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Donor-derived cell-free DNA predicts allograft failure and mortality after lung transplantation

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ABSTRACT

Background: Allograft failure is common in lung-transplant recipients and leads to poor outcomes including early death. No reliable clinical tools exist to identify patients at high risk for allograft failure. This study tested the use of donor-derived cell-free DNA (%ddcfDNA) as a sensitive marker of early graft injury to predict impending allograft failure.
Methods: This multicenter, prospective cohort study enrolled 106 subjects who underwent lung transplantation and monitored them after transplantation for the development of allograft failure (defined as severe chronic lung allograft dysfunction [CLAD], retransplantation, and/or death from respiratory failure). Plasma samples were collected serially in the first three months following transplantation and assayed for %ddcfDNA by shotgun sequencing. We computed the average levels of ddcfDNA over three months for each patient (avddDNA) and determined its relationship to allograft failure using Cox-regression analysis.
Findings: avddDNA was highly variable among subjects: median values were 3·6%, 1·6% and 0·7% for the upper, middle, and low tertiles, respectively (range 0·1%–9·9%). Compared to subjects in the low and middle tertiles, those with avddDNA in the upper tertile had a 6·6-fold higher risk of developing allograft failure (95% confidence interval 1·6–19·9, p = 0·007), lower peak FEV1 values, and more frequent %ddcfDNA elevations that were not clinically detectable.
Interpretation: Lung transplant patients with early unresolving allograft injury measured via %ddcfDNA are at risk of subsequent allograft injury, which is often clinically silent, and progresses to allograft failure.
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1. Introduction

Allograft failure occurs in lung-transplant recipients at higher rates than in other solid-organ transplant recipients [1–3]. This difference is primarily due to the frequent development of chronic lung allograft dysfunction (CLAD), which when severe, nearly always leads to death [1]. CLAD has several clinical phenotypes, the most predominant being bronchiolitis obliterans syndrome (BOS) which causes lung obstruction,

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Research in context

Evidence before this study

Lung transplant patients have the shortest survival of any other solid organ transplantation primarily due to a high incidence of chronic rejection (also called chronic lung allograft dysfunction – CLAD). Several therapies have been attempted but are generally ineffective. The clinical course of CLAD is therefore progressive with irreversible allograft injury that ultimately leads to allograft failure. Perhaps interventions at earlier stages before allograft injury becomes irreversible may delay or even prevent the development of CLAD and improve lung transplant outcomes. To-date, no reliable clinical predictive biomarker exists. Several biomarkers have been proposed but remain limited for one or more of the following reasons: requirement of invasive procedures such as bronchoscopy to obtain samples, poor specificity or sensitivity, and/or detection CLAD with significant time-lag to the irreversible clinical manifestations. This study proposes a non-invasive blood test as a potential predictive biomarker. Our overarching hypothesis is that allograft injury early after transplantation predictive of CLAD and other poor outcomes. Two clinical observations support this hypothesis. First, early post-transplant complications like primary graft dysfunction show a strong relationship with CLAD suggesting that allograft injury early after transplantation is a precursor of CLAD. Second, lung transplant patients undergo rigorous monitoring with bronchoscopies along with transbronchial biopsy, spirometry and other testing to detect and treat acute complications with the goal of preventing CLAD. Yet, CLAD still occurs an alarmingly high rate leading us to suspect the existence of allograft injury that is undetectable clinically and by monitoring tools. Testing this hypothesis require quantification of allograft injury early after transplantation. Unfortunately, the limitations of available clinical tools make them unreliable to quantitate early allograft injury. Histopathology, the current goal standard, is semi-quantitative at best and limited by low sensitivity, invasiveness and high variability. Spirometry, another monitoring tool, is limited by low sensitivity owing to large pulmonary reserve. Recently, our group introduced a sensitive genomic blood test that reliably quantitates allograft injury from infection, acute rejection and other complications. This test takes advantage of the wide genomic difference between transplant donors and recipients, as well as the sensitivity of genome sequencing to identify and quantify circulating donor-derived cell-free DNA – ddcfDNA. The test is broadly applicable across transplantation and has been used to detect acute rejection. In this study, we leverage the sensitivity of %ddcfDNA to quantitate allograft injury (both clinically-detected and clinically-silent) in the early post-transplant period and determine its relationship to allograft failure (CLAD or death).

Added value of this study

We monitored lung transplant patients for the development of CLAD or death. Their serially collected plasma samples in the early post-transplant period were used to quantify %ddcfDNA. The average %ddcfDNA in the early post-transplant period of 3-months, which we designate as avddDNA, was variable between patients. Levels of avddDNA correlated with early post-transplant clinical risk factors like older age and primary graft dysfunction. Patients with high avddDNA, signifying high early post-transplant allograft injury, showed 6·6-fold higher hazard of developing CLAD or death than those with lower avddDNA levels. More than half of these patients showed no clinical complication over the early post-transplant period. Patients with high avddDNA also showed lower lung function than those with low avddDNA levels. At a molecular level, they continued to show high %ddcfDNA levels beyond the early post-transplant period. Surprisingly, only one-third of the high %ddcfDNA levels were detected clinically or by monitoring tools, the rest were clinically unrecognized.

Implications of all the available evidence

We show that avddDNA is a potential predictive biomarker for CLAD and death. If validated, this blood test could be used to non-invasively risk stratify patients for CLAD as early as the initial three-months of transplantation. With clinical versions of the test soon becoming available, such a marker would therefore provide early time-points to intervene, perhaps preventing irreversible clinical manifestations and CLAD sets-in. We also show that most injury in lung transplant patients go unrecognized clinically or by existing monitoring tools and therefore go untreated. Identifying the triggers of this previously unrecognized allograft injury may open new avenues to intervene and prevent the development of CLAD and other poor outcomes. This warrants further investigation. Together, our results indicate that cumulative injury early after transplantation predicts subsequent allograft injury and is a precursor for downstream poor outcomes. These findings are potentially application across transplantation since the assay is equally reliable in these other transplant settings.

and restrictive allograft syndrome (RAS) which restricts lung expansion and function [1,2,4]. Allograft failure is thought to result from accumulation of injury from various sources, including primary-graft dysfunction (PGD), antibody-mediated rejection (AMR), acute cellular rejection (ACR), infections, and others causes [1,5–7]. These risk factors also predispose to allograft failure in other solid organs transplantation. PGD occurs in the early post-transplant period, and its strong association with allograft failure suggest that early post-transplant allograft injury is a risk factor for subsequent development of CLAD and allograft failure. The risk of allograft failure is strongest for patients with PGD that persists beyond 72 h after transplantation, making persistent PGD a potential proxy for ongoing allograft injury.

We therefore hypothesize that persistent injury in the early post-transplant period is a precursor of ongoing allograft injury and allograft failure. A sensitive, and direct quantitative method that can accurately quantify early allograft injury may therefore predict allograft failure and would enable earlier and more accurate identification of patients most at risk of severe CLAD, allograft failure and premature death. These patients may benefit from more closer monitoring. Unfortunately, current clinical tools, spirometry and histopathology on transbronchial biopsies are semi-quantitative, invasive, and/or are limited by poor sensitivity [8] making them unreliable to assess early post-transplant allograft injury.

Advances in genome sequencing technologies now offer an opportunity to overcome the limitations of sensitivity associated with current clinical methods. However, to our knowledge, no prior study has assessed whether ddcfDNA is marker of chronic, ongoing allograft injury that is ultimately irreversible. In the transplantation setting, where donor and recipient have different genomes, these methods can be applied to discriminate and quantitate plasma donor-derived cell-free DNA (ddcfDNA) as a marker of allograft injury. Cell free DNA (cfDNA) is released by dying cells into the bloodstream and is therefore a direct measure of organ injury, correlating with disease severity or disease state in various clinical scenarios including sepsis [9], trauma [10], and cancer [11]. Cell-free DNA has a short half-life of 15 min [12] and thus provides assessments of organ injury at a high temporal resolution. Detection of %ddcfDNA using unbiased whole-genome sequencing is

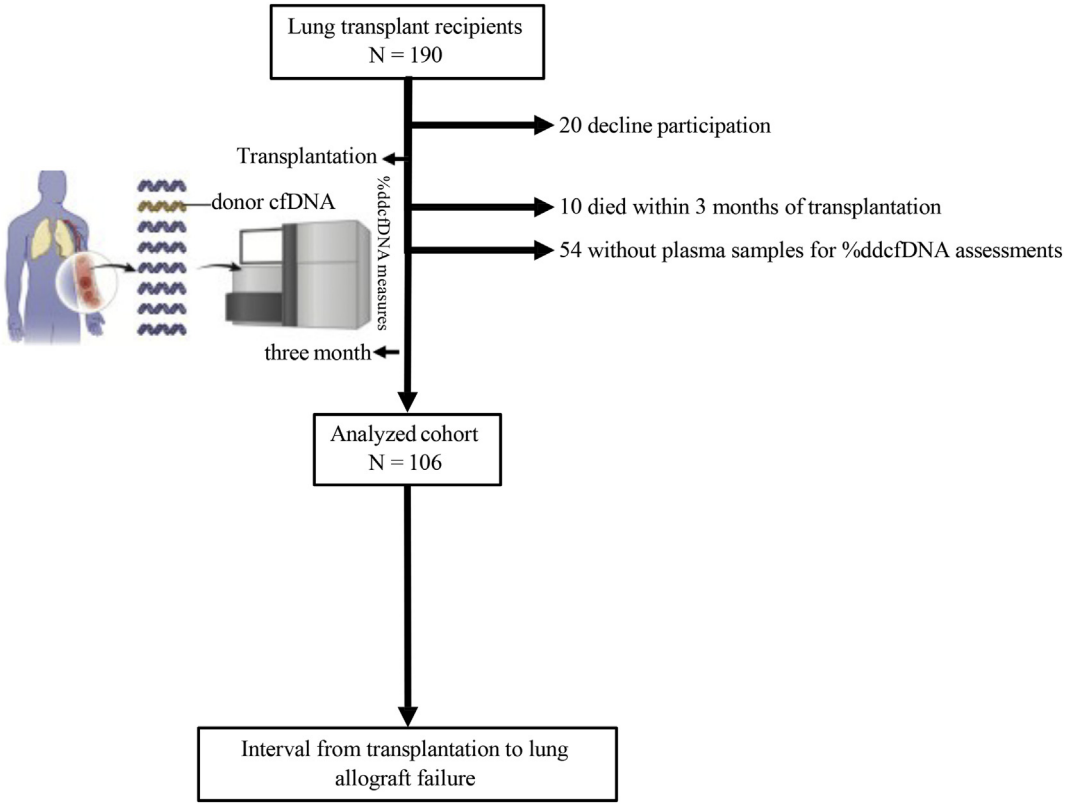


Fig. 1. Study design. Subjects were excluded if they died within three months of transplantation or did not provide plasma samples for %ddcfDNA assessment. 106 subjects were included. Plasma samples collected within the initial three months following transplantation were assessed for %ddcfDNA by shotgun sequencing. The average %ddcfDNA (avddDNA) was calculated as the predictive marker, and its relationship to the primary outcome was assessed. The primary outcome was lung-allograft failure (a composite outcome including death from respiratory causes, retransplantation and/or severe chronic lung allograft dysfunction-CLAD defined by ISHLT criteria1). The secondary outcome was all-cause mortality.

highly reproducible [13], and this method has been shown to accurately quantify microbial nucleic acids, enabling concurrent monitoring of infectious complications following transplantation [14] and changes in microbiome architecture.

The ddcfDNA is sensitive to detect acute rejection in lung [14], heart [15], liver [16] and kidney [17] transplants. Following lung transplantation, %ddcfDNA values ≥1% have been shown to detect severe acute allograft rejection with 100% sensitivity [14], and this may represent a clinically relevant %ddcfDNA threshold. This high sensitivity provides an opportunity to assess early post-transplant injury and determine its relationship with downstream allograft injury and allograft failure. In this study, we examined the following research questions: 1. What recipient and donor factors are related to %ddcfDNA? 2. how %ddcfDNA is related to downstream allograft injury and allograft failure?

2. Methods

2.1. Study design

Subjects awaiting lung transplantation in two ongoing cohort studies were included. The first study, Genome Transplant Dynamics (GTD) study (NCT01985412), commenced in 2010 and is a single-center study at Stanford University Hospital, California, investigating the test characteristics of %ddcfDNA to detect acute rejection. The second study is the Genomic Research Alliance for Transplantation (GRAfT) study (NCT02423070), which commenced recruitment in 2015 at three-centers (the Johns Hopkins Hospital and the University of Maryland Medical Center, Maryland, and Inova Fairfax Hospital, Virginia). In both studies, subjects were at least 18 years of age and enrolled while awaiting lung transplantation. Subjects were monitored prospectively after transplantation. We excluded subjects for whom no plasma samples were available for %ddcfDNA assay. 108 subjects had sufficient samples to compute avddDNA and were included to determine the relationship between covariates and avddDNA. Two of these subjects died within 3 months and were excluded for outcome regression analyses, leaving 106 subjects. The study design is summarized in Fig. 1. The enrollment details for the institutions involved in the study are included in Supplementary Table 1a. The study design was approved by the Institutional Review Board at each institution. The patient monitoring plan is detailed in the Supplementary Methods section, and the immunosuppression regimen is outlined in Supplementary Table 1b.

2.2. Outcome measures

The primary outcome of the study was defined as the time from transplantation to first detection of allograft failure, represented by any of the three endpoints: severe CLAD (as defined by the International Society for Heart and Lung Transplantation criteria [1]), retransplantation, and death from respiratory causes. These three endpoints represent the most severe clinical complications that arise from a failing lung allograft. Additional information on the rationale for selecting this composite primary outcomes and definition of CLAD is provided in Supplementary Methods. The secondary outcomes were defined as the time from transplantation to all-cause mortality and CLAD-free survival. All outcomes were adjudicated by transplant physicians blinded to %ddcfDNA measurements.

2.3. Clinical covariables and measurements

Clinical covariates were recorded throughout the study. Before transplantation, donor and recipient demographics, smoking history, cytomegalovirus serology status and other variables were recorded, including donor cause of death and history of chest trauma; and recipient reason for transplantation and lung allocation score. In the immediate

Table 1
Donor and recipient covariates by avddDNA tertiles (n = 108).

Variables	Total	Low tertile	Middle tertile	Upper tertile
Recipient covariates				
Age Mean (SD)	54·61 (15·11)	51·56 (14·26)	55·06 (15·48)	57·22 (15·44)
Sex n(%)				
Male	58(54)	18(50)	18(50)	22(61)
Female	50(46)	18(50)	18(50)	14(39)
Race n(%)				
Non-Caucasian	19(18)	8(24)	3(9)	8(23)
Caucasian	85(82)	26(76)	32(91)	27(77)
Obesity BMI ≥30 Kg/m2 n (%)				
No	90(84)	31(89)	30(83)	29(81)
Yes	17(16)	4(11)	6(17)	7(19)
Smoking history n(%)				
Never	77(71)	27(75)	26(72)	24(67)
Past	31(29)	9(25)	10(28)	12(33)
Transplantation reason n(%)				
COPD	22(20)	5(14)	9(25)	8(22)
CF	18(17)	9(25)	4(11)	5(14)
ILD	52(48)	13(36)	20(56)	19(53)
PAH	3(3)	1(3)	1(3)	1(3)
Sarcoidosis/others	13(12)	8(22)	2(6)	3(8)
LAS mean(SD)	47·10 (16·71)	41·19 (10·92)	48·69 (17·88)	51·12 (18·90)
Donor covariates				
Age mean(SD)	36·36 (15·09)	39·75 (15·18)	35·67 (15·89)	33·67 (13·90)
Sex n(%)				
Male	65(60)	22(61)	22(61)	21(58)
Female	43(40)	14(39)	14(39)	15(42)
Race n(%)				
Non-Caucasian	28(27)	12(35)	6(17)	10(29)
Caucasian	75(73)	22(65)	29(83)	24(71)
Obesity BMI ≥30 Kg/m2 n (%)				
No	78(74)	28(78)	25(74)	25(69)
Yes	28(26)	8(22)	9(26)	11(31)
Smoking history n(%)				
Never	98(95)	33(97)	33(97)	32(91)
Past	5(5)	1(3)	1(3)	3(9)
Chest trauma n(%)				
No	103(98)	35(97)	34(100)	34(97)
Yes	2(2)	1(3)	0	1(3)
Cause of death n(%)				
Head trauma or GSW	44(41)	12(33)	16(44)	16(46)
CVA	42(39)	18(50)	12(33)	12(34)
Anoxia or overdose	18(17)	5(14)	7(19)	6(17)
Others	3(3)	1(3)	1(3)	1(3)
Mismatch covariates				
Sex n(%)				
Match	71(66)	24(67)	22(61)	25(69)
Mismatch	37(34)	12(33)	14(39)	11(31)
Race n(%)				
Match	67(62)	17(47)	26(72)	24(67)
Mismatch	41(38)	19(53)	10(28)	12(33)
HLA mismatch mean(SD)	9·70 (2·04)	9·27 (1·49)	9·95 (2·36)	9·90 (2·20)
Sensitized recipients n(%)	41(37)	14(37)	17(50)	9(25)
CMV status n(%)				
D + R+	40(42)	14(44)	14(42)	12(39)
D + R−	22(23)	10(31)	7(21)	5(16)
D−R+	19(20)	5(16)	5(15)	9(29)
D−R−	15(16)	3(9)	7(21)	5(16)
Peri-transplant covariates				
Average ischemic time mean(SD)	276·17 (62·02)	282·14 (53·26)	265·58 (67·14)	281·74 (64·56)
Type of transplantation n(%)				
Bilateral	84(79)	30(86)	30(83)	24(67)
Single	23(21)	5(14)	6(17)	12(33)
Induction n(%)				
No	70(65)	24(69)	26(72)	20(56)
Yes	37(35)	11(31)	10(28)	16(44)
PGD grade 3 n(%)				
No	78(78)	27(82)	27(79)	24(73)
Yes	22(22)	6(18)	7(21)	9(27)
DSA within 3 months	43(41)	13(35)	13(38)	17(50)
Acute rejection episodes mean(SD)	1·81 (1·92)	1·89 (2·34)	1·22 (1·17)	2·31 (2·07)
Study cohort n(%)				
GRAfT	54 (50)	16(44)	16(44)	22(61)
GTD	54 (50)	20(56)	20(56)	14(39)

COPD = chronic obstructive lung disease, CF = cystic fibrosis, ILD = interstitial lung disease, PAH = pulmonary arterial hypertension, LAS = lung allocation score obtained closest to transplantation· GSW = gunshot wound, overdose = death from drug overdose, HLA = human leukocyte antigen CMV status = cytomegalovirus IgG status, D+ = donor positive for CMV IgG, D- = donor negative for CMV IgG, R+ = recipient positive for CMV IgG, R- = recipient negative for CMV IgG, PGD grade 3 = Primary graft dysfunction grade 3 defined by International Society of Heart and Lung grading criteria, DSA = donor-specific antibodies.

post-transplant period, arterial blood gas and chest x-ray data on Day 3 after transplantation were recorded to defined PGD status. Use of induction therapy was also documented. In the post-transplantation period, serial data obtained as part of routine clinical care including spirometry, histopathology, donor-specific antibodies, microbiological tests, chest radiographs and other laboratory test were recorded to define the primary and secondary endpoints as well as clinical complications of ACR, AMR or infections. The methods and schedules for collecting these data, as well as definitions of these variables is detailed in the Supplementary Methods.

2.4. %ddcfDNA measurement

Plasma samples were collected serially (Supplementary Fig. 1) and % ddcfDNA was measured using a previously described shotgun-sequencing method [14]. In summary, genomic DNA isolated from donor and recipient pre-transplant blood was genotyped, and the data for the two samples were compared to identify single-nucleotide polymorphisms (SNPs). Following the transplant, cfDNA was isolated from plasma samples to generate a DNA library for shotgun sequencing. The cfDNA sequence reads were then surveyed for the presence of donor and recipient SNPs and %ddcfDNA was calculated as the percentage of donor SNPs to total (recipient and donor) SNPs [14,15]. Number of samples analyzed for each cohort, as well as sequencing data characteristics are represented in Supplementary Table 2.

2.5. Calculating three-month average %ddcfDNA (avddDNA)

The early post-transplantation decay kinetics %ddcfDNA were analyzed separately for double- and single-lung transplants using previously described method that showed a two-step logarithmic decay [14]. Since median %ddcfDNA values for single-lung transplants were half the median values for double-lung transplants, we multiplied the %ddcfDNA by two to correct for single-lung transplantation. To compute average %ddcfDNA within the initial three-month period, we plotted individual %ddcfDNA versus time and calculated the area under the curve (AUC) between days 14 and 90 (inclusive). Plasma samples from days 0–13 were analyzed, but the %ddcfDNA values were excluded from our calculations to limit any contamination by cfDNA released during transplant surgery. We thus calculated the average %ddcfDNA (*avddDNA*) by dividing the AUC of %ddcfDNA by 77 (to represent the 13 to 90-day period). More detail is provided in the Supplementary Methods.

2.6. Statistical analysis

Descriptive analyses were conducted. The distribution of the continuous variables for donor and recipient factors (e.g. recipient age, donor age, lung allocation score (LAS), ischemic time, number of rejection episodes, number of HLA mismatches) were checked. Since each fell within the limit of the cutoff for normality, we represent the mean across the three tertiles of avddDNA. For categorical variables, we represent the frequency (Table 1). We also compared the mean of continuous variables with independent sample *t*-tests and frequencies of categorical variables with Chi-square or Fisher's Exact tests (if any cell size<10) between the analyzed and excluded cohorts (Supplementary Table 1c) and between the GRAfT and GTD cohorts (Supplementary Table 1d), separately.

Next, we assessed variables that are related to avddDNA. The skewness for avddDNA is 1·9, falling within the limit of the cutoff for normality [18]. So, we used a linear regression analyses to determine the relationship between each variable (independent variables) and avddDNA (continuous dependent variable). Variables with $p < 0.2$ were included into a multivariate regression model. In addition to inclusion of the covariates based on statistical significance, we also included variables that were related to the predictor (type of transplantation) or that were clinically relevant to the outcome (severe PGD) regardless of the p-values. We also include study cohort (GTD vs. GRAfT) since the two cohorts showed different rates of the primary and secondary outcomes. The univariate and multivariate analyses are presented in Table 2.

The principal analysis was a time-to-event analysis, or survival with avddDNA and other factors, as predictor and allograft failure as outcome (Table 3). Survival analysis was repeated separately for each of the two secondary outcomes – CLAD-free survival and all-cause mortality (Supplementary Table 3). For all the subjects, the time from transplantation to last clinical follow up visit ranges from 0.47–63 months. Since the predictor was measured over the initial 3 months of transplantation, subjects with follow-up of 3 months or less were excluded.

The primary outcome, allograft failure, is defined as such if the subject had at least one of three conditions: severe CLAD, re-transplantation or death from respiratory causes. The survival time was calculated from the date of transplantation until the earliest date out of the three conditions (for subjects with events) or last clinical follow up (for censored subjects). The subjects were censored if they did not have the event by the last clinical follow up.

There are two secondary outcomes. The 1st outcome is defined if the subject had at least one of the two conditions: any grade of CLAD or death from any cause. The survival time was calculated from the date of transplantation until the earliest date out of the two conditions or the last clinical follow-up. Not meeting any of the two outcomes (any grade CLAD or death) defines CLAD-free survival or being alive and CLAD-free, and was censored if subjects did not have the event by the last clinical follow-up. The 2nd outcome is all-cause mortality defined as death of any cause. The survival time was calculated from the date of transplantation until death or last clinical follow-up visit. The subjects were censored if they did not have the event (death) by the last clinical follow-up.

For each survival outcome, Kaplan-Meier method was used to estimate the survival function by the three tertiles of avddDNA. Log-rank tests were used to test the differences in survival by the tertiles. The proportional hazard assumption was tested on the basis of Schoenfeld residuals.

The proportional hazard assumption was held for each outcome. Univariate Cox regression model was first conducted to estimate the Hazard Ratio (HR) of each outcome of interest in relation to avddDNA and other variables, including avddDNA as a continuous variable first and then as a categorical variable, separately. For the multivariate analysis, we first included avddDNA as a continuous variable to estimate the adjusted Hazard Ratios (adjusted HRs, model 1), adjusting for the covariates based on the statistical significance if the p value was <0.20 in the univariate analysis or based on clinical relevance (PGD grade 3, cohort study, single vs. bilateral) following purposeful selection algorithm [19]. We then repeated the Cox regression model for each outcome using avddDNA as a categorical variable based on tertiles (upper-, middle-, and low-tertile avddDNA) with the low-tertile as the reference group (model 2). Statistical analyses were performed using GraphPad Prism 7 graphing (GraphPad Software) [20] and the STATA 12·0 statistical package (StataCorp LLC) [21].

2.7. Power analyses

The principal analysis assessed the relationship of avddDNA to allograft failure. The priori sample size calculation suggested that, given the standard deviation of avddDNA as 1·9, a sample size of 106 will allow us to have enough power (> 0·80) to detect a significant regression coefficient for avddDNA if the regression coefficient is >0·2, assuming that the overall failure in the survival analyses as 50%.

2.8. ddcfDNA and clinical assessments beyond three months

To assess allograft injury beyond the initial three months of transplantation, we used both clinical (spirometry, histopathology, microbiology) and molecular (%ddcfDNA) tools. Mean FEV1 and %ddcfDNA for each avddDNA tertile were calculated and compared at arbitrary three-month intervals; values within two weeks on either side of each arbitrary interval were included. Based on previously published data for detecting acute rejection [14] we focused on episodes in which % ddcfDNA was ≥1% to determine whether elevated %ddcfDNA levels coincided with clinical indicators of allograft injury. These clinical indicators included ACR, AMR, and/or clinical infection and were considered episodes if they were separated by at least 2 weeks. We time-matched the episodes of elevated %ddcfDNA to clinical data and assigned them as clinically detectable if they were within two weeks of a clinical event or clinically silent if outside of this time range. Clinical definitions of AMR, ACR, and clinical infections are summarized in the Supplementary Methods.

3. Results

3.1. Participant characteristics and clinical outcomes

Of the 190 subjects approached for this study, 20 subjects declined participation, ten died within three months of transplantation and a further 52 subjects lacked plasma samples for avddDNA measurements, leaving a final cohort of 108 subjects for analysis – 54 from GTD and 54 from GRAfT. The analyzed cohort of 108 subjects had an average age at transplantation of 54·1 years. Interstitial lung disease was the most common reason for transplantation (46·3%) followed by cystic fibrosis (20·4%). Disease severity index defined by lung allocation score at transplantation showed an average of 47·3 (Table 1). Comparisons of the analytic and excluded cohorts and of the GTD and GRAfT cohorts are shown in Supplementary Table 1c and d.

Median follow-up time was 36·3 months. Two of the 108 subjects died within 3-months were excluded leaving 106 subjects for further analysis. Of the 106 subjects, 43·5% (n = 46) reached the primary outcome (death from respiratory causes = 27, CLAD but no respiratory death or re-transplantation = 14, and re-transplantation = 5), 41·7% (n = 45) reached the secondary outcome of all-cause mortality, and 41·5% of the cohort were alive and free from CLAD. Causes of death were respiratory failure (27/45), followed by sepsis (14/45), and others (4/45: colon cancer, myocardial infarction, pulmonary embolism, post-transplant lymphoproliferative disorder). Of the 18 subjects who died from non-respiratory causes, the majority (14/18) had concurrent acute rejection or evidence of severe CLAD by the time of death.

Table 2
Relationship between recipient and donor covariates and avddDNA (n = 108).

Variables	Coefficient	Unadjusted regression coefficient 95% CI	p	Coefficient	Adjusted regression coefficient 95% CI	p
Recipient covariates						
Age	0·03	0–0·05	0·023	0·02	−0·05–0·07	0·973
Sex						
Male	Reference					
Female	−0·431	−1·17–0·31	0·256			
Race						
Non-Caucasian	Reference					
Caucasian	−0·77	−1·75–0·21	0·121	−0·37	−1·81–1·08	0·611
Obesity BMI ≥30 Kg/m2						
No	Reference					
Yes	1·29	0·3–2·28	0·011	1·20	−0·17–2·58	0·085
Smoking history						
Never	Reference					
Past	0·78	−0·03–1·59	0·058	0·15	−1·51–1·81	0·857
Transplantation reason						
COPD	Reference					
CF	−1·08	−2·29–0·14	0·083	−0·79	--2·78–1·21	0·432
ILD	−0·25	−1·22–0·73	0·617	−0·48	−1·82–0·86	0·476
PAH	−0·36	−2·72–2·20	0·760	−0·75	−3·81–2·30	0·621
Sarcoidosis/others	−0·89	−2·23–0·45	0·193	−0·99	−2·89–0·92	0·303
LAS	0·03	0·01–0·06	0·005	0·03	0·002–0·06	0·038
Donor covariates						
Age	−0·03	−0·05–0	0·039	−0·01	−0·05–0·02	0·357
Sex						
Male	Reference					
Female	0·34	−0·42–1·09	0·376			
Race						
Non-Caucasian	Reference					
Caucasian	−0·12	−0·98–0·74	0·789			
Obesity BMI ≥30 Kg/m2						
No	Reference					
Yes	0·09	−0·77–0·95	0·837			
Smoking history						
Never	Reference					
Past	−0·03	−1·71–1·65	0·968			
Chest trauma						
No	Reference					
Yes	1·23	−1·37–3·83	0·350			
Cause of death						
Head trauma or GSW	Reference					
CVA	−0·79	−1·86–0·28	0·147	−0·275	−1·63–1·08	0·685
Anoxia or overdose	−0·78	−1·61–0·05	0·065	−1·41	−3·34–0·52	0·149
Others	−0·35	−2·63–1·94	0·765	−0·32	−3·10–2·45	0·816
Mismatch covariates						
Sex						
Match	Reference					
Mismatch	−0·06	−0·84–0·72	0·882			
Race						
Match	Reference					
Mismatch	0·23	−0·54–0·99	0·558			
Sensitized recipient						
No	Reference					
Yes	0·02	−0·21–0·24	0·880			
HLA mismatch	−0·56	−1·32–0·21	0·153	−0·83	−1·81–0·14	0·091
CMV status						
D + R+	Reference					
D−R+	−0·66	−1·67–0·36	0·202	−0·43	−1·86–0·55	0·510
D + R−	1·00	−0·07–2·06	0·067	0·25	−1·01–1·51	0·697
D−R−	0·09	−1·06–1·25	0·873	−0·14	−1·79–1·14	0·851
Peri-transplant covariates						
Average ischemic time	0·00	−0·01–0·01	0·498			
Type of transplantation						
Bilateral	Reference					
Single	0·57	−0·33–1·47	0·216	−0·21	−1·40–0·97	0·719
Induction						
No	Reference					
Yes	0·55	−0·23–1·32	0·167	1·18	−2·84–5·20	0·559
PGD grade 3						
No	Reference					
Yes	1·09	0·2–1·97	0·017	0·31	−1·06–1·69	0·647
DSA within 3 months	0·02	−0·17–0·21	0·834			
Acute rejection episodes	0·59	−0·13–1·30	0·107	0·61	−0·39–1·60	0·225

Table 2 (*continued*)

Variables	Coefficient	Unadjusted regression coefficient	p	Coefficient	Adjusted regression coefficient	p
		95% CI			95% CI	
Study cohort						
GRAfT	Reference					
GTD	−0·68	−1·41–0·05	0·067	0·13	−4·26–4,51	0·954

Unadjusted and adjusted linear regression models were performed in relation to avddDNA. The adjusted model included variables with p-value <0·2 on crude analysis, type of transplantation, PGD and study cohort· COPD = chronic obstructive lung disease, CF = cystic fibrosis, ILD = interstitial lung disease, PAH = pulmonary arterial hypertension, LAS = lung allocation score obtained closest to the date of transplantation· GSW = gunshot wound, overdose = death from drug overdose, PGD grade 3 = Primary graft dysfunction grade 3 defined by International Society of Heart and Lung grading criteria· CMV status = cytomegalovirus IgG status, D+ = donor positive for CMV IgG, D- = donor negative for CMV IgG, R+ = recipient positive for CMV IgG, R- = recipient negative for CMV IgG.

Table 3
Relationship between avddDNA, donor, and recipient covariates and allograft failure (n = 106).

Variables	HR	Unadjusted hazard ratio (HR)	p	HR	Model 1 adjusted HR		HR	Model 2 adjusted HR	
		95% CI			95% CI	p		95% CI	p
Continuous avddDNA	1·34	1·16–1·55	<0·001	1·39	1·07–1·80	0·015			
avddDNA tertiles									
Lowest	Reference								
Middle	1·33	0·62–2·83	0·465				2·44	0·89–6·71	0·084
Highest	3·30	1·59–6·87	0·001				6·63	1·59–19·92	0·007
Recipient covariates									
Age	1·00	0·98–1·02	0·868						
Sex									
Male	Reference								
Female	0·60	0·33–1·08	0·089	1·22	0·44–3·39	0·699	1·22	0·44–3·39	0·719
Race									
Non-Caucasian	Reference								
Caucasian	0·52	0·26–1·06	0·071	0·25	0·06–1·00	0·049	0·19	0·04–0·83	0·027
Obesity BMI ≥30 Kg/m2									
No	Reference								
Yes	1·47	0·71–3·05	0·296						
Smoking history									
Never	Reference								
Past	0·65	0·3–1·42	0·282						
Transplantation reason									
COPD	Reference								
CF	0·91	0·36–2·26	0·835						
ILD	1·06	0·51–2·22	0·870						
PAH	1·59	0·20–12·62	0·658						
sarcoidosis/others	1·86	0·71–4·88	0·204						
LAS	1·01	0·99–1·03	0·102	1·02	0·99–1·04	0·202	1·02	0.99–1.04	0·202
Donor covariates									
Age	1·01	0·99–1·03	0·209						
Sex									
Male	Reference								
Female	0·57	0·31–1·05	0·070	0·44	0·17–1·18	0·105	0·51	0·19–1·37	0·178
Race									
Non-Caucasian	Reference								
Caucasian	1·04	0·53–2·01	0·919						
Obesity BMI ≥30 Kg/m2									
No	Reference								
Yes	1·16	0·59–2·3	0·672						
Smoking history									
Never	Reference								
Past	0·00	cannot be estimated	1·000						
Chest trauma									
No	Reference								
Yes	0·81	0·11–5·93	0·839						
Cause of death									
Head trauma or GSW	Reference								
CVA	0·95	0·52–1·72	0·861	1·12	0·45–2·77	0·813	0·90	0·36–2·22	0·818
Anoxia or overdose	0·09	0·01–0·67	0·019	0·20	0·02–2·16	0·185	0·08	0·01–0·09	0·040
Others	1·24	0·29–5·32	0·765	1·20	0·13–11·15	0·875	1·33	0·15–12·23	0·800
Mismatch covariates									
Sex									
Match	Reference								
Mismatch	0·65	0·34–1·25	0·294						
Race									
Match	Reference								
Mismatch	1·07	0·59–1·92	0·832						

(*continued on next page*)

Table 3 (*continued*)

Variables	HR	Unadjusted hazard ratio (HR)	p	HR	Model 1 adjusted HR		HR	Model 2 adjusted HR	
		95% CI			95% CI	p		95% CI	p
Sensitized recipient									
No	Reference								
Yes	0·72	0·39–1·32	0·282						
HLA mismatch	0·98	0·84–1·15	0·803						
CMV status									
D + R+	Reference								
D + R−	0·72	0·34–1·51	0·382	3·72	0·87–6·36	0·031	4·35	1·24–15·25	0·022
D−R+	0·40	0·17–0·96	0·039	1·03	0·31–2·10	0·961	1·09	0·35–3·35	0·881
D−R−	0·72	0·29–1·77	0·468	2·46	0·58–6·23	0·191	3·64	0·86–15·44	0·080
Peri-transplant covariates									
Average ischemic time	1·00	0·99–1·00	0·643						
Type of transplantation									
Bilateral	Reference								
Single	0·51	0·22–1·2	0·124	0·35	0·08–1·49	0·156	0·20	0·04–1·01	0·051
Induction									
No	Reference								
Yes	0·47	0·2–1·06	0·068	0·49	0·03–8·25	0·621	1·24	0·09–16·91	0·874
PGD grade 3									
No	Reference								
Yes	0·88	0·41–1·9	0·744	2·58	0·50–13·28	0·258	6·46	1·45–28·85	0·015
DSA within 3 months	1·22	0·66–2·25	0·516						
Acute rejection episodes	1·2	1·06–1·35	0·004	1·44	1·14–1·33	0·002	1·44	1·13–1·83	0·003
Study cohort									
GRAfT	Reference								
GTD	2·27	1·11–4·61	0·024	1·81	0·10–31·47	0·684	1·74	0·31–104·29	0·236

Two adjusted regression models were performed, one with avddDNA as continuous variable (Model 1) and the other with avddDNA treated as a category variable based on tertiles (Model 2). The adjusted model included variables with p-value <0·2 on unadjusted analysis, type of transplantation, PGD and study cohort. COPD = chronic obstructive lung disease, CF = cystic fibrosis, ILD = interstitial lung disease, PAH = pulmonary arterial hypertension, LAS = lung allocation score obtained closest to the date of transplantation· GSW = gunshot wound, overdose = death from drug overdose, PGD grade 3 = Primary graft dysfunction grade 3 defined by International Society of Heart and Lung grading criteria. CMV status = cytomegalovirus IgG status, D+ = donor positive for CMV IgG, D- = donor negative for CMV IgG, R+ = recipient positive for CMV IgG, R- = recipient negative for CMV IgG. Note: For crude HR, only avddDNA tertiles and continuous avddDNA are significantly (ps < 0·05) related to the outcome after Bonferroni adjustment.

3.2. %ddcfDNA measured in the early post-transplantation period

We analyzed 1145 plasma samples, 9·7 samples/patient, (Supplementary Table 2a) for %ddcfDNA. Of these, 505 (4·7 samples/patient) were within three months post-transplantation. Results of the sequencing analysis are provided in Supplementary Table 2b. Immediately after transplantation, %ddcfDNA levels were high and then followed a two-step logarithmic decay: an initial fast decay and then a slower decay to a low, stable level. Although levels of %ddcfDNA were higher for bilateral- than for single-lung transplantation immediately after surgery (43·3% vs. 23·5%, p < 0·001), the %ddcfDNA decay kinetics were similar (double transplant fast half-life = 0·9 days, slow half-life = 10·3 days; single transplant fast half-life = 1·0 days, slow half-life = 12·9 days).

We observed three distinct patterns of %ddcfDNA decay during the initial three months after transplantation that varied among individual subjects as described below. To stratify subjects by level of %ddcfDNA and the decay pattern during this early post-transplant period, we calculated each patient's average %ddcfDNA over the first three months following transplantation (avddDNA, Fig. 2a). The distribution of avddDNA values is shown in Fig. 2b. The cohort was divided into avddDNA three groups or tertiles (low, middle, upper) which correlated with the three distinct %ddcfDNA decay pattern observed. All three groups showed high immediate post-transplant %ddcfDNA, but variable % ddcfDNA decay. Subjects with avddDNA in the low tertile (n = 36, median avddDNA = 0·7%, range = 0·1%–1·0%) displayed a rapid % ddcfDNA decline to a low, stable level by one-month post-transplantation (Fig. 2c, dotted line; Supplementary Fig. 2, Subjects 1–5). The subjects with avddDNA in the middle tertile (Fig. 2c, dashed line; Supplementary Fig. 2, Subjects 6–10) showed slow decay initially, but their %ddcfDNA levels reached a stable level comparable to the first group by three months after transplantation. A third group with avddDNA in the upper tertile (median 3·6%, range = 2·2%–9·8%) showed even slower %ddcfDNA decay (Fig. 2c, solid line; Supplementary Fig. 2, Subjects 11–15) with persistently elevated %ddcfDNA levels compared to the other two groups. Subjects with avddDNA in the upper tertiles had higher LAS score and number of acute rejection episodes (Table 1) than the middle or low avddDNA groups.

3.3. Relationship between donor and recipient covariates and avddDNA

Subjects in the upper avddDNA showed the following pre-transplant characteristics compared to subjects in the low avddDNA tertile: older age (57.2 vs. 51.6 years), higher LAS score (51.1 vs. 41.2), higher frequency of previous smoking (33% vs. 29%), and higher frequency of CMV $D^{-}R^{+}$ status (29% vs. 16%). Interstitial lung disease was more common (53% vs. 36%) while cystic fibrosis was less common (14% vs. 25%) as reasons for transplantation for the upper than low avddDNA tertile. At transplantation, subjects in the upper avddDNA tertile received more single lung transplantation (33% vs. 21%) and induction therapy (44 vs. 31%) than subjects in the low avddDNA tertile. After transplantation, they developed PGD grade 3 (27% vs. 18%) and DSA (50% vs. 37%) more frequently, and also showed more episodes of acute rejection (2.3 vs. 1.9 episodes) than subjects in the low avddDNA tertile (Table 1).

We next performed linear regressions to identify factors that correlate with higher avddDNA levels. Pre-transplant factors including recipient age, obesity, LAS score, as well as donor age correlated with higher avddDNA levels on univariate analysis. Only recipient LAS was positively associated with higher avddDNA after multivariate analysis (Table 2). In the early post-transplantation period, use of induction therapy or ischemic time did not correlate with avddDNA levels. The presence of PGD grade 3 at 72 h [22] correlated higher avddDNA levels than no grade 3 PGD (Table 2) on univariate but not on multivariate analysis. After adjusting for lung mass by doubling %ddcfDNA values

for single-lung transplantations, single- and double-lung transplantations showed comparable avddDNA levels.

3.4. Relationship between average %ddcfDNA (avddDNA), clinical covariates and outcomes

To establish any links between avddDNA, donor covariates or recipient covariates and the primary or secondary outcomes of the study, we conducted Cox models, including avddDNA as a continuous or categorical variable (avddDNA tertiles) for outcome separately. In the unadjusted univariate analysis, both avddDNA and avddDNA tertile were significantly related to the primary outcome (ps ≤ 0.001 for avddDNA and upper tertile vs. low tertile). The significance held after Bonferroni adjustment (ps < 0.05 for avddDNA and higher tertile vs. low tertile). After adjusting for relevant covariates, avddDNA showed a significant correlation with the primary outcome: a 1% increase in avddDNA increased the risk of allograft failure 1·4-fold (95% CI 1·1–1·5, p = 0·015). Subjects in the upper avddDNA tertile had a 6·6-fold greater risk of allograft failure compared to the subjects in the low avddDNA tertile (HR = 6·6; 95% CI 1·6–19·9, p = 0·007, Fig. 3a, Table 3). Their median time to develop allograft failure was 25 months compared to 42 and 45 months for subjects in the middle and low avddDNA tertiles respectively (Supplementary Table 4). On multivariate analysis with categorical avddDNA, the following factors also increased the risk of reaching the primary endpoint: non-Caucasian race, PGD grade 3, single lung transplant, CMV D^+R^- status, higher number acute rejection episodes (Table 3). Donor cause of death from anoxia or drug overdose reduced the risk of the primary outcome. The primary outcome was higher in the GTD than in the GRAfT cohort on univariate analysis, but not after adjusting for donor and recipient covariates. Multivariate analysis with continuous avddDNA is presented in Table 3.

The secondary outcome of CLAD-free survival, defined as no CLAD or no death, showed a strong association to avddDNA. A 1% increase in avddDNA increased the risk of CLAD/death by 1·5 (95% CI 1·2–1·9, p = 0·001). Subjects in the upper avddDNA tertile showed a 7·8-fold higher risk of CLAD/death compared to the subjects in the avddDNA low tertile (95% CI 2·2–27·7, p = 0·001, Fig. 3b, Supplementary Table 3a). Their median time to reach this endpoint was 14 months compared to 36 and 32 months for subjects in the middle and low avddDNA tertiles respectively. The avddDNA was equally predictive of CLAD-free survival when the GRAfT and GTD cohorts were analyzed separately (GRAfT, log rank chi square = 7·4, p = 0·006, GTD, log

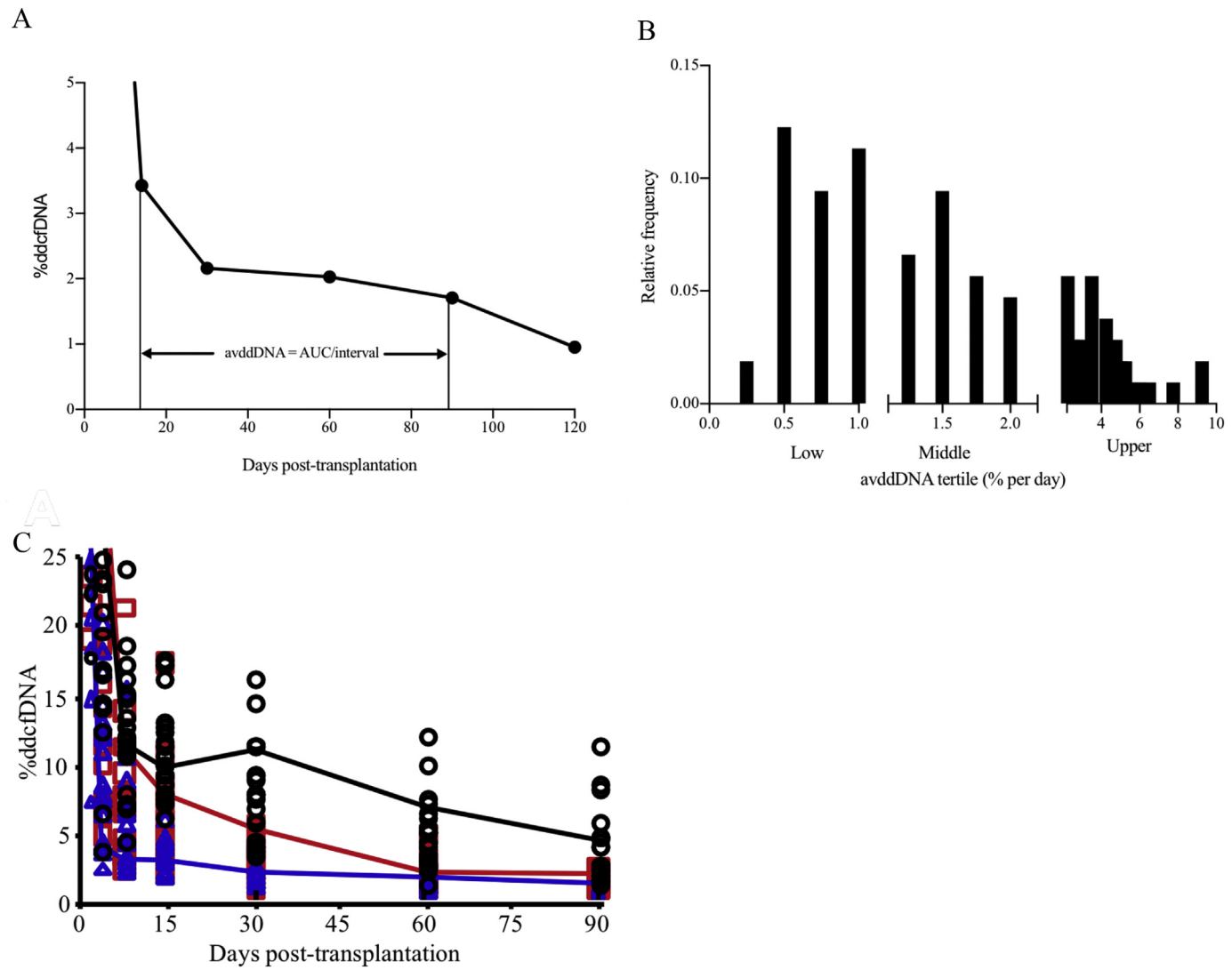
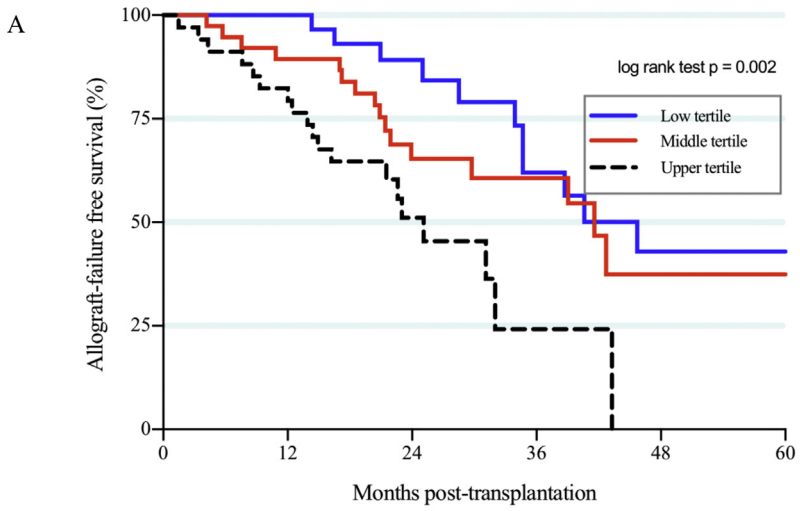
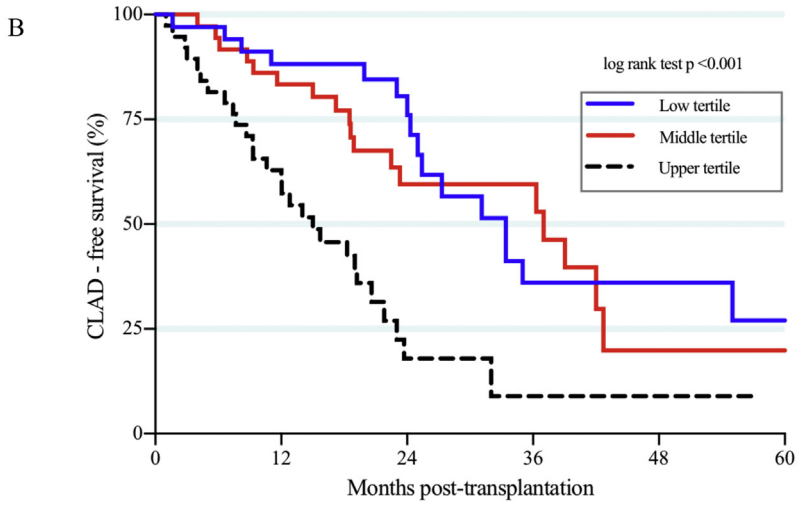


Fig. 2. Calculation of the predictive biomarker: average %ddcfDNA (avddDNA). (A) %ddcfDNA vs. time curve for a representative patient used to calculate the predictive measure, avddDNA, which is the area under the curve (AUC) divided by 77 days (the interval during days 14-90, inclusive). (B) Frequency distribution of avddDNA values. The cohort was divided into upper, middle and low tertiles based on avddDNA values. The x-axis breaks represent the avddDNA tertiles. (C) Individual subjects %ddcfDNA values over time separated by avddDNA tertiles of upper (black dots, black solid line), middle (red dots, red solid line) and low (blue dots, blue solid line) tertiles. Trends of %ddcfDNA for a representative subject in each avddDNA is represented by solid lines. Plots for 15 individual subjects, five representatives for each group, are shown in Supplementary Fig. 3.



	Number of subjects at risk at x-axis mark					
Month post-transplant	0	12	24	36	48	60
avddDNA tertile						
Low	36	36	19	13	7	3
Middle	35	32	17	11	5	2
Upper	35	28	12	2	0	0



	Number of subjects at risk at x-axis mark					
Month post-transplant	0	12	24	36	48	60
avddDNA tertile						
Low	36	31	18	8	6	4
Middle	35	31	16	9	3	3
Upper	35	23	5	2	2	1

Fig. 3. Relationship of average %ddcfDNA (avddDNA) and outcomes. Kaplan-Meier curves for time to allograft failure (A) and CLAD-free survival (B) for the upper-, middle-, and low avddDNA tertiles. The table below the curves shows the number of subjects at risk in each avddDNA tertile at different timepoints. P-values determined by log-rank test. Cox-regression analyses of avddDNA in relationship to the primary and secondary endpoints are presented in Table 3 and Supplementary Table 3a

rank chi square = 10·9, p = 0·001). Female sex, non-Caucasian race, recipients transplanted for ILD, higher number of episodes of acute rejection and being in the GTD cohort also increased the risk of CLAD/death on multivariate analysis that included avddDNA as categorical variable. With chronic obstructive lung disease as reference, cystic fibrosis was the only reason for transplantation associated with the primary outcome. Multivariate analysis that includes avddDNA as continuous variables is represented in Supplementary Table 3a.

All-cause mortality also showed a strong association with avddDNA; a 1% increase in avddDNA increased the risk of death from any cause by 1·5-fold (95% CI 1·2–1·8, p = 0·001, Supplementary Table 3b). Subjects in the upper avddDNA tertile showed of a 3·9-fold higher risk of

all-cause mortality compared to the subjects in the low avddDNA tertile (95% CI 1·4–10·1, p = 0·006, Supplementary Table 3b). The median survival for the upper avddDNA tertile was 31 months, compared to 42 and 55 months for the middle and low avddDNA tertiles respectively (Supplementary Table 4). In addition to avddDNA, PGD grade 3 and being in the GTD cohort increased the risk all-cause mortality on multivariate analysis (Supplementary Table 3). Multivariate analysis that includes avddDNA as continuous variables is represented in Supplementary Table 3b.

3.5. Relationship between avddDNA, PGD, and outcomes

We further assessed the relationship of avddDNA and allograft failure in subjects with PGD grade 3, the most studied early risk factor for allograft failure. Twenty-one of 106 subjects had PGD grade 3, of these, seven subjects were in the avddDNA upper tertile, eight subjects were in the middle tertile, and six were in the low tertile. Subjects with grade 3 PGD in the upper tertile were at higher risk of allograft failure (5/7 or 71·4%) than those in the middle (3/8 or 37·5%) and low tertiles (1/6 or 16·6%).

3.6. %ddcfDNA trends beyond three months after transplantation

To further investigate our hypothesis that early unresolving allograft injury sets the stage for subsequent allograft injury, we studied % ddcfDNA levels beyond the early post-transplantation period from 3 to 15 months. Subjects in the low tertile for avddDNA showed continuous stable %ddcfDNA levels until 12 months after transplantation, after which we observed a slow secondary increase (Fig. 4a). By contrast, subjects in the upper avddDNA tertile showed persistently higher % ddcfDNA levels compared to other tertiles. Their %ddcfDNA levels nadir at six months post-transplantation and thereafter, the median % ddcfDNA increased for the remainder of the study period.

3.7. Clinical manifestations of elevated ddcfDNA episodes

We next investigated whether the episodes of elevated %ddcfDNA levels (%ddcfDNA ≥1%) manifested clinically. We matched elevated % ddcfDNA episodes to clinically-diagnosed episodes and found that two-thirds of elevated %ddcfDNA episodes (n = 138) were clinically silent (Fig. 4b) and only coincided with a third of the elevated %ddcfDNA

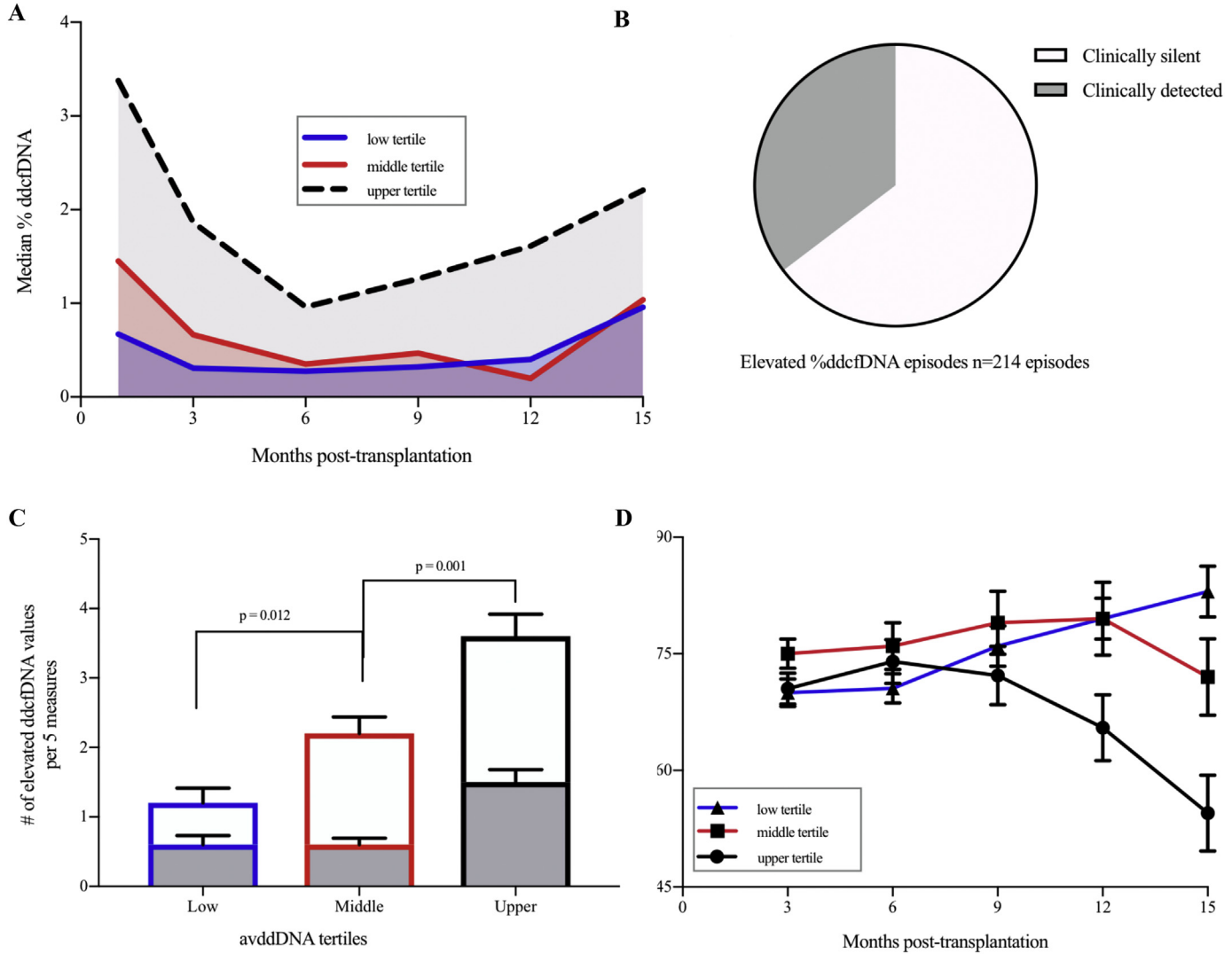


Fig. 4. Molecular (%ddcfDNA) and clinical (FEV1) measurements of allograft injury beyond the early post-transplantation period (a) Median %ddcfDNA trends for the upper-, middle-, and low tertiles of avddDNA up to 18 months post- transplantation (b) Episodes of elevated %ddcfDNA (defined as 1% %ddcfDNA) were time-matched to clinical events of acute rejection (AMR orACR) or clinical infections for each avddDNA tertile. Those which coincided with a clinical event were classified as "clinically-detected" (grey filled), and those episodes that did not coincide with a clinical event were classified as "clinically-silent" (no fill). (c) The number of clinically-detected and clinically-silent episodes per five %ddcfDNA measurements is shown (average for each tertile). Clinically-detected (grey filled) and clinically-silent (no filled) elevated %ddcfDNA episodes are represented separately. Error bars represent standard error. P-values were determined using Student t-test. (d) Average FEV1 over time for each avddDNA tertile. Error Bars represent standard error.

episodes ACR (n = 21), AMR (n = 22), clinical infection (n = 33). The remaining clinically-silent and clinically apparent %ddcfDNA episodes were predominant in subjects in the avddDNA upper tertile compared to the other two tertiles (Fig. 4c). Of the 33 clinical infection episodes with elevated %ddcfDNA, respiratory viruses were more commonly detected (n = 19) than bacterial (n = 8) or fungal infections (n = 6).

We also consider microbial colonizers as potential triggers for clinically-silent %ddcfDNA elevations. 46·2% of the cohort harbored an organism classified as a colonizer. %ddcfDNA at time points with colonizers were similar to non-rejection control time-points. However, when CLAD-associated colonizers (pseudomonas, staphylococcus) were considered separately, we observed higher %ddcfDNA levels than non-rejection time-points(1·1% vs. 0·3%, p = 0·056).

3.8. Relationship of avddDNA level to lung function

We analyzed 3899 spirograms to study lung function over time for the upper, middle, and low avddDNA tertiles. Subjects in the upper tertile had a median peak FEV1 of 74% of predicted values, occurring on average, six months after transplantation. Thereafter, the median FEV1 of this tertile showed a sustained decline, ultimately leading to CLAD. Over the same time period, the FEV1 of subjects in the middle and low avddDNA tertiles continued to increase: to 80% at 12 months in the middle tertiles, and 84% at 18 months in the low tertiles (Fig. 4d).

4. Discussion

In this proof-of-concept study, we used a novel genomic-derived biomarker, %ddcfDNA, to characterize post-transplantation trends of lung-allograft injury that lead to allograft failure and death. Our results demonstrate that the average %ddcfDNA in the early post-transplantation period (avddDNA) correlated with the development of allograft failure and all-cause mortality (Fig. 3).

Adoption of any new predictive biomarker requires an evaluation of its correlation with and benefit compared to existing clinical measures. We found that traditional risk factors of poor outcomes including older recipients, prior smoking history, CMV D^+R^- status, severe PGD, number of rejection episodes and other variables (Table 1) were more common in subjects in the upper than lower avddDNA tertiles. We also correlated our genomic markers to spirometry, a clinical measure of allograft function. We found that subjects with high avddDNA levels subsequently showed lower lung function and more clinical complications (acute rejection or infections) than subjects with lower avddDNA levels. At a molecular level, these subjects show more frequent elevated % ddcfDNA levels beyond the early post-transplantation period, suggesting that early unresolving allograft injury sets the stage for further allograft injury and dysfunction. Only one-third of these elevated %ddcfDNA episodes were associated with acute rejection or clinical infection. The remainder were not coincident to any signs detectable by histopathology, spirometry, clinical examination or by any other clinical tests. These episodes of clinically-silent elevations in %ddcfDNA could represent early detection of injury that progresses to pathologically overt changes. Our recent analysis of subjects with AMR supports this hypothesis; we observed a sustained rise in %ddcfDNA for several weeks to months before clinical or histological manifestations of AMR became apparent [23]. Similarly, in the current study, subjects with avddDNA in the upper tertile showed an early and lower peak FEV1 levels, (Fig. 4) and a greater risk of progression to allograft failure. Thus, these clinically silent events detected by %ddcfDNA and not by previously described clinical tools could offer earlier detection of pathological changes. This molecular assay therefore offers earlier time points to potentially intervene with treatments like extracorporeal phototherapy and others to reduce or prevent subsequent CLAD and allograft failure. Potential biological triggers of these clinically-silent increases in %ddcfDNA may include alloantibodies [23], acid reflux [24], and occult infection; these warrant further investigation.

The current study indicates that we could stratify subjects into tertiles of risk, even subjects with primary Graft Dysfunction (PGD) [22], the most studied early risk indicator of allograft failure. PGD has the advantage of assigning risk allograft risk within 72 h of transplantation, as opposed to avddDNA, which is computed over three months. However, use of avddDNA enabled better stratification of the PGD subjects for the risk of allograft failure than use of PGD alone. We acknowledge that the lack of correlation may be due to the small sample size of our study in relation to prior studies [5]. Further, our study evaluated PGD on Day 3 and not Day 1 or 2, and therefore potentially underestimated the incidence of PGD. The interaction between PGD and avddDNA deserves careful analysis in a larger sample size. With more powerful computing now available, we anticipate that future studies will use machine learning tools to derive equally predictive avddDNA with less time lag.

In our patient cohort, respiratory failure was the predominant cause of death. The strong correlation observed between avddDNA and our secondary outcome of all-cause mortality therefore adds support to our central hypothesis that early and unresolving allograft injury is an early indicator of allograft failure. The relationship between avddDNA and all-cause mortality in other cohorts where respiratory failure is a less predominant cause of death [25] deserves further evaluation. However, the relationship we observed between avddDNA and allograft failure is likely to be similar even in the latter cohort.

Limitations of our study include the inability to assess CLAD in subjects who were too unwell to undergo spirometry assessments. However, these subjects rapidly progressed to death and their adverse outcome was therefore captured in our composite endpoint. Also, we observed lower survival in our cohort compared to some large registries [25]. The low survival was primarily in GTD cohort due to a high incidence of CLAD secondary to antibody-mediated rejection. However, avddDNA was predictive in the GRAfT and GTD cohorts with different mortality rates suggesting the high mortality is not a limitation to generalizability of our findings. Further, variability in number of samples analyzed per patient may introduce bias in computing avddDNA. The variable number of samples per patient was due primarily to differences in the number clinically-indicated bronchoscopies and therefore reflect usual post-transplant care rather than sampling bias. Nonetheless, such difference may introduce analytic bias. To reduce potential bias, we selected the AUC method to compute avddDNA. This approach normalizes for number of sample analyzed and fixed the interval of interest to 3 months for all patients. Further, only subjects with at least 3 samples analyzed within the interval of interest were included.

Future studies should address these limitations, validate our findings, and concurrently employ machine learning algorithm to compute avddDNA measurement with less time lag than the three months used in this study. Such future study would require a larger cohort size and more frequent %ddcfDNA measurements. The earlier measures may enable assessments of the risk of allograft failure in the initial 3 months after transplantation. This risk was not assessed in our analysis where subjects who died within 3 months of transplantation were excluded from the study. If validated, avddDNA would enable the stratification subjects at risk of allograft failure in routine clinical practice. Routine clinical use of the %ddcfDNA test is highly feasible since the test is reproducible and transferrable [13]. Further, commercial versions of the test will soon be available for clinical use. Using this test to identify high-risk subjects early could allow tailored monitoring, manipulation of their immunosuppression regimens or other therapies in the hope of delaying or even avoiding allograft failure.

In summary, from our proof-of-concept study, we propose avddDNA as a predictive marker for allograft failure and premature death. To our knowledge, this is the first report of a method to detect and quantify clinically silent, but likely pathological, events preceding allograft

failure. These underlying events, uncovered through our analysis of % ddcfDNA, may help to explain the unacceptably high rate of CLAD that remains the Achilles heel of lung transplantation. Our findings require further validation in larger cohorts, but nevertheless lay the foundation for future clinical studies. Mechanistic studies will also be needed, to investigate the pathological importance of clinically-silent allograft injury. Future studies may also evaluate our hypothesis in other solid organs transplantation, in which assessments of %ddcfDNA have shown similar performance characteristics for detecting acute complications [13,15–17].

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Author's contributions

SA-E, KKK and HA-E conceived the study and designed analyses. UF, IT, PDS, HL, SDN, JBO, AWB, AI recruited and monitored patients. IT, PDS, SDN, JBO, AWB, KP, AI adjudicated for outcomes. GJB and CM performed consensus histopathology reads. AD, JW, HL, UF, NG, DG, AM, KB collected biological samples and clinical data and prepared data tables. KKK, SRQ, IDV measured ddcfDNA for GTD. SG, MK, JZ, IT measured ddcfDNA for GRAfT. SA-E and YW performed statistical analysis and compile results. SA-E wrote initial manuscript draft, all authors reviewed the manuscripts and revisions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2018.12.029.

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