 1: Microbiologic results included in the Infection Cohort

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