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Clinical outcomes from the Assessing Donor-derived cell-free DNA Monitoring Insights of kidney Allografts with Longitudinal surveillance (ADMIRAL) study

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The use of routine monitoring of donor-derived cell-free DNA (dd-cfDNA) after kidney transplant may allow clinicians to identify subclinical allograft injury and intervene prior to development of clinically evident graft injury. To evaluate this, data from 1092 kidney transplant recipients monitored for dd-cfDNA over a three-year period was analyzed to assess the association of dd-cfDNA with histologic evidence of allograft rejection. Elevation of dd-cfDNA (0.5% or more) was significantly correlated with clinical and subclinical allograft rejection. dd-cfDNA values of 0.5% or more were associated with a nearly three-fold increase in risk development of *de novo* donor-specific antibodies (hazard ratio 2.71) and were determined to be elevated a median of 91 days (interquartile range of 30-125 days) ahead of donor specific antibody identification. Persistently elevated dd-cfDNA (more than one result above the 0.5% threshold) predicted over a 25% decline in the estimated glomerular filtration rate over three years (hazard ratio 1.97). Therefore, routine monitoring of dd-cfDNA allowed early identification of clinically important graft injury. Biomarker monitoring complemented histology and traditional laboratory surveillance strategies as a prognostic marker and risk-stratification tool post-transplant. Thus, persistently low dd-cfDNA levels may accurately identify allograft quiescence or absence of injury, paving the way for personalization of immunosuppression trials.

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The deployment of nucleic acid–based noninvasive biomarkers within routine clinical care reflects a paradigm shift in traditional monitoring after kidney transplant. Current clinical management of transplant relies on detection of functional injury (elevated creatinine), therapeutic drug monitoring, and selectively screening for harmful donor-specific antibodies (DSAs). In the absence of clinical signs, clinicians seeking to identify subclinical allograft injury and intervene prior to development of irreversible damage, were forced to rely on invasive allograft biopsies, which have inherent limitations from sampling error and variation in interpretation.[1]

Routine monitoring with donor-derived cell-free DNA (dd-cfDNA) after solid organ transplantation has been shown to accurately identify and characterize allograft injury,[1–3] correlate with pathologic findings,[4–6] and assess response to therapy including treatment of rejection.[7,8] Importantly, evaluation in dd-cfDNA have been demonstrated to occur ahead of clinically apparent organ injury.[9,10] Consequently, allograft monitoring with plasma dd-cfDNA levels can support noninvasive identification of pathologies including cellular and humoral allograft rejection, viral injury, and drug toxicity.[3,6] dd-cfDNA can also be employed in the setting of acute allograft injury to guide further diagnostic testing and assess improvement following clinical intervention.[7] The routine use of dd-cfDNA to detect,

characterize, or exclude ongoing allograft injury is a valuable addition in current post-transplant surveillance.

While the effectiveness of dd-cfDNA has been established in clinical trials, its utility in routine clinical practice has not been well described. The ADMIRAL study (Assessing AlloSure Dd-cfDNA, Monitoring Insights of Renal Allografts with Longitudinal Surveillance; NCT04566055), is a large, multicenter, observational cohort study of kidney transplant recipients monitored with dd-cfDNA for ≤3 years. The purpose of this study was to validate clinical trial data by documenting the effectiveness of dd-cfDNA in identifying allograft rejection and subclinical changes in a real-world setting and evaluate the relationship between dd-cfDNA measurements and nonimmune allograft injury. Additionally, ADMIRAL aimed to characterize the relationship between elevation in dd-cfDNA and important predictors of long-term graft survival, including estimated glomerular filtration rate (eGFR) and formation of *de novo* donor-specific antibodies (dnDSAs).

METHODS

Study population

A total of 1092 adult kidney transplant recipients across 7 transplant centers were monitored with AlloSure dd-cfDNA (CareDx Inc) as part of their standard of care. Data was collected between June 1, 2016, and January 31, 2020. An Institutional Review Board waiver of informed consent was obtained, and the study was performed in accordance with international standards and was not part of a larger study. Patients were managed prospectively with dd-cfDNA as part of post-transplant care where data captured was retrospectively examined. Clinical events (e.g., rejection, infection) and routine laboratory testing (creatinine, DSAs) were determined using the center's electronic medical records. A full list of data collected is provided in Supplementary Table S1. Patients who had contraindications to dd-cfDNA monitoring were excluded. Exclusions include pregnancy, multiple organ recipients, monozygous twin-to-twin transplant, and patients with prior bone marrow transplantation. No exclusions from the analysis and no withdrawal of patients were made as the use of dd-cfDNA was medically necessary as part of the standard of care.

AlloSure dd-cfDNA methodology

dd-cfDNA was measured at regular intervals based on each center's standard of care practice and was used both as part of surveillance testing and acutely as a diagnostic aid in patients with clinically evident graft dysfunction. A list of center management protocols is provided in Supplementary Table S2. Venous blood was collected in Streck Cell-Free DNA BCT tubes and 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.[11] The targeted next-generation sequencing assay employs highly polymorphic single nucleotide polymorphisms to quantify dd-cfDNA without need for separate genotyping of the recipient or the donor.[11]

Diagnosis of graft dysfunction and biopsy-defined rejection

Results of protocol surveillance and for-cause kidney transplant biopsies were captured. Indications for for-cause biopsy included change in creatinine, worsening proteinuria, development of dnDSA,

or a combination of these. Initial clinical management was performed based on local biopsy interpretation at the discretion of the patient's transplant provider. Biopsy reports were subsequently examined centrally by a single pathologist (LB), masked to the dd-cfDNA score, for study analysis. Centrally interpreted biopsy results were reported using the Banff 2019 classification scheme. Banff lesion scores were recorded and discrepancies between local and central reporting were identified. If no Banff scores or clinical diagnosis was provided on the biopsy report, or if other pathologies were reported, these rejections were excluded for the purposes of the rejection analysis. In cases of disagreement, central interpretation was included in the analysis. Mixed rejection was captured and classified as antibody-mediated rejection (ABMR) and the T cell–mediated rejection (TCMR) group did not include borderline cases. A detailed breakdown of the biopsy findings is provided in Supplementary Table S3.

Other concomitant pathologic diagnoses, such as calcineurin inhibitor toxicity, glomerulopathy, or acute tubular injury or acute tubular necrosis (or both) were also captured and used for the injury analysis. They were not included in the rejection analysis. For patients diagnosed with allograft rejection, the decision to treat was made according to each center's clinical protocol. eGFR changes, dnDSAs, and future rejection events were also captured, along with all dd-cfDNA levels that were drawn per each center's standard protocol, before, during, and after acute events.

A paired biopsy was defined as a biopsy occurring ≤30 days after dd-cfDNA measurement. This inclusion period reflects the logistical complexity of getting patients scheduled for and completing allograft biopsy. Biopsy results were included in the analysis only if there was no intervention performed between the time of the dd-cfDNA measurement and biopsy. A histogram of days between dd-cfDNA sampling and biopsy is shown in Supplementary Figure S1.

Statistical analyses

Descriptive statistics were used for patient demographics and distribution of dd-cfDNA measurements obtained from blood samples at the time of clinical events. In the analysis, the discriminatory power was considered at previously published thresholds of 0.5% and 1%,[3,4] to calculate the performance characteristics of the assay (sensitivity, specificity, negative predictive value, positive predictive value). Subsequently patients were categorized as high dd-cfDNA (≥0.5%) versus low dd-cfDNA (<0.5%) for further analysis.

Comparisons between the high and low dd-cfDNA groupings were evaluated via Fisher's exact test for categorical variables and Student's *t*-test for continuous variables. Nonparametric comparisons of dd-cfDNA cumulative distributions between dichotomized groupings were evaluated via Kolmogorov-Smirnov 2-sample tests. The area under the receiver-operating characteristic curve (AUROC) was used to determine the discriminating accuracy of dd-cfDNA and other parameters of interest. Cumulative distributions curves were used to examine the relationship between dd-cfDNA level and the clinical indication for the allograft biopsy (for-cause vs. surveillance).

Multivariate logistic regression was used to determine which independent covariates were predictive of high dd-cfDNA measurements (see Supplementary Table S4 for model variables). Potential confounding factors were evaluated to ensure interpretation was robust. Statistical analysis was performed in R (R Core Team).

Patients and samples were included into nonmutually exclusive groups for the purpose of analysis based on the data available as shown in the flow diagram in Figure 1. The subsets of the ADMIRAL cohort included in the correlational analyses for each of the
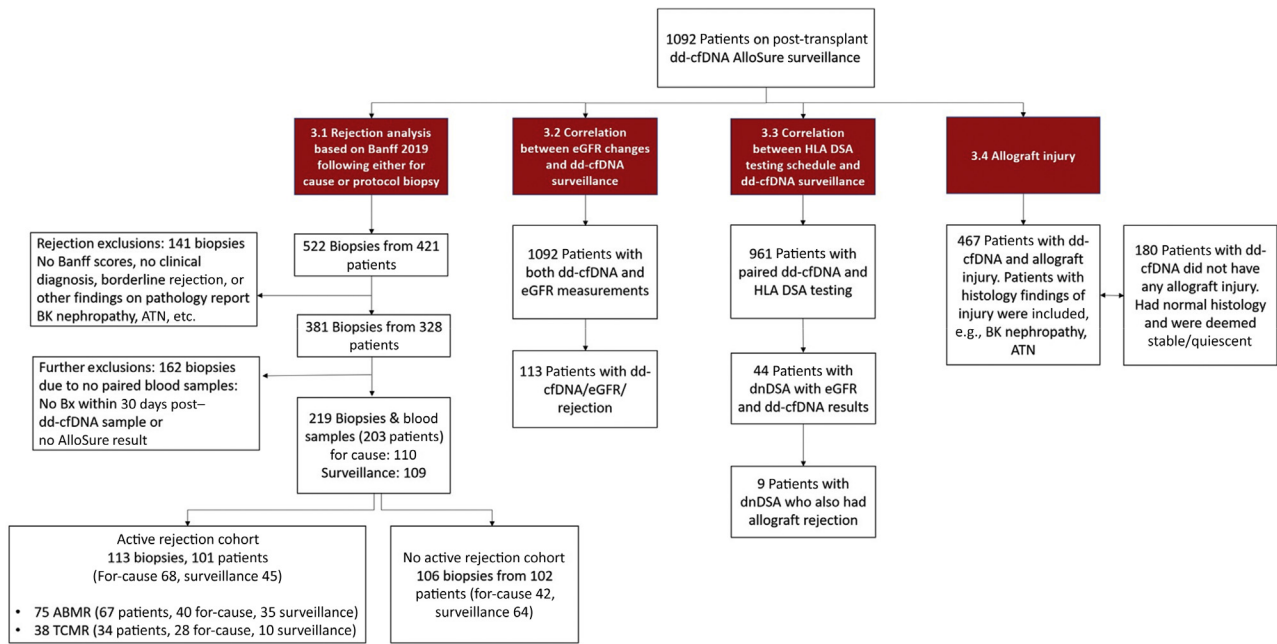
Figure 1 | Consort flow diagram showing the total data and how it was used to perform all the analyses in the study. ATN, acute tubular necrosis; Bx, biopsies; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibody; dnDSA, *de novo* donor-specific antibody; eGFR, estimated glomerular filtration rate; HLA, human leukocyte antigen.

questions has been outlined in this figure, including the total biopsies taken and the breakdown of results used for analysis.

dd-cfDNA and eGFR analyses. Kidney function was determined by eGFR calculated using the Modification of Diet in Renal Disease equation. dd-cfDNA and eGFR for each month was assessed where present, and then was partitioned into clusters as part of an unsupervised machine learning assessment to ascertain the relationship between eGFR and dd-cfDNA using Spearman rank correlation as an alternative to regression. If <1 eGFR measurement was available each month, the average was taken. Wong *et al.*[12] provided an update to the analytical variation and intrapatient variation of AlloSure dd-cfDNA. This was used to calculate the serial delta change between dd-cfDNA results associated with pathology using the methods outlined by Lund *et al.*[13] Analytical variation was defined as 2.7%, intrapatient variation = 61%, and the index of individuality = 0.23%.[12] K-means clustering[14] was used; distinct clusters representing time points allowed the formation of time horizons from 0 to 3 years post-transplant. The machine learning algorithm partitioned data into monthly clusters that were predetermined by minimizing the sum of squared distance using key features such as ethnicity, sex, age at transplant, evidence of BK virus infection, dd-cfDNA score, presence of DSAs, allograft rejection, and creatinine. Intracluster noise reduction strategies were applied to exclude interference of detection limits value. Spearman rank correlation was then used to measure the degree of association between eGFR and dd-cfDNA, with the correlation coefficient applied to determine the strength of the relationship. The clusters generated provided 3 different time horizons for assessment: 0 to 4 months, 4 to 12 months, and 12 to 36 months. More information is provided in Supplementary Methods.

dd-cfDNA and dnDSA analyses. The relationship between dd-cfDNA and development of dnDSAs was assessed in patients with paired dd-cfDNA and human leukocyte antigen (HLA) DSA testing

(both tests drawn at the same time). All patients started with a nonidentified DSA. Patients were defined as dnDSA-positive if there was evidence of new DSA detected at a level defined as positive by the local transplant program as part of the post-transplant surveillance. Reports were then centrally read. Mean fluorescence intensity of >500 was agreed to be positive, for both HLA class 1 and class 2, and was used for this analysis.[15] Freedom from dnDSAs was assessed using Kaplan-Meier analysis, and once patients developed DSAs, they were censored. Patients were categorized as having high dd-cfDNA (any measurement ≥0.5%) or a low dd-cfDNA (all dd-cfDNA in timeline measurement <0.5%). A multivariate statistical model and Cox proportional-hazard was used to evaluate the association of dd-cfDNAs with the development of dnDSAs (see Supplementary Table S5 for model variables).

Quiescence and allograft injury assessment. The value of dd-cfDNA as a marker of quiescence was retrospectively assessed using both biopsy and dnDSA measurement through longitudinal observation. Allograft quiescence was defined as the absence of injury. Injury included out-of-range tacrolimus level (<4 ng/ml, >12 ng/ml), BK viremia, dnDSA-positive, urinary tract infection, proteinuria, allograft rejection, or recurrent focal segmental glomerulosclerosis, as confirmed by paired biopsy ≤30 days after dd-cfDNA measurement.

RESULTS

The demographic characteristics of the 1092 ADMIRAL study patients are largely like the US adult transplant population reported to the United Network of Organ Sharing (UNOS) registry (Table 1). The ADMIRAL cohort was composed of a numerically higher percentage of African American recipients (28% vs. 24%; *P* = 0.78) and fewer retransplant candidates (8% vs. 13%; *P* = 0.16). There was also a higher proportion

Table 1 | Demographics of the ADMIRAL cohort compared to UNOS 2020–2021 published data

Patient variables	ADMIRAL	UNOS 2020–2021	P value (Fisher exact)
Sex, %			
Female	40	39	0.54
Male	60	61	0.71
Race, %			
Caucasian	48	55	0.46
African American	28	24	0.78
Hispanic	17	14	0.81
Asian	5	5	0.92
Other	2	3	0.73
Age at transplantation, yr			
Mean	49.5	46.7	0.16
Minimum–maximum range	17–84	0–96	0.22
Retransplant, %	8	13	0.16
Weight, kg	84	77	0.34
Height, cm	170	168	0.46
Median eGFR, ml/min per 1.73 m^2	69	73	0.52
Median serum creatinine, mg/dl	1.52	1.63	0.12
cPRA, %			
Mean	34	Not available	NA
>80%	16		
Range	1–96	Not available	NA
Median number of AlloSure tests per patient	6	Unknown	NA
Donor variables			
Age, yr			
Mean	40.7	37.7	0.92
Range	0–72	0–88	0.75
Sex, %			
Female	45	46	0.86
Male	55	54	0.67
Relation, %			
Unrelated	94	67	0.03
Deceased, %	94	68	0.04

ADMIRAL, Assessing AlloSure Dd-cfDNA, Monitoring Insights of Renal Allografts with Longitudinal Surveillance; cPRA, calculated panel reactive antibody; eGFR, estimated glomerular filtration rate; NA, not applicable; UNOS, United Network of Organ Sharing.

Table 2 | Demographics of the ADMIRAL rejection cohort compared to the no rejection cohort

Patient variables	No rejection (n = 979)	Rejection (n = 113)	P values
Sex, %			
Female	47	53	0.74
Male	52	48	0.65
Race, %			
Asian	0	100	0.01
Black	30	70	0.04
Caucasian	62	38	0.05
Hispanic	59	41	0.41
Other	0	100	0.01
Age at transplantation, yr			
Mean	50.7	45.8	0.69
Minimum–maximum range	22–78	12–75	NA
Retransplant, %	14	25	0.88
Weight, kg			
Mean	80.2	84	0.67
Height, cm			
Mean	168.2	169.7	0.89
eGFR, ml/min per 1.73 m^2			
Median	53.3	47	0.41
Serum creatinine, mg/dl			
Median	1.37	1.55	0.34
Number of AlloSure tests per patient			
Median	6	6	0.99
cPRA, %			
Mean	34	41	0.78
Range	1–96	3–100	NA
Donor variables			
Age, yr			
Mean	42.8	40.4	0.62
Range	22–55	19–49	NA
Sex, %			
Female	20	828	0.37
Male	80	72	0.51
Relation, %			
Unrelated	69	67	0.73
Deceased, %			
DCD	53	73	0.51
DBD	47	27	0.42

ADMIRAL, Assessing AlloSure Dd-cfDNA, Monitoring Insights of Renal Allografts with Longitudinal Surveillance; cPRA, calculated panel reactive antibody; DBD, donation after brain death; DCD, donation after cardiac death; eGFR, estimated glomerular filtration rate; NA, not applicable.

of deceased donor recipients in this study compared with in the UNOS registry (94% vs. 68%; *P* = 0.04).

Association of dd-cfDNA level and acute rejection

The analytic sample included 5,873 dd-cfDNA measurements from 1092 patients. Figure 1 describes the study cohort. The association between dd-cfDNA levels and the presence of any allograft rejection status was assessed using 219 biopsies from 203 patients with biopsy-paired dd-cfDNA results. Among the 219 biopsies, 110 were for-cause biopsies and the remaining 109 were surveillance biopsies performed under center-specific clinical protocols. The 113 biopsies (68 for-cause and 45 surveillance) from 101 patients were classified as acute rejection biopsies. The demographic characteristics of the acute rejection study patients are summarized in Table 2, while data on rejection is summarized in Supplementary Table S3. Of the local biopsies, 16% were rescored by the central pathologist (LB).

There was no statistically significant difference in the median creatinine in patients with a no rejection biopsy (1.38 mg/dl; interquartile range [IQR]: 1.07–1.96 mg/dl) and patients with Banff-defined rejection (1.57 mg/dl; IQR: 1.28–2.18 mg/dl); *P* = 0.096. The AUROC for creatinine was 0.492 (95% CI: 0.38–0.59). In comparison, the median dd-cfDNA level among patients with a no rejection biopsy was 0.23% (IQR: 0.19%–0.64%), which was significantly lower than the median dd-cfDNA in patients with biopsies demonstrating defined cellular or humoral rejection (1.6%; IQR: 0.68%–2.6%); *P* < 0.0001. The AUROC for all rejection dd-cfDNA was 0.798 (95% CI: 0.72–0.87), which was significantly higher than the AUROC of creatinine; *P* < 0.001 (Figure 2). The Youden's index for dd-cfDNA was 0.69%.

dd-cfDNA levels differed significantly between patients with ABMR and TCMR; *P* < 0.001. ABMR was diagnosed in
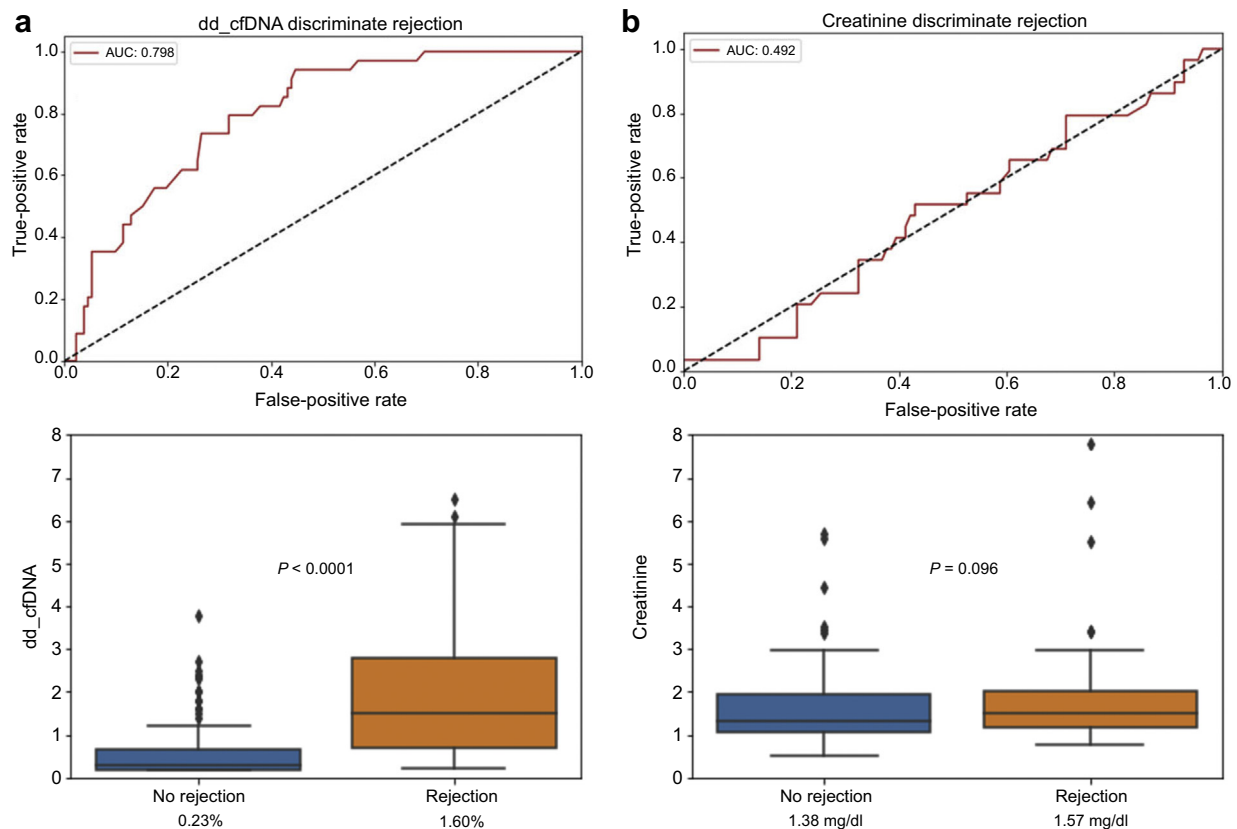
Figure 2 | Box and whisker plot showing the median donor-derived cell-free DNA (dd-cfDNA) and creatinine levels observed in patients with and without allograft rejection. (**a**) The ROC analysis for dd-cfDNA: area under the receiver-operating characteristic curve (AUROC) 0.798, with a median of 0.23% seen in patients with no rejection and 1.6% in patients with allograft rejection; $P < 0.0001$. (**b**) The ROC analysis for creatinine: AUROC 0.492, with a median creatinine of 1.38 mg/dl in patients with no rejection versus 1.57 mg/dl in patients with allograft rejection; $P = 0.096$.

75 biopsies (40 for-cause, 35 surveillance) from 67 patients. Among these patients, compared to nonrejection patients, the median dd-cfDNA was 1.8%; $P < 0.001$. TCMR was diagnosed in 38 biopsies (28 for-cause, 10 surveillance) from 34 patients. Patients with TCMR, compared to nonrejection patients, had a median dd-cfDNA value of 0.7%; $P < 0.001$. The median dd-cfDNA in patients with borderline TCMR (t1 i1: Banff scores) was 0.20% (IQR: 0.19%–0.25%). More information on performance and distributions of rejection groups is provided in Supplementary Figures S2, S3, and S4.

Clinical indication for biopsy was determined to have a significant impact on measured dd-cfDNA level regardless of pathologic findings. In patients without rejection, dd-cfDNA levels were significantly higher in patients undergoing a for-cause biopsy (0.34%; IQR: 0.19%–1.2%) than in patients undergoing a surveillance biopsy (0.23%; IQR: 0.19%–0.42%); $P = 0.038$. Similarly, median dd-cfDNA in patients diagnosed with ABMR in the for-cause biopsies was higher (2.2%; IQR: 1.5%–3.7) than ABMR diagnosed in patients in the surveillance biopsy group (0.91%; IQR: 0.47%–1.65%); $P = 0.0004$. The median dd-cfDNA in patients with TCMR biopsy diagnosis (excluding borderline) was 1.3% (IQR: 0.53%–3%) in the for-cause biopsy group and 0.52% (IQR:

0.34%–1.4%) in the surveillance biopsy group ($P = 0.2802$). All surveillance biopsies showing rejection had significantly higher dd-cfDNA than nonrejection biopsies did; $P < 0.001$. See Supplementary Figure S5 for cumulative distributions.

dd-cfDNA discriminates among biopsies showing no rejection, any rejection, ABMR, and TCMR biopsies (Table 3). Test characteristics differed by diagnostic threshold (0.5% vs. 1%) and identified pathology (any rejection, ABMR, TCMR). A 1% increase of dd-cfDNA was associated with a 3.3-fold increase in the risk of any rejection ($P < 0.001$), with an overall rejection hazard ratio (HR) of 1.89 (95% CI: 1.77–2.17).

Association of dd-cfDNA elevation and eGFR progression
The median number of eGFR and dd-cfDNA results per patient was 11 (IQR: 8–22) and 6 (IQR: 4–10), respectively. eGFR patterns over the first 4 months post-transplant, were erratic, with no clear trend identified. Analysis of kidney function between month 12 and month 36 demonstrated a correlation between the elevation of dd-cfDNA and subsequent decline in kidney function (Spearman correlation coefficient R: −0.84; $P = 0.01$) (Figure 3). Elevations in dd-cfDNA (≥0.5%) were associated with significant eGFR decline at 3 years post-transplant. Persistently

Table 3 | Performance characteristics of AlloSure dd-cfDNA to discriminate allograft rejection

Diagnosis (threshold %)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
All rejection (0.5)	78	71	50	90
All rejection (1.0)	58	82	54	84
ABMR (0.5)	79	59	42	88
ABMR (1.0)	65	75	49	85
TCMR (0.5)	75	50	36	84
TCMR (1.0)	45	63	31	76

ABMR, antibody-mediated rejection; dd-cfDNA, donor-derived cell-free DNA; NPV, negative predictive value; PPV, positive predictive value; TCMR, T cell–mediated rejection.
Nonrejection pathologies were excluded. These data compare 113 rejection biopsies with 106 no-rejection biopsies.

elevated dd-cfDNA (>1 result ≥0.5%) nearly doubled the risk of a 25% decline in eGFR (HR: 1.97; 95% CI: 1.39–2.68); $P = 0.041$.

## Relationship between dd-cfDNA level and identification of dnDSAs

The 961 patients with paired dd-cfDNA and HLA DSA results had no preexisting DSAs. The median calculated panel reactivity antibody was 37% (IQR: 11%–77%). The median number of paired DSAs and dd-cfDNA samples per patient was 5 (IQR: 3–9). Of these patients, dnDSAs were found in 44 patients (4.6%), 19 with class I and 25 with class II, 9 of whom also had histologic evidence of allograft rejection. dd-cfDNA >0.5% was associated with a nearly 3-fold elevation in the risk of future dnDSA formation (HR: 2.71; $P = 0.001$) (Figure 4). In a multivariable analysis, every 1% increase in the dd-cfDNA level was associated with a 20% increase in the risk of dnDSAs (HR: 1.19; $P = 0.004$). Serial examination of dd-cfDNA values in patients who developed dnDSAs demonstrated a median 121% (IQR: 69%–183%) increase in dd-cfDNA from prior dd-cfDNA results, which occurred a median of 91 days (IQR: 30–125 days) preceding detection of dnDSAs. Furthermore, dd-cfDNA remained elevated in all cases with measurable dnDSAs.

Association of dd-cfDNA elevation and graft injury

A composite state of graft injury defined as ≥1of the following events: tacrolimus level (<4 ng/ml, >12 ng/ml), BK viremia, dnDSA-positive, urinary tract infection, proteinuria, allograft rejection, or recurrent focal segmental glomerulosclerosis was identified in 467 patients. dd-cfDNA was measured up to 30 days ahead of injury event. Another subset of 180 patients without any of these events or evidence of kidney allograft injury were grouped under the quiescent category. Shown in Figure 5, the median dd-cfDNA level in the quiescent (noninjury) patients was 0.21% (95% CI: 0.19–0.34), while the median dd-cfDNA for patients with active injury was 0.51% (95% CI: 0.48%–1.2%); $P < 0.0001$. dd-cfDNA measurement had an AUROC of 0.727 (95% CI: 0.71–0.88). The Youden's index for dd-cfDNA threshold was 0.79%. The median creatinine in patients with quiescence was 1.32 mg/dl (95% CI: 1.16–1.65 mg/dl), which was not statistically different from patients meeting graft injury criteria (median creatinine: 1.48 mg/dl; 95% CI: 1.1–2.71 mg/dl); $P = 0.08$. The AUROC for creatinine was 0.575 (95% CI: 0.52–0.62). Shown in Table 4, a dd-cfDNA threshold value of 0.5% has a positive predictive value of 77.5% and negative predictive value of 71.6% for graft injury. In addition to the absolute value, the delta change in dd-cfDNA was associated with allograft injury. A median increase of 149% (IQR: 94–161) between serial results is indicative of graft injury ($P = 0.02$).

DISCUSSION

The large, multicenter, ADMIRAL cohort study independently validated the observation that dd-cfDNA detects both clinically evident and subclinical ABMR and TCMR in a real-world application of dd-cfDNA monitoring. dd-cfDNA was significantly more predictive of ongoing graft injury than the current standard-of-care measures of serum creatinine. In addition, elevated dd-cfDNA was associated with declining eGFR and the development of dnDSAs. Most importantly, low dd-cfDNA predicted allograft quiescence, which can

*AS = dd-cfDNA (AlloSure)
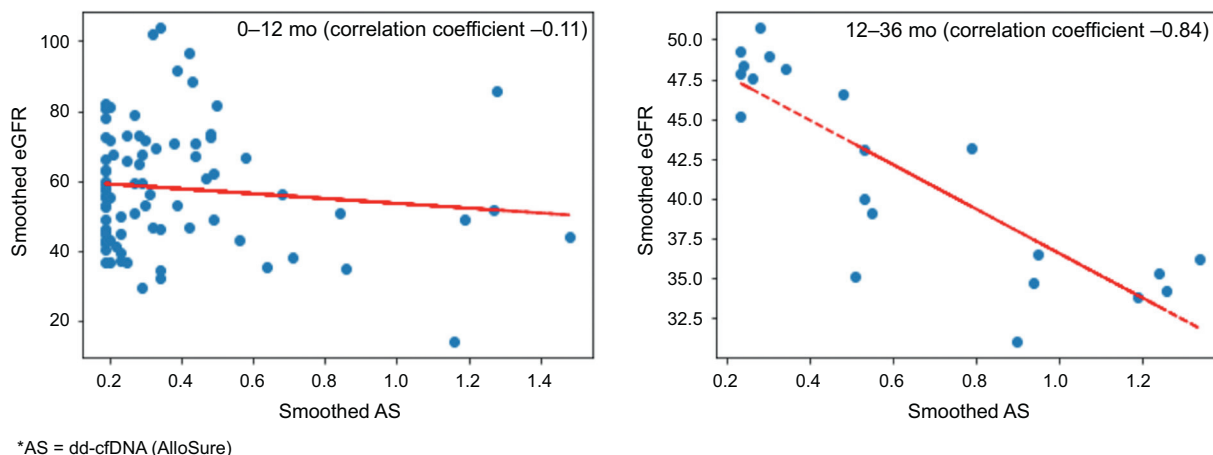
Figure 3 | eGFR trends for 1092 patients over the first 36 months post-transplant with a significant decline between 12 and 36 months (Spearman coefficient of −0.84). dd-cfDNA, donor-derived cell-free DNA.
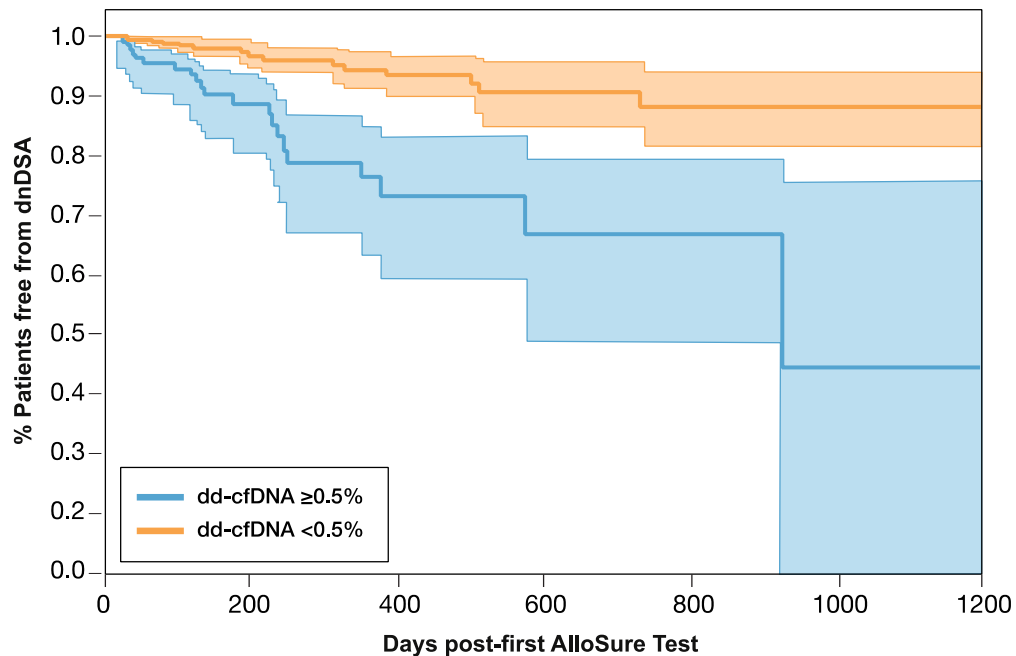
Days post–1st	0–200	200–400	400–600	600–800	800–1000	>1000
dd-cfDNA	Number at risk					
Low (<0.5)	808	801	797	796	796	796
	(Censored event of dnDSA)	7	4	1		
High (≥0.5)	153	139	123	122	121	121
	(Censored event of dnDSA)	14	16	1	1	

Figure 4 | Free from donor-specific antibody (DSA) model, with Cox proportional hazard showing risk of *de novo* DSAs (dnDSAs). There were 44 events observed from 961 patients, with 153 patients starting with donor-derived cell-free DNA (dd-cfDNA) levels ≥0.5% compared with 808 patients with dd-cfDNA <0.5%. Analysis of dichotomized groups at 0.5% level of dd-cfDNA showed a hazard ratio of 2.71 ($P = 0.001$). Patients with rejection were not included in the analysis or other events leading to censoring (death, loss to follow-up, etc.).

substantially reduce the need for protocol biopsies. The definition of injury used was not exhaustive, with other pathologies potentially impacting allograft survival. Notably, patients monitored with dd-cfDNA (AlloSure) had higher levels of dd-cfDNA, which correlated with both alloimmune and nonalloimmune causes of injury. Screening using dd-cfDNA is important, as early identification of injury in post-transplant surveillance is critical for optimization of investigation and treatment. Hence the utility of dd-cfDNA as an injury surveillance tool has potential to impact clinical decision making.

Patients with ABMR had levels of dd-cfDNA that were markedly higher in patients with both clinical rejection (2.2% vs. 0.34%) and subclinical rejection (0.91% vs. 0.23%). Similarly, patients with clinically evident (1.30% vs. 0.34%) and subclinical (0.52% vs. 0.23%) TCMR had statistically significant increases in dd-cfDNA compared to patients without evidence of rejection. These results demonstrate utility of dd-cfDNA in subclinical rejection to be a useful

leading indicator of injury, where the elevation of allograft injury in absence of clinical changes is less pronounced but still abnormal when compared to stable patients. dd-cfDNA elevations correlated with TCMR grades greater than borderline (Banff 2019 classification), with the median dd-cfDNA increasing with the severity of rejection grades: 1A (0.78%), 1B (1.3%), 2A (3.68%). The median dd-cfDNA in patients with borderline TCMR was 0.20%, with wide 95% CIs, suggesting heterogeneous injury within this diagnosis. Furthermore, many borderline rejections are being treated by transplant providers without clear evidence of clinical benefit. Similar findings have been reported with histology diagnosing significantly more borderline TCMR than tissue-based gene transcript assessment and median dd-cfDNA of 0.33%.[16]

The delta between serial dd-cfDNA was also associated with clinically significant events including dnDSA formation and allograft injury. These results suggest the need to consider a deviation from baseline, in combination with an elevation of dd-cfDNA above a threshold of 0.5%, to identify significant
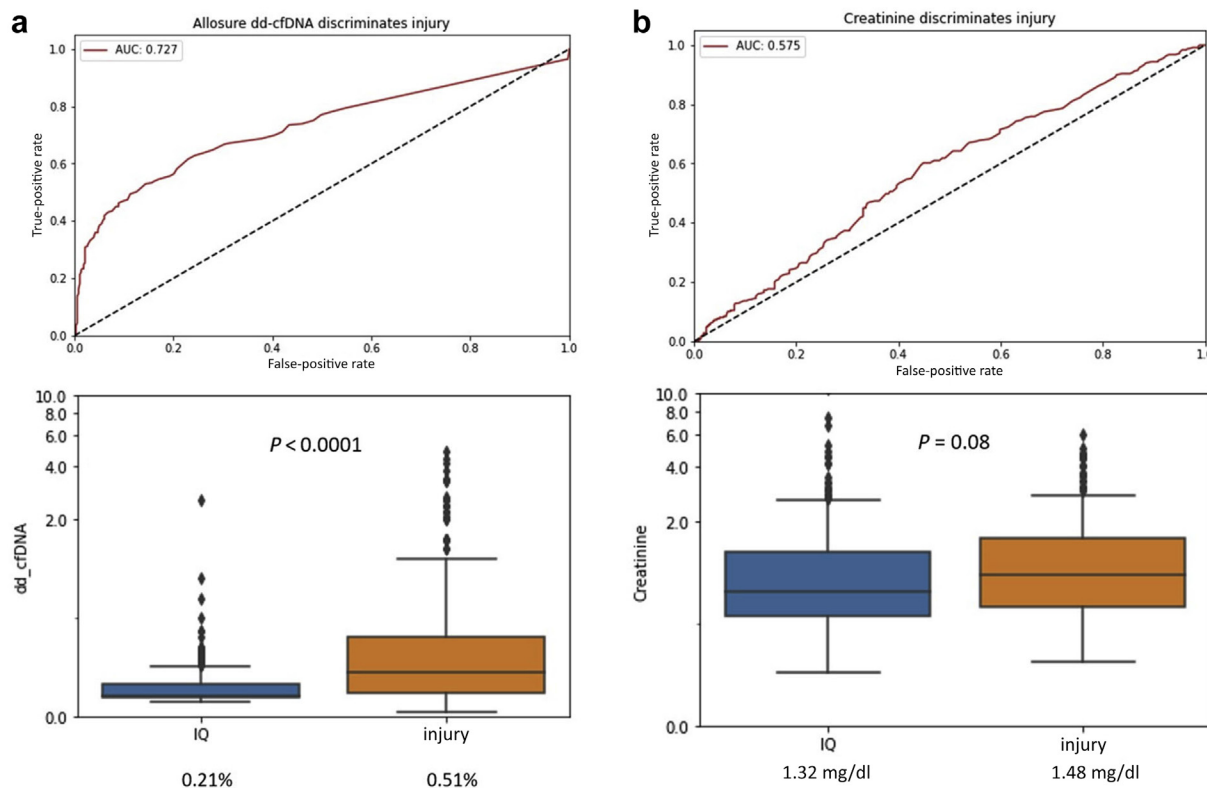
Figure 5 | Box and whisker plot showing the median donor-derived cell-free DNA (dd-cfDNA) and creatinine levels for 647 patients, of which 467 patients developed allograft injury and 180 patients were clinically stable with immune quiescence (IQ). (a) The ROC analysis for dd-cfDNA: area under the receiver-operating characteristic curve (AUROC) 0.727, with a median of 0.21% seen in patients with no injury and 0.51% in patients with injury; $P < 0.0001$. (b) The ROC analysis for creatinine: AUROC 0.572, with a median creatinine of 1.32 mg/dl in patients with no injury versus 1.48 mg/dl in patients with injury; $P = 0.08$.

graft injury. This finding has previously been shown by Stites et al.[4] While a measured level >0.5% or the increase of 149% from baseline (or both) does not definitively prove injury, these changes suggest patients should have intensive surveillance, further diagnostic study, potential intervention, or a combination of these. Given the optimal threshold for allograft rejection was determined at 0.69%, the relative change of dd-cfDNA is very important to consider in combination with the absolute number.

The ADMIRAL study confirmed the correlation between dd-cfDNA level and rejection established by the Circulating

Table 4 | Consideration of dd-cfDNA as a molecular marker of injury where the absence of injury is identified as quiescence

AlloSure dd-cfDNA (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
0.2	78.3	47.7	50	86.9
0.3	64.8	72.1	60.5	76.1
0.4	53.7	84.1	69	73.3
0.5	45.1	91.4	77.5	71.6
1.0	21.3	98.9	93.1	65.5

dd-cfDNA, donor-derived cell-free DNA; NPV, negative predictive value; PPV, positive predictive value.
This is a comparison of 167 patients with injury against 180 patients who were quiescent.

Donor-Derived Cell-Free DNA in Blood for Diagnosing Acute Rejection in Kidney Transplant Recipients (DART) study (NCT02424227).[3] In the DART study, a 1% threshold was used to discriminate between rejection and no rejection. ADMIRAL suggests that interpretation of serial change in dd-cfDNA level is also important in the interpretation of injury. These new data suggest that considering a median dd-cfDNA elevation of 149% from baseline signals a change from quiescence to potential injury. For most patients this seems to be an absolute elevation from baseline of ≥0.24% (IQR: 0.19%–0.39%). In other studies, Anand et al.[17] demonstrated that an increase in dd-cfDNA of ≥141% was associated with abnormal pathology, supporting the 149% threshold reported here. These data suggest that routine post-transplant surveillance with dd-cfDNA, which uses both serial changes and absolute thresholds (e.g., 0.5%), will increase the sensitivity to detect addressable injury in a timely fashion and in the absence of clinical symptoms.[18]

Allograft injury is multifactorial with pathology other than alloimmune damage resulting in dd-cfDNA elevations. Allograft damage can result from recurrent disease, calcineurin inhibitor toxicity, or infection, each of which requires directed intervention. Therefore, the ability to discriminate allografts free from clinical and subclinical injury is very

important and is broadly known as "allograft quiescence." dd-cfDNA <0.5% was strongly correlated with allograft quiescence, potentially reducing the need for an invasive procedure. Conversely, elevations in dd-cfDNA were specific and predictive of the study composite diagnosis of allograft injury. Thus, routine monitoring with dd-cfDNA may allow clinicians to risk stratify post-transplant patients, identify those with graft injury in need of potential further intervention and those without injury who may benefit from reduction in immunosuppression to avoid long-term drug-induced comorbidity.[19]

Development of dnDSAs has been correlated with decreased allograft survival, even in the absence of clinically evident ABMR.[20] However, while many patients develop dnDSAs, not all dnDSAs result in significant allograft injury. In a recent prospective, multicenter study of 123 patients who were biopsied after the development of dnDSAs in the absence of clinical rejection, only 41% had pathologic evidence of humoral rejection.[21,22] In a single-center study from the Mayo Clinic, 967 patients were monitored with dnDSA screening and protocol biopsies.[23] At a median follow-up of 4.2 years, 7% of the patients developed dnDSAs, 20% of patients had biopsy evidence of borderline or more severe acute cellular rejection, and only 32.5% had evidence of either active or chronic active ABMR at time of dnDSA detection. From the DART study, Jordan *et al.*[24] identified 87 patients with kidney transplants with 90 clinically indicated biopsies along with paired dd-cfDNA and DSA testing In patients with dnDSAs with ABMR, the average dd-cfDNA was 2.9% compared with 0.34% in patients with dnDSAs without ABMR, and 0.29% in patients without dnDSAs. In this observational cohort, 60.7% of DSA-positive patients did not have elevated dd-cfDNA and therefore did not appear to have evidence of antibody-mediated allograft injury. This supports previous findings where long-term allograft survival was not compromised in the setting of non–complement-binding DSAs.[25] These data suggest that dd-cfDNA may provide crucial incremental information that could complement dnDSA monitoring, by identifying clinical and subclinical ABMR in patients with kidney transplants. Molecular sensitization as the causal injury that drives antibody formation remains an interesting prospect as does the concept of antibodies being absorbed by the allograft before being seen by Luminex, causing molecular injury.[26] Furthermore, the temporal observation between dd-cfDNA elevations and dnDSAs warrants further investigation to assess both the etiology of dnDSA formation[26] and the potential for therapeutic intervention. Huang *et al.*[27] previously demonstrated histologic features of ABMR in patients with elevations in dd-cfDNA that did not have any appreciable HLA antibodies. In addition, non-HLA transplantation immunity revealed by lymphocytotoxic antibodies has been well published with Crespo *et al.*[28] showing the importance of AT1R in patients with ABMR who are DSA-negative.[29] Thus, the utility of dd-cfDNA in the assessment of non-HLA DSAs needs to be considered and, although not performed in this analysis, is

planned from patients with stored serum. With the pathogenicity of non-HLA DSAs still being determined, the use of dd-cfDNA in its assessment may be a useful tool for future studies.[30]

Both Clayton *et al.*[31] and Faddoul *et al.*[32] have reported that a decline in eGFR is superior to other surrogate measures of long-term kidney transplant outcomes. A 30% decline in eGFR between years 1 and 3 after kidney transplant is strongly associated with risks of subsequent death and death-censored allograft failure.[31,32] ADMIRAL extends our understanding of the correlation between changes in dd-cfDNA level and long-term graft outcomes. Higher levels of dd-cfDNA were correlated with subsequent declining eGFR (correlation coefficient: −0.84), suggesting that early identification of injury before traditional functional changes occur could impact graft survival. The mechanisms of injury are clearly multifactorial but suggest that elevated dd-cfDNA may identify patients who would benefit from further investigation.

By using results from routine clinical care, our findings represent the largest prospective cohort of kidney transplant recipients undergoing surveillance with dd-cfDNA published to date. The limitations of this study primarily reflect its observational, real-world design. Comparison with UNOS data suggest that clinical determination did not bias inclusion of patients across these 7 centers and that this cohort truly represents the wider transplant population. As clinicians were unblinded with regard to dd-cfDNA measurements and other clinical data, clinical treatment may have altered the natural history of disease and affected the correlations reported. In addition, logistical constraints led to dd-cfDNA levels and biopsies not always being concurrently obtained. To account for these barriers, we allowed biopsies done ≤30 days after dd-cfDNA levels to be considered as paired results. While it is possible that subclinical rejection may have resolved prior to biopsy, this effect would most likely have biased the study toward the null finding and thus should not invalidate the findings reported here. Verification bias is a consideration as biopsies were performed locally and not all read or acted on centrally. However, with data showing consistent patterns despite this heterogeneity, the results identify clear direction for future work. Missing values causing ascertainment bias in the absence of a control group in the prediction model analysis is also a consideration, but we feel the large sample size limits this, where longitudinal serial samples allow patients to be their own control. Another potential limitation is that testing is more frequently performed in the first year of transplant. Therefore, there is a natural ascertainment and selection bias as alloimmune injury and infection are more common during this period; however, this follows the routine clinic schedule so again it reflects real-life practice. Further investigation is needed to establish the optimal interval of monitoring as there is clear multifactorial value considering dd-cfDNA as part of the clinical assessment of the patient. Finally, heterogeneity of dd-cfDNA levels between patients, underlying pathology, effect of interventions impacting the

degree of association between dd-cfDNA measurements, and clinical evidence need to be considered. In the future, Bayesian probability evaluation incorporating knowledge of the patient's past clinical course and current presentation needs to be considered in modeling algorithms to reduce the impact of this heterogeneity.

These findings suggest an expanded role of dd-cfDNA in clinical practice, supporting its use in post-transplant patient standard-of-care management, complementing histology and traditional surveillance strategies as an important prognostic marker and risk-stratification tool. Achieving allograft quiescence is vital to improving long-term outcomes, as both immune- and non-immune–mediated injury leads to accelerated graft loss. Our findings further expand the base of knowledge on interpretation of dd-cfDNA levels in various clinical contexts, showing broader utility as a leading indicator ahead of clinical presentations of allograft injury, formation of dnDSAs, eGFR decline, and subclinical rejection. Additional interventional studies[19] are underway to help better define how the information provided by dd-cfDNA can be used to guide clinical practice and decisions regarding immunomodulation, management of infection, treatment of all types of rejection, and control or even prevent the formation of dnDSAs.

AUTHOR CONTRIBUTIONS
LB, DAA, TKW-D, and SD drafted or revised the manuscript and participated in the results analysis. GG, AP, SA, ES, JSB, and TA drafted or revised the manuscript, acquired data, and participated in the results analysis. IM, VB, MW, ADG, and HM acquired data. PJ acquired data and contributed to drafts. JZ, WT, and KQ participated in the results analysis. RW revised the manuscript.

SUPPLEMENTARY MATERIAL
Supplementary File (Word)
Figure S1. Histogram for the number of days between donor-derived cell-free DNA (dd-cfDNA) sampling and biopsy.
Figure S2. Distributions of donor-derived cell-free DNA (dd-cfDNA) in no rejection, antibody-mediated rejection (ABMR), and T cell–mediated rejection (TCMR) groups.
Figure S3. Performance of donor-derived cell-free DNA (dd-cfDNA) in allograft rejection.
Figure S4. Distribution of delta change results for stable patients and those with injury.
Figure S5. Cumulative distribution of donor-derived cell-free DNA (dd-cfDNA) levels in biopsies.
Table S1. Data variables collected: donor (D) and recipient (R) variables.
Table S2. Center standard-of-care protocols.
Table S3. Biopsy Banff 2019 lesion scores used in the rejection analysis.
Table S4. Univariate and multivariate regression model for allograft rejection.
Table S5. Univariate and multivariate regression model for *de novo* donor-specific antibodies (dnDSAs).
Supplementary Methods.

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