Received: 31 August 2018 | Revised: 1 February 2019 | Accepted: 24 February 2019

DOI: 10.1111/ajt.15339

ORIGINAL ARTICLE

AJT

Noninvasive detection of graft injury after heart transplant using donor-derived cell-free DNA: A prospective multicenter study

Kiran K. Khush[1] | Jignesh Patel[2] | Sean Pinney[3] | Andrew Kao[4] | Rami Alharethi[5] | Eugene DePasquale[6] | Gregory Ewald[7] | Peter Berman[8] | Manreet Kanwar[9] | David Hiller[10] | James P. Yee[11] | Robert N. Woodward[10] | Shelley Hall[12] | Jon Kobashigawa[2]

[1]Division of Cardiovascular Medicine, Stanford University, Stanford, California

[2]Cedars-Sinai Smidt Heart Institute, Los Angeles, California

[3]Mount Sinai Medical Center, New York, New York

[4]St. Luke's Hospital Mid America Heart Institute, Kansas City, Missouri

[5]Intermountain Healthcare, Salt Lake City, Utah

[6]University of California, Los Angeles, California

[7]Washington University School of Medicine, Saint Louis, Missouri

[8]Tampa General Hospital, Tampa, Florida

[9]Allegheny General Hospital, Pittsburgh, Pennsylvania

[10]Research and Development, CareDx, Brisbane, California

[11]Clinical Research, CareDx, Brisbane, California

[12]Baylor University Medical Center, Dallas, Texas

Correspondence
Kiran K. Khush
Email: kiran@stanford.edu

Funding information
CareDx

Standardized donor-derived cell-free DNA (dd-cfDNA) testing has been introduced into clinical use to monitor kidney transplant recipients for rejection. This report describes the performance of this dd-cfDNA assay to detect allograft rejection in samples from heart transplant (HT) recipients undergoing surveillance monitoring across the United States. Venous blood was longitudinally sampled from 740 HT recipients from 26 centers and in a single-center cohort of 33 patients at high risk for antibody-mediated rejection (AMR). Plasma dd-cfDNA was quantified by using targeted amplification and sequencing of a single nucleotide polymorphism panel. The dd-cfDNA levels were correlated to paired events of biopsy-based diagnosis of rejection. The median dd-cfDNA was 0.07% in reference HT recipients (2164 samples) and 0.17% in samples classified as acute rejection (35 samples; $P = .005$). At a 0.2% threshold,

Abbreviations: ACR, acute cellular rejection; AMR, antibody-mediated rejection; AR, acute rejection; AUC, area under the curve; cfDNA, cell-free DNA; CLIA, Clinical Laboratories Improvements Act; dd-cfDNA, donor-derived cell-free DNA; D-OAR, Donor-Derived Cell-Free DNA-Outcomes AlloMap Registry; LVEF, left ventricular ejection fraction; NPV, negative predictive value; NR, no rejection; OPTN, Organ Procurement and Transplantation Network; PPV, positive predictive value.

dd-cfDNA had a 44% sensitivity to detect rejection and a 97% negative predictive value. In the cohort at risk for AMR (11 samples), dd-cfDNA levels were elevated 3-fold in AMR compared with patients without AMR (99 samples, $P = .004$). The standardized dd-cfDNA test identified acute rejection in samples from a broad population of HT recipients. The reported test performance characteristics will guide the next stage of clinical utility studies of the dd-cfDNA assay.

KEYWORDS
biomarker, clinical research/practice, heart (allograft) function/dysfunction, heart transplantation/cardiology

1 | INTRODUCTION

Acute rejection (AR), including acute cellular rejection (ACR) and antibody-mediated rejection (AMR), continues to be a complication after heart transplant (HT).[1] AR is a major cause of hospitalization and graft dysfunction during the early years post HT. However, symptoms may develop late in the process of graft damage, which underscores the need for routine rejection surveillance. Endomyocardial biopsy remains the primary means for rejection monitoring, but it is associated with patient discomfort, expense, and potentially serious procedural complications.[2-4] Noninvasive peripheral gene expression testing via the AlloMap assay (CareDx, Inc., Brisbane, CA) has been widely adopted for AR surveillance by HT centers in the United States. This gene expression assay can provide a high (>99%) negative predictive value[5] and has proved to be useful for ruling out ACR. However, the gene expression profiling test has a limited positive predictive value (PPV) for cellular rejection, and the assay was not designed to detect AMR. For these reasons, the development of a more comprehensive, noninvasive assay for the surveillance of AR (both ACR and AMR) remains of great interest and clinical importance.[6]

Donor-derived cell-free DNA (dd-cfDNA), detected in the blood of transplant recipients, has been proposed as a noninvasive marker of graft injury, which can be caused by ACR as well as acute AMR. Early dd-cfDNA studies were based on the hypothesis that AR causes cell death in the allograft, which leads to increased levels of dd-cfDNA in the recipient's bloodstream.

Data from single-center studies have shown that elevated dd-cfDNA levels can detect AR after HT, and an increase in levels can occur before rejection is detected on endomyocardial biopsy.[7-9] Shotgun whole-genome sequencing has been used to detect and quantify dd-cfDNA, but the complexity and cost of the analyses limit its application as a clinically relevant surveillance tool. Targeted quantification of dd-cfDNA provides a more rapid and cost-effective surveillance strategy, but also requires genotyping of the transplant donor, which can be impractical.

More recently, a targeted amplification, next-generation sequencing assay (AlloSure®; CareDx, Inc.) has been analytically and clinically validated to quantify the percentage of dd-cfDNA in transplant recipients' blood without the need for donor or recipient genotyping.[10] This assay was shown to detect ACR in HT in a multicenter retrospective case-control study[10] and to detect active rejection in kidney transplant in a prospective, multicenter trial.[11]

This study is the first to report a large, prospective, multicenter clinical validation of the ability of a standardized dd-cfDNA assay to detect AR in HT recipients.

We conducted the Donor-Derived Cell-Free DNA-Outcomes AlloMap Registry (D-OAR) study to examine the characteristics of dd-cfDNA in a routine, clinical surveillance setting with HT recipients at 26 centers across the United States. The objective of D-OAR was to determine the test performance of dd-cfDNA for the detection of ACR, AMR, and graft dysfunction.

2 | METHODS

2.1 | D-OAR study design

The D-OAR (NCT02178943) is an observational, prospective, multicenter registry that aimed to evaluate clinical outcomes in HT recipients who were receiving regular allograft rejection surveillance.[12] The primary objective was to determine whether the dd-cfDNA level in an HT recipient's blood can differentiate rejection from the absence of rejection, as determined by endomyocardial biopsy interpretation. Secondary objectives were to determine whether graft dysfunction in the absence of rejection is associated with increased dd-cfDNA levels and to characterize dd-cfDNA levels in stable patients who have no evidence of AR.

Eligible study subjects were HT recipients ≥ 15 years and > 55 days posttransplant who were undergoing AlloMap gene expression profiling for rejection surveillance. Multiorgan transplant recipients were excluded.

Pretransplant and serial data after transplant, including clinical status, hospitalizations, diagnostic tests (including endomyocardial biopsies, AlloMap scores, and echocardiograms), immunosuppressive maintenance therapy and drug levels, and posttransplant adverse events, were collected. The D-OAR study included 26 HT centers in the United States.[12] The primary study

outcomes were AR (ACR and AMR) and graft dysfunction, as defined here later.

Between July 2014 and September 2016, blood specimens were collected for quantification of dd-cfDNA levels at each surveillance visit that occurred for AlloMap testing. The regular surveillance schedule for testing was determined by each participating center's standard of care. Following a protocol amendment in September 2016 (through 2018), the dd-cfDNA specimen was drawn only when a patient had a clinical suspicion of rejection and a planned "for-cause" biopsy. Surveillance biopsies and biopsies performed for cause were analyzed separately in addition to the combined analysis, to determine whether the 2 clinical settings render differing relationships with regard to cell-free DNA. Two follow-up dd-cfDNA specimens were collected within 8 weeks after "for-cause" biopsies in patients who were treated for rejection and/or graft dysfunction.

2.2 | Cedars-Sinai study

A parallel single-center study was conducted at Cedars-Sinai Medical Center from March 2016 to May 2017. HT recipients identified as high risk for the development of AMR were enrolled and followed longitudinally. The inclusion criteria were pretransplant PRA ≥ 10%, presence of donor-specific antibodies at the time of transplant, any posttransplant detection of DSA or biopsy-proved AMR, or "for-cause" biopsy due to reduced left ventricular ejection fraction (LVEF). Samples were collected beginning 14 days posttransplant from patients 18 years or older. This cohort included 110 samples from 33 patients and information on dd-cfDNA and biopsy grades were collected for ACR and AMR. The objective of this study was to correlate dd-cfDNA levels in patients with pAMR ≥ 1 in this independent sample set.

2.3 | Blood samples and dd-cfDNA measurements

Venous blood was collected in Streck Cell-Free DNA BCT tubes before the performance of endomyocardial biopsies and was shipped to the central Clinical Laboratories Improvements Act–certified laboratory at CareDx, Inc. Details of the standardized specimen processing and analytical methods to determine the percentage of dd-cfDNA (AlloSure®) have been published.[10] The targeted next-generation sequencing assay uses highly polymorphic single nucleotide polymorphisms to quantify dd-cfDNA without the need for separate genotyping of the recipient or the donor.[10] All measurements were performed by laboratory technicians unaware of the clinical identity of the samples.

2.4 | Diagnosis of graft dysfunction and of biopsy-defined rejection

Information was collected on the number of, and clinical indication for, endomyocardial biopsies for each patient. Biopsies were graded according to the International Society for Heart and Lung Transplantation revised classification scheme for ACR[13] and pathologic diagnosis of AMR.[14] Biopsy interpretation was performed by the pathologist at the participating transplant center.

The AR group was defined as transplant recipients whose blood samples indicated either ACR (grade 2R or 3R), AMR (pAMR grade 1, 2, or 3), or mixed rejection (satisfying the requirements of both ACR and AMR). For analysis by type of rejection, mixed rejections were pooled with AMR. The no rejection (NR) group was defined as recipients whose samples had no biopsy evidence of AR (ACR grade 0R or 1R and AMR grade pAMR0). The graft dysfunction group was defined as recipients whose blood samples had no biopsy evidence of AR and who had ≥ 1 of the following: LVEF < 40% or a decrease of ≥ 25% from the prior visit.

2.5 | Reference population

The reference population was defined as all D-OAR patients with samples that did not indicate ACR (grade 2R or 3R), AMR (pAMR grade 1, 2, or 3), or mixed rejection during the course of the study. For sensitivity, we also define a restricted population in which patients with samples with any ACR grade 1R as well as dd-cfDNA samples not paired with biopsy were excluded (146 patients [214 samples]).

2.6 | Statistical analyses

The primary objective of the statistical analysis was to determine whether the dd-cfDNA level in an HT recipient's blood can differentiate AR from NR, as determined by local pathologists' endomyocardial biopsy diagnostic classification. Surveillance biopsies and biopsies performed for cause were analyzed separately, to determine whether the 2 clinical settings render differing relationships with regard to dd-cfDNA testing. The blood was collected for dd-cfDNA assays within 3 days before endomyocardial biopsy. Wilcoxon rank sum testing was used to compare dd-cfDNA values associated with AR with dd-cfDNA values associated with NR. Further comparisons were performed of ACR vs no ACR in patients without AMR, and of AMR vs no AMR. Performance characteristics of dd-cfDNA to diagnose AR were computed, including sensitivity, specificity, PPV, and NPV.

Additional analyses assessed the correlation of dd-cfDNA to graft dysfunction in the absence of AR. The graft dysfunction group and the no–graft dysfunction group were compared by using the Wilcoxon rank sum test. An additional objective was to characterize the reported values of dd-cfDNA in the reference population, including median levels and normal ranges.

Power calculations were performed as follows. An effect size of 0.83 was estimated from a prior HT study of dd-cfDNA in reference cases of biopsy-based AR compared with control cases with NR.[10] We assumed that the [mean log(dd-cfDNA) AR – [mean log(dd-cfDNA) NR]/(standard deviation of log (dd-cfDNA)] = 0.83. Accordingly, there is a 90% power to demonstrate a significant

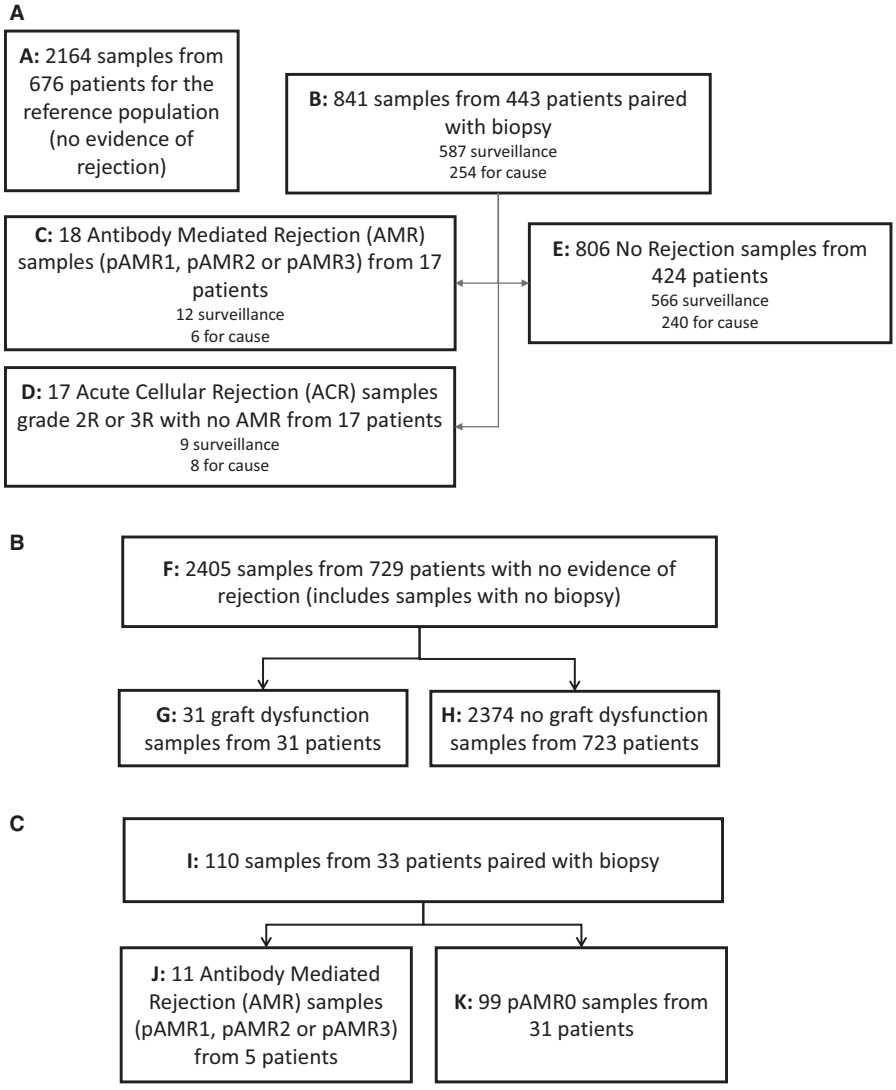

FIGURE 1 CONSORT diagrams for Donor-Derived Cell-Free DNA-Outcomes AlloMap Registry (D-OAR). A, Reference population (Box A) includes all 2164 D-OAR samples from patients with no clinical signs or symptoms of rejection and no biopsy-based evidence of rejection (no rejection [NR] = no acute cellular rejection [ACR] grade ≥ 2R or antibody-mediated rejection [AMR] grade ≥ pAMR1). Eight hundred forty-one samples (Box B) had biopsy paired with donor-derived cell-free DNA (dd-cfDNA) results, of which 18 had AMR, including 2 mixed rejections (Box C), 17 had ACR (Box D), and 806 had NR (ACR grade 0R or 1R and AMR grade pAMR0, Box E). B, The 2405 D-OAR samples that did not have a rejection diagnosis (Box F) include 31 graft dysfunction samples (left ventricular ejection fraction [LVEF] < 40 or drop in LVEF of ≥ 25% from previous visits, Box G) and 2374 no graft dysfunction samples (Box H). C, The 110 samples from Cedars-Sinai patients (Box I) include 11 associated with biopsy evidence of AMR (Box J) and 99 with NR (Box K)

difference in dd-cfDNA in 17 cases of AR compared with dd-cfDNA levels in 323 NR cases, from a total of 340 visits, assuming ACR and/or AMR would be discovered at 5% of biopsy surveillance visits.

3 | RESULTS

3.1 | D-OAR patients

From September 2014 to October 2017, 740 HT recipients were enrolled at 26 clinical sites (Figure 1A; Table S1, participating sites) from which 2447 plasma dd-cfDNA level samples were drawn. Blood samples were drawn for dd-cfDNA quantification from 55 days to >5 years posttransplant. Most (81%) samples were drawn within the first year posttransplant, and 13% were drawn during the second year (Figure S1). Eight hundred forty-one dd-cfDNA results were paired with a biopsy, of which 587 biopsies were performed for routine rejection surveillance and 254 biopsies were "for cause" based on clinical suspicion (Table 1, Figure 1A).

3.2 | Reference population

The reference population (Table 1, Figure 1A, Box A) was composed of 676 patients who contributed 2164 samples and who had no clinical signs or symptoms of AR during the course of the study. The mean age at enrollment was 54 years, 73% were > 50 years old, 75% were male, 70% were white, and the most common indications for transplant were dilated cardiomyopathy (49%) and ischemic cardiomyopathy (32%). Patients were enrolled at a median of 170 days posttransplant (IQR 116-249 days), and 3 samples were drawn, on average, per patient. This reference population is demographically similar to, but slightly older, than HT patients included in the Organ Procurement and Transplantation Network data,[15] who are 74% male and 73% white, and 57% were > 50 years old at time of transplant.

The median dd-cfDNA level in this reference population was 0.07% (IQR 0.03%-0.14%), as shown in Figure 2A. The 97.5th percentile was 1.29%. Sensitivity analysis on the restricted population (all samples from patients with any ACR grade 1R, as well as dd-cfDNA samples not paired with biopsy, were excluded)

TABLE 1 Demographic and clinical characteristics of the reference population

Variable	Reference	All biopsies	NR	AR	*P* (AR vs NR)
No. of samples	2164	841	806	35	
No. of patients	676	443	409	34	
Samples per patient	3.2	1.9	2.0	1.0	
Pretransplant diagnosis					.201
Congenital	18 (3%)	13 (3%)	11 (3%)	2 (6%)	
Ischemic cardiomyopathy	216 (32%)	139 (31%)	125 (31%)	14 (41%)	
Multiple	14 (2%)	10 (2%)	10 (2%)	0 (0%)	
Nonischemic cardiomyopathy	329 (49%)	221 (50%)	209 (51%)	12 (35%)	
Other	92 (14%)	55 (12%)	50 (12%)	5 (15%)	
Retransplant	7 (1%)	5 (1%)	4 (1%)	1 (3%)	
Race					.605
Asian	20 (3%)	9 (2%)	8 (2%)	1 (3%)	
Black	112 (17%)	59 (13%)	54 (13%)	5 (15%)	
White	474 (70%)	326 (74%)	303 (74%)	23 (68%)	
Hispanic	50 (7%)	34 (8%)	31 (8%)	3 (9%)	
Other	20 (3%)	15 (3%)	13 (3%)	2 (6%)	
Male sex	505 (75%)	330 (74%)	305 (75%)	25 (74%)	.841
Cytomegalovirus serologic status					.523
D^-:R^-	113 (17%)	70 (16%)	66 (16%)	4 (12%)	
D^-:R^+	127 (19%)	76 (17%)	70 (17%)	6 (18%)	
D^+:R^-	171 (25%)	122 (28%)	115 (28%)	7 (21%)	
D^+:R^+	235 (35%)	147 (33%)	131 (32%)	16 (47%)	
Unknown	30 (4%)	28 (6%)	27 (7%)	1 (3%)	
Mechanical support					.381
None	326 (48%)	204 (46%)	184 (45%)	20 (59%)	
Left ventricular assist device	297 (44%)	201 (45%)	189 (46%)	12 (35%)	
Temporary circulatory support	42 (6%)	37 (8%)	35 (9%)	2 (6%)	
Total artificial heart	11 (2%)	1 (0%)	1 (0%)	0 (0%)	
Age at enrollment, y	54 ± 13	54 ± 12	54 ± 12	55 ± 13	.721
LVEF at enrollment, %	59 ± 9	59 ± 9	59 ± 9	61 ± 7	.393
Days posttransplant at enrollment	292 ± 537	299 ± 391	280 ± 311	523 ± 892	0<0.001
Height, cm	174.6 ± 9.9	175 ± 9.6	174.9 ± 9.7	176.2 ± 9.2	.495
Weight, kg	84.9 ± 18.3	86.7 ± 18.8	86.4 ± 18.4	90.7 ± 23.5	.221

AR, acute rejection; NR, no rejection; LVEF, left ventricular ejection fraction.

showed similar characteristics: the median dd-cfDNA level was 0.07% (IQR 0.03%-0.12%). There was no statistical difference between the assessment of the reference population and the restricted population (P = .925, Kolmogorov–Smirnov test). The levels of dd-cfDNA in HT recipients' blood remain very low and stable during the first 2 years posttransplant, in the absence of AR (Figure 2B, P = .182). The median dd-cfDNA intrapatient variability (CV_I) is 70%, and the interpatient coefficient of variation (CV_G) of patient median values is 86%, based on 350 patients from the reference population who had at least 3 test results per patient.

3.3 | Clinical events

3.3.1 | Rejection

Of the total of 841 endomyocardial biopsies performed in study subjects and paired with dd-cfDNA, there were 17 biopsy-proved ACRs (grade 2R or 3R, no AMR), 18 AMRs (grade pAMR1 or pAMR2 including 2 mixed rejections), and 806 NR samples (Figure 1A, Boxes B-E). The greatest number of biopsies (384) and the most AR cases (14) occurred within the first 6 months post-transplant. Ten ARs were diagnosed in 279 biopsies performed

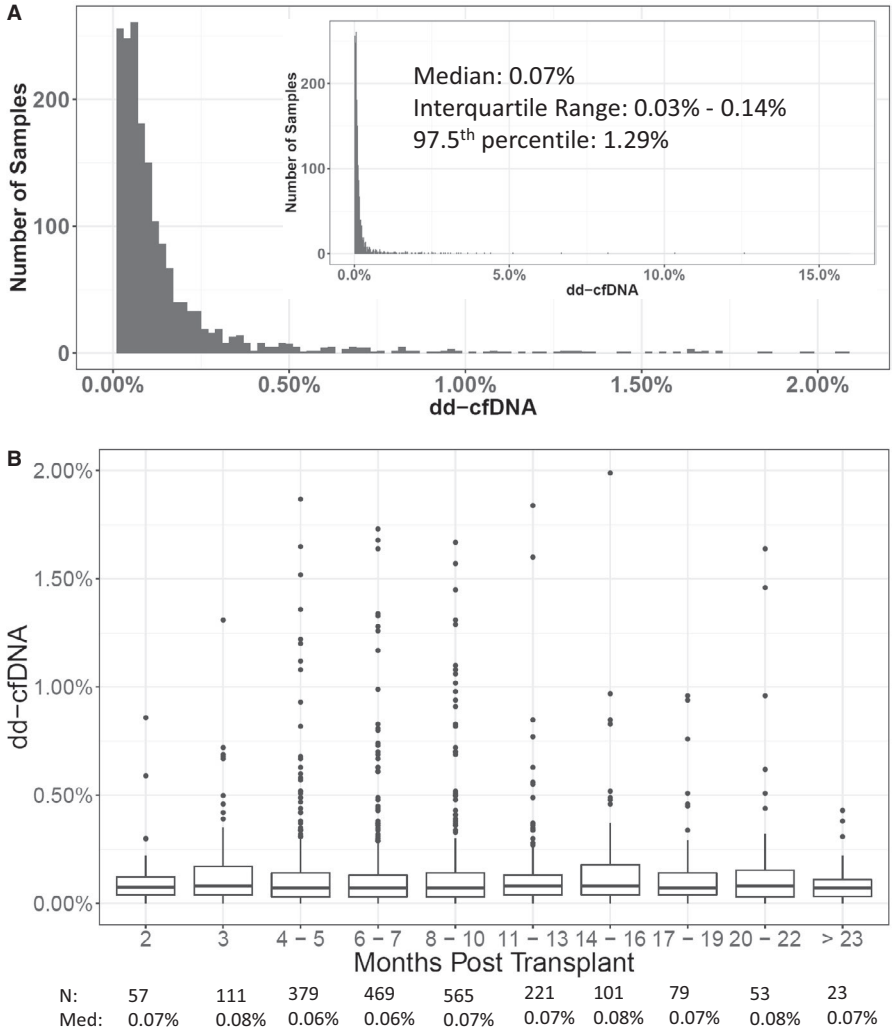

FIGURE 2 The donor-derived cell-free DNA (dd-cfDNA) levels in heart transplant reference population. A, Analysis includes 2164 Donor-Derived Cell-Free DNA-Outcomes AlloMap Registry samples. The median dd-cfDNA level was 0.07% (IQR, 0.03%-0.14%). The 97.5th percentile was 1.29%. B, The dd-cfDNA levels by time posttransplant. Levels of dd-cfDNA in the reference population are stable from 55 days to 2 years posttransplant (P = .182)

during months 6 through 12, 4 ARs occurred in 122 biopsies during year 2, and 4 additional ARs occurred in 51 biopsies during years 3 to 5 posttransplant. Three additional AR cases were diagnosed in 5 biopsies after 5 years posttransplant.

The dd-cfDNA levels differed significantly between patients with and without AR (Figure 3A). The median level of dd-cfDNA in patients with AR was significantly higher (0.17%) than in the group of patient specimens without rejection (0.07%, P < .001). Median dd-cfDNA levels were 0.17% for both ACR and AMR.

ACR grade 1R had a similar median level (0.08%) to grade 0 biopsies (0.07%), whereas ACR 2R (moderate) had a median dd-cfDNA level of 0.15%, and ACR 3R (severe) had a median dd-cfDNA level of 0.30% (Figure 3B). dd-cfDNA levels differentiated ACR grade ≥ 2R (P = .004) from NR. Biopsies graded as pAMR0 had median dd-cfDNA levels of 0.07%, whereas the median was 0.12% in pAMR1 and 0.25% in pAMR2 (Figure 3C).

The fractions of true- and false-positive results for dd-cfDNA to detect AR are shown in Figure S2. The area under the curve (AUC) was 0.64 (95% confidence interval 0.52 to 0.75). With a cutoff of 0.2%, the dd-cfDNA assay had 80% specificity and 44% sensitivity to differentiate AR from NR. The PPV was 8.9%, and the NPV was 97.1%. When examined by type of rejection, the PPV for ACR detection was 4.8% with an NPV of 98.6%, whereas for AMR, the PPV was 4.2% and the NPV was 98.6%.

3.4 | Surveillance biopsy analysis

Of the 587 surveillance group samples, there were 21 ARs, including 9 ACRs and 12 AMRs. Each of these cases was from a unique patient. The median level of dd-cfDNA in surveillance samples paired with rejection was 0.15% (IQR 0.04%-0.23%) and 0.07% in samples paired with NR (IQR 0.02%-0.14%). The sample size was too small to detect a statistically significant difference in median dd-cfDNA levels (P = .140, Figure 3D). Sensitivity was 38.1% (21.4%-54.5%), specificity was 84.0% (81.2-86.7%), PPV was 8.1% (4.7%-11.9%), NPV was 97.3% (96.7%-98.0%), and AUC was 60.5% (46.0%-74.2%) for identifying rejection.

3.5 | For-cause biopsy analysis

Of the 254 "for-cause" samples, there were 14 ARs, including 8 biopsy-proved ACRs from unique patients (ACR 2R or 3R) and 6 AMRs

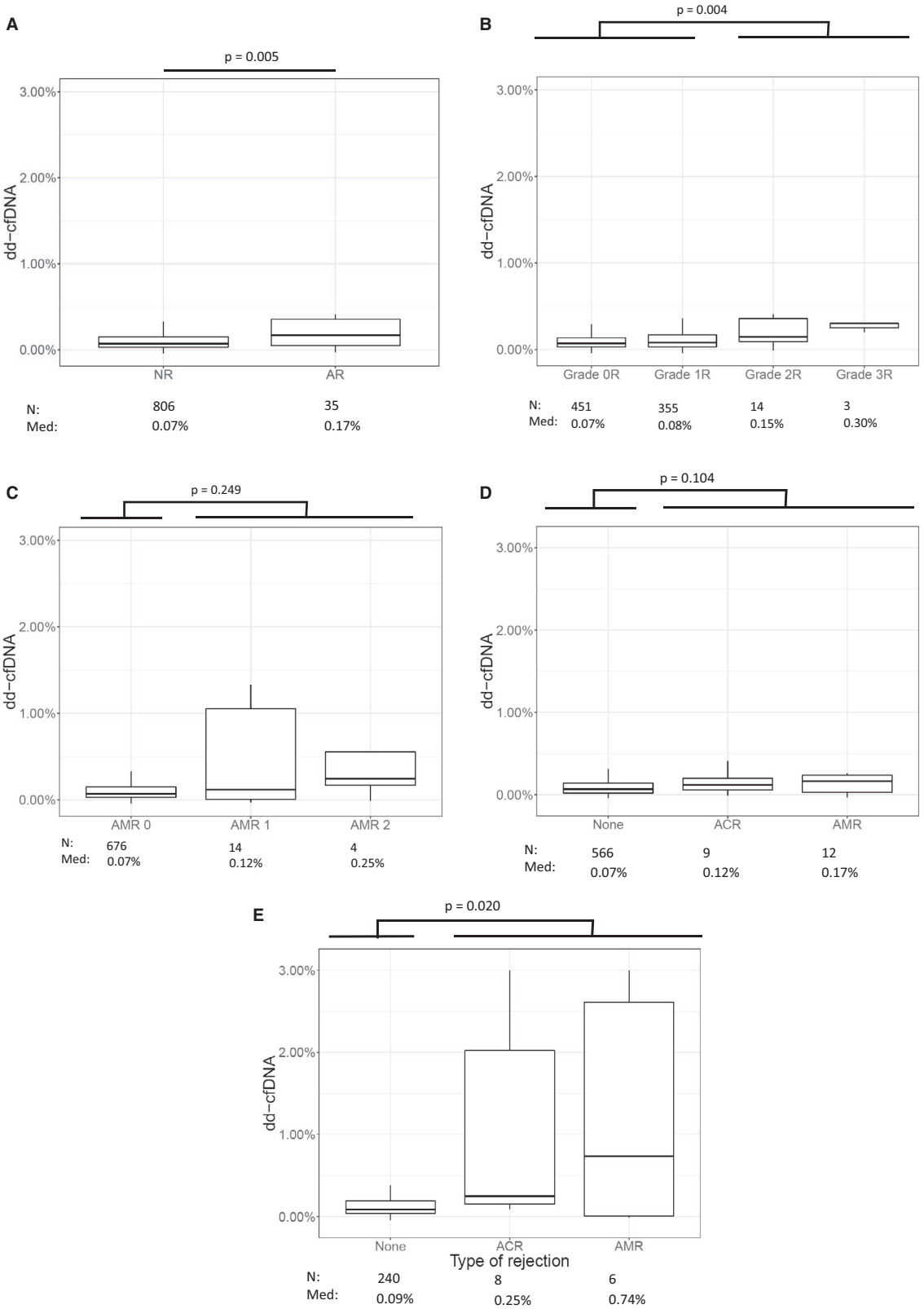

FIGURE 3 The donor-derived cell-free DNA (dd-cfDNA) level correlates with acute rejection (AR). A, Sample sizes and median dd-cfDNA levels for samples with (AR) and without (NR) AR. B, Sample sizes and median dd-cfDNA levels by ACR grade, for patients not diagnosed with AMR. C, Sample sizes and median dd-cfDNA levels by AMR grade, including patients with mixed rejection. D, Sample sizes and median dd-cfDNA levels for samples associated with surveillance biopsy with NR, ACR grade ≥ 2R, and AMR grade ≥ pAMR1 (including mixed rejection). E, Sample sizes and median dd-cfDNA levels for samples associated with for-cause biopsy with NR, ACR grade ≥ 2R, and AMR grade ≥ pAMR1 (including mixed rejection)

(pAMR1 or pAMR2). The median level of dd-cfDNA in for-cause samples paired with rejection was 0.25% (IQR 0.10%-0.31%) and 0.09% in samples paired with NR (IQR 0.04%-0.19%). Statistically different median dd-cfDNA levels were observed in patients with and without rejection (Figure 3E, $P = .02$). Sensitivity was 53.8% (33.3-75.1%), specificity was 76.1% (71.6%-80.2%), PPV was 11.6% (7.6%-16.6%), NPV was 96.6% (95.2%-98.2%), and AUC was 68.5% (48.0%-86.4%) for identifying rejection.

3.6 | Graft dysfunction

Thirty-one graft dysfunction events paired with dd-cfDNA occurred in 31 unique study subjects (Figure 1B, Box F-H). The dd-cfDNA levels (median 0.07%) were not significantly different from the median levels in the reference population. However, when the %dd-cfDNA is plotted against either LVEF or LVEF change, those with lowest LVEF generally had the highest dd-cfDNA levels (Figure 4). Of those with both low LVEF and a ≥ 25% reduction of LVEF from the prior visit (red circles in Figure 4), the median dd-cfDNA value was 0.53% (n = 8, $P = .007$ compared with no AR or graft dysfunction; clinical details are given in Table S2).

3.7 | Cedars-Sinai study

Among 110 samples from 33 patients in the Cedars-Sinai study, there were 99 samples from 33 patients with pAMR0, 3 samples from 3 patients with pAMR1, and 8 samples from 3 patients with pAMR2 (Figure 1C, Box I-K). Sixty-seven percent of the patients were nonwhite, and the average age was 56 years. The patients with AMR were younger than those without AMR (47 vs 58 years). Moderate or severe ACR was not diagnosed in any of the patients: 25 patients had 53 grade 0R results and 22 patients had 57 grade 1R results. Patients with pAMR1 or pAMR2 had higher median dd-cfDNA levels (0.50%) than those with pAMR0 (0.16%) ($P = .004$) (Figure 5). The dd-cfDNA level of pAMR0 was more than double that observed for pAMR0 in D-OAR; nevertheless, dd-cfDNA could still differentiate patients with pAMR1 or pAMR2 from those without AMR with sensitivity of 88.0%, specificity of 61.5%, PPV of 20.2%, and NPV of 97.9% at a threshold of 0.2%.

4 | DISCUSSION

This report presents the results of a large, multicenter, prospective study that was designed to establish the performance characteristics of a well-validated, fully standardized dd-cfDNA assay in a broad population of HT recipients in the United States. The size and design of the study estimated that the number of AR events was sufficient to demonstrate statistically significant performance characteristics of the assay. Critically, the dd-cfDNA measurement is an analytically validated assay in a College of American Pathologists-accredited, CLIA-certified reference laboratory.[10]

In the study population of patients who had received an HT at least 55 days before enrollment, dd-cfDNA testing detected AR with an AUC of 0.64 and provided an estimated NPV of 97.1% and PPV of 8.9%. These results, from a contemporary HT patient population that includes patients with both ACR and AMR, validate prior reports of the performance characteristics of this assay in a population in which only ACR was characterized.[10] We demonstrated that dd-cfDNA levels are significantly higher in patients with AR compared with patients with no biopsy evidence of rejection. The current report is strengthened

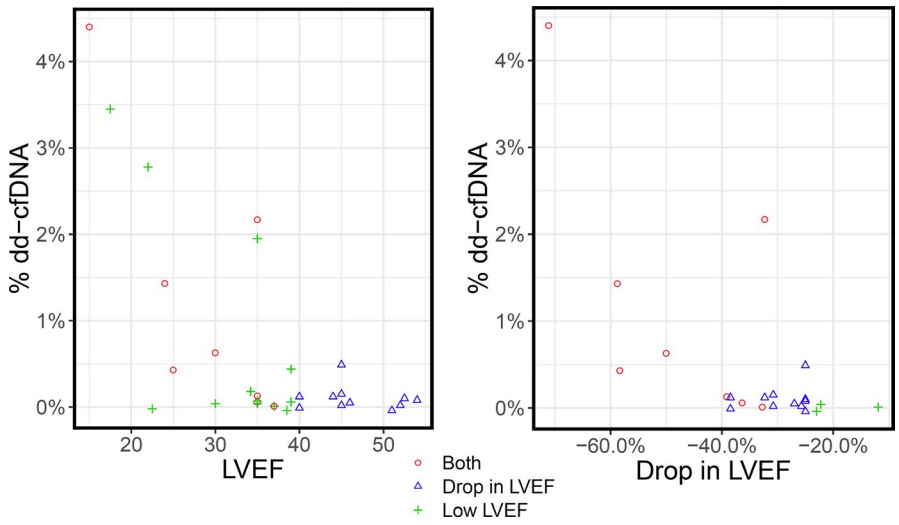

FIGURE 4 Association of donor-derived cell-free DNA (dd-cfDNA) with graft dysfunction without biopsy evidence of rejection. Thirty-one patient samples with either no biopsy or no acute rejection (grade 0 or grade 1R acute cellular rejection and pAMR0) within 3 days of the dd-cfDNA sample. Graft dysfunction was defined as left ventricular ejection fraction (LVEF) < 40% (green cross, n = 12, median 0.07%), a drop in LVEF ≥ 25% from the prior visit (blue triangle, n = 11, median 0.08%), or both (red circle, n = 8, median 0.53%). Left, LVEF. Right, drop in LVEF. One patient (without a time-matched biopsy) had dd-cfDNA of 1.4% measured 4 days before a biopsy revealed grade 3R rejection

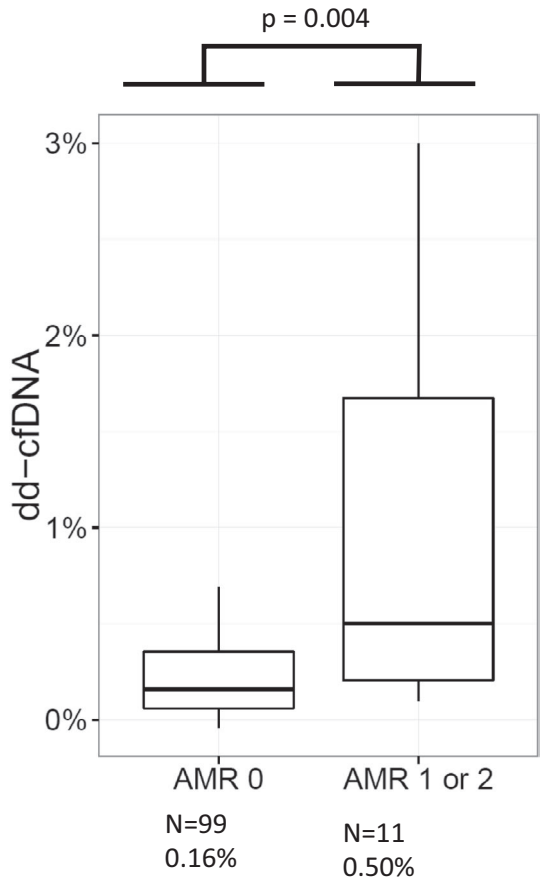

FIGURE 5 Donor-derived cell-free DNA (dd-cfDNA) correlates with antibody-mediated rejection (AMR) in the Cedars-Sinai study. Sample sizes and median dd-cfDNA levels for samples from patients with AMR grade pAMR0 and AMR grade ≥ pAMR1. Samples with pAMR1 or higher have elevated levels of dd-cfDNA

by inclusion of an independent patient set (Cedars-Sinai cohort) that confirms the ability of dd-cfDNA to detect AMR. dd-cfDNA levels are also correlated with the presence of graft dysfunction, especially in patients with a large (≥25%) drop in LVEF. These results confirm the hypothesis that cell-free DNA is released from cells within the donor organ during episodes of significant graft injury. Reassuringly, episodes of grade 1R (mild) ACR, which is usually considered clinically irrelevant, were not correlated with elevated dd-cfDNA levels. These results seem to confirm the clinical suspicion that grade 1R (mild) ACR does not result in significant graft injury. The natural progression of ACR grade 1R is not well defined, but we observed that the majority of patients did not progress to clinically overt rejection. In another recent study,[16] the composite outcome of death, retransplant, rejection with hemodynamic compromise (defined as LVEF ≤ 40% or a drop ≥ 25% compared with baseline or use of inotropic drugs or mechanical support), and nonspecific graft dysfunction (hemodynamic compromise without evidence of rejection) occurred in 103 patients during follow-up, and the occurrence of this composite endpoint at 1, 5, and 10 years was 4%, 15%, and 23%, respectively, whereas grade 1R ACR was found in 40.7% (456/1118) of biopsies performed between 2 and 6 months posttransplant.

In the reference population of stable HT recipients free of rejection, dd-cfDNA is present at very low levels (median 0.07%). This is in contrast to stable kidney transplant recipients, in whom dd-cfDNA is detected at a median of 0.21% by using the AlloSure® assay.[11] Differences in baseline dd-cfDNA levels may reflect differences in the rate of cell turnover within the allograft. This is also in contrast to the median dd-cfDNA level of 0.16% in pAMR0 patients in the Cedars-Sinai study. This difference is likely due to the difference in patient populations between the 2 cohorts; the stable patients in D-OAR were thought to be at low risk for AR and tended to have less allograft injury than the patients in the Cedars-Sinai study, who were all allosensitized patients.

Prior studies have also shown that dd-cfDNA levels may begin to rise weeks to months before AR is diagnosed on endomyocardial biopsy.[8,17] These elevated levels represent the early graft injury that occurs before myocyte damage is apparent on histology. Surveillance with dd-cfDNA may therefore detect early rejection and thereby trigger augmentation of immunosuppression to prevent a more severe rejection event that may result in irreversible graft damage. This early detection of graft injury in the setting of a negative biopsy may account for some of the false-positive results seen in this study. Similarly, the relatively low PPV of 8.9% for the detection of AR reflects the low prevalence of rejection in this clinically stable patient population. Patients were enrolled in the D-OAR study while undergoing routine AlloMap peripheral gene expression testing for rejection surveillance. In general, patients who undergo AlloMap testing tend to be "low risk" clinically—they have not had recent rejection events and are not highly allosensitized. Only 2.2% of the biopsies performed in D-OAR patients were positive for ACR and 2.1% were positive for AMR. The incidence of treated rejection in the first year posttransplant, as reported by the International Society for Heart and Lung Transplantation thoracic transplant registry, is currently 13%.[1] Early rejection events may have occurred in the first 2 months posttransplant, before patients were eligible to enroll in D-OAR.

Additionally, many patients were enrolled later than 2 months posttransplant and were tested over several years, during which time the prevalence of AR is very low. The distribution of tests was 81% in the first year and 14% in the second year (Figure S1).

Another consideration when evaluating dd-cfDNA test performance is that endomyocardial biopsy is not a true "gold standard" for the diagnosis of AR. There are many limitations of the biopsy, including sampling error and interobserver variability in biopsy interpretation. A prior study that compared expert panel (core) biopsy interpretation with locally assigned grades showed that 52% of local ≥ 2R ACRs were assigned lower grades (no significant rejection) by the panel and that overall agreement for the diagnosis of ACR between panel and local reads was only 28.4%.[18] dd-cfDNA, which directly assesses damage to the transplanted organ, may therefore be a more objective and accurate assay for graft injury than the traditional biopsy.

The dd-cfDNA noninvasive monitoring test can reduce biopsy utilization and save health care costs, as has been modeled for the AlloMap test.[19] It has been estimated that the cost-effectiveness of a blood-based biomarker compared with endomyocardial biopsy for

the diagnosis of acute allograft rejection may result in a cost saving of $27,244 and quality adjusted life year gain of 0.046 on average during the first 5 years posttransplant.[20]

The limitations of this study include (1) the change in protocol designed to increase the number of rejection events. To adjust for this change in study methods, we have analyzed the "surveillance" and "for-cause" results separately. (2) Concurrent dd-cfDNA results were available for only 58% of biopsy specimens. (3) The only analyte quantified by the assay used in this study is the fraction of dd-cfDNA in the total cfDNA in the recipient's plasma. It is possible that conditions unrelated to AR, such as increased turnover or death of recipient cells (as seen in trauma[21] and sepsis[22]), can result in elevated total cfDNA levels and thereby reduce the donor fraction. Nevertheless, dd-cfDNA levels have previously been shown by multiple independent groups to be elevated in the setting of AR after heart,[8-10] kidney,[7,11] liver,[7,23,24] and lung[25] transplant. (4) The Cedars-Sinai cohort was small and was from a single center. Also, only AMR events were observed in this cohort, likely due to the patient selection criteria. However, the results are consistent with the D-OAR study and confirm the ability of dd-cfDNA to detect graft damage. (5) With the recommended cutoff of 0.2%, there will be some false-positive results; however, the potential negative impact of these may be mitigated if the clinician considers other clinical information about the patient (including symptoms, signs, and imaging results) before deciding whether a biopsy should be performed.

dd-cfDNA is measured in plasma from a blood draw and therefore can be performed frequently after HT. This, combined with the potential of the assay to detect early signs of graft damage,[8,17] opens the door to more personalized titration of immunosuppressive therapies. Immunosuppressive medications such as corticosteroids and calcineurin inhibitors could potentially be weaned faster in patients with no evidence of graft injury and augmented in patients who demonstrate a rise in dd-cfDNA levels. Thus, more effective immunosuppression could be administered to recipients at higher risk of AR, whereas the side effects and toxicities of these medications could be avoided in stable patients. Additionally, this test may be performed if there is clinical suspicion for rejection or graft injury, before deciding on the need for endomyocardial biopsy. HT recipients may present with nonspecific symptoms, such as dyspnea, that could be due to graft dysfunction or opportunistic infection. The dd-cfDNA result may thereby help to focus subsequent diagnostic testing. This is especially useful in patients who are receiving anticoagulation therapy, with anatomic challenges, or with other contraindications to biopsy procedures.

As with all laboratory tests, clinical evaluation of the patient must be factored into the interpretation of test results. The dd-cfDNA test results may not eliminate the need for biopsy, but a high level may increase the probability of a positive biopsy result and would provide further justification for initiating clinical treatment of AR. The high NPV of the assay, on the other hand, would reduce the need for biopsies in patients with low suspicion for AR.

In summary, this study establishes the performance of the dd-cfDNA assay to detect acute rejection and graft dysfunction after HT in a large and diverse patient cohort in the United States. These results set the stage for subsequent clinical utility studies of the dd-cfDNA assay in HT patient management.

ACKNOWLEDGMENTS

The authors would like to thank Preethi Prasad and Theresa Wolf at CareDx, Inc., the research coordinators at the participating transplant centers for supporting the study conduct as well as sample and data collection, and the many heart transplant recipients who selflessly participated in this study.

DISCLOSURE

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. Dr Khush is an advisor to CareDx and has received research support from CareDx. Drs Yee, Woodward, and Hiller are employees of CareDx. Drs Hall and Kobashigawa serve as co-PIs and as advisors (consultants) to CareDx. Drs Pinney, Kao, Alharethi, DePasquale, Ewald, Berman, and Kanwar were co-PIs in the DOAR study. Dr Patel has no conflicts of interest to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1. Lund LH, Khush KK, Cherikh WS, et al. The Registry of the International Society for Heart and Lung Transplantation: thirty-fourth Adult Heart Transplantation Report-2017; Focus Theme: allograft ischemic time. *J Heart Lung Transplant*. 2017;36(10):1037-1046.
2. Baraldi-Junkins C, Levin HR, Kasper EK, Rayburn BK, Herskowitz A, Baughman KL. Complications of endomyocardial biopsy in heart transplant patients. *J Heart Lung Transplant*. 1993;12(1 Pt 1):63-67.
3. Bhat G, Burwig S, Walsh R. Morbidity of endomyocardial biopsy in cardiac transplant recipients. *Am Heart J*. 1993;125(4):1180-1181.
4. Williams MJ, Lee MY, DiSalvo TG, et al. Biopsy-induced flail tricuspid leaflet and tricuspid regurgitation following orthotopic cardiac transplantation. *Am J Cardiol*. 1996;77(15):1339-1344.
5. Pham MX, Teuteberg JJ, Kfoury AG, et al. Gene-expression profiling for rejection surveillance after cardiac transplantation. *N Engl J Med*. 2010;362(20):1890-1900.
6. Parent MC, Clarke BA, Khush KK. Non-invasive Tools for Monitoring Acute Cardiac Allograft Rejection: State of the Art. In: Leone O, Angelini A, Bruneval P, (eds). *Pathology of Cardiac Transplantation-A Clinical-Pathological Perspective*. Cham, Switzerland: Springer International Publishing AG; 2017.
7. Beck J, Oellerich M, Schulz U, et al. Donor-derived cell-free DNA is a novel universal biomarker for allograft rejection in solid organ transplantation. *Transplant Proc*. 2015;47(8):2400-2403.

8. De Vlaminck I, Valantine HA, Snyder TM, et al. Circulating cell-free DNA enables noninvasive diagnosis of heart transplant rejection. *Sci Transl Med*. 2014;6(241):241ra277.
9. Hidestrand M, Tomita-Mitchell A, Hidestrand PM, et al. Highly sensitive noninvasive cardiac transplant rejection monitoring using targeted quantification of donor-specific cell-free deoxyribonucleic acid. *J Am Coll Cardiol*. 2014;63(12):1224-1226.
10. Grskovic M, Hiller DJ, Eubank LA, et al. Validation of a clinical-grade assay to measure donor-derived cell-free DNA in solid organ transplant recipients. *J Mol Diagn*. 2016;18(6):890-902.
11. Bloom RD, Bromberg JS, Poggio ED, et al. Cell-free DNA and active rejection in kidney allografts. *J Am Soc Nephrol*. 2017;28(7):2221-2232.
12. ClinicalTrials.gov. https://clinicaltrials.gov/ct2/show/NCT02178943?term=D-OAR&rank=1. Accessed March 9, 2018.
13. Stewart S, Winters GL, Fishbein MC, et al. Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. *J Heart Lung Transplant*. 2005;24(11):1710-1720.
14. Berry GJ, Burke MM, Andersen C, et al. The 2013 International Society for Heart and Lung Transplantation Working Formulation for the standardization of nomenclature in the pathologic diagnosis of antibody-mediated rejection in heart transplantation. *J Heart Lung Transplant*. 2013;32(12):1147-1162.
15. Organ Procurement and Transplantation Network (OPTN). https://optn.transplant.hrsa.gov/data/view-data-reports/national-data/. Accessed April 30, 2018.
16. Moayedi Y, Foroutan F, Miller RJH, et al. Risk evaluation using gene expression screening to monitor for acute cellular rejection in heart transplant recipients. *J Heart Lung Transplant*. 2019;38(1):51-58.
17. Snyder TM, Khush KK, Valantine HA, Quake SR. Universal noninvasive detection of solid organ transplant rejection. *Proc Natl Acad Sci U S A*. 2011;108(15):6229-6234.
18. Crespo-Leiro MG, Zuckermann A, Bara C, et al. Concordance among pathologists in the second Cardiac Allograft Rejection Gene Expression Observational Study (CARGO II). *Transplantation*. 2012;94(11):1172-1177.
19. Evans RW, Williams GE, Baron HM, et al. The economic implications of noninvasive molecular testing for cardiac allograft rejection. *Am J Transplant*. 2005;5(6):1553-1558.
20. Hollander Z, Mohammadi TH, Assadian S, et al. Cost-effectiveness of a blood-based biomarker compared to endomyocardial biopsy for the diagnosis of acute allograft rejection [abstract]. *J Heart Lung Transplant*. 2016;35(4):S53.
21. Naumann DN, Hazeldine J, Dinsdale RJ, et al. Endotheliopathy is associated with higher levels of cell-free DNA following major trauma: a prospective observational study. *PLoS ONE*. 2017;12(12):e0189870.
22. Long Y, Zhang Y, Gong Y, et al. Diagnosis of sepsis with cell-free DNA by next-generation sequencing technology in ICU patients. *Arch Med Res*. 2016;47(5):365-371.
23. Beck J, Bierau S, Balzer S, et al. Digital droplet PCR for rapid quantification of donor DNA in the circulation of transplant recipients as a potential universal biomarker of graft injury. *Clin Chem*. 2013;59(12):1732-1741.
24. Schutz E, Fischer A, Beck J, et al. Graft-derived cell-free DNA, a noninvasive early rejection and graft damage marker in liver transplantation: a prospective, observational, multicenter cohort study. *PLoS Med*. 2017;14(4):e1002286.
25. De Vlaminck I, Martin L, Kertesz M, et al. Noninvasive monitoring of infection and rejection after lung transplantation. *Proc Natl Acad Sci U S A*. 2015;112(43):13336-13341.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Khush KK, Patel J, Pinney S, et al. Noninvasive detection of graft injury after heart transplant using donor-derived cell-free DNA: A prospective multicenter study. *Am J Transplant*. 2019;19:2889–2899. https://doi.org/10.1111/ajt.15339