ARTICLES

Biological Variation of Donor-Derived Cell-Free DNA in Renal Transplant Recipients: Clinical Implications

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Background: Previous studies have demonstrated that donor-derived cell-free DNA (dd-cfDNA) found in circulating blood of transplant recipients may serve as a noninvasive biomarker of allograft rejection. To better interpret the clinical meaning of dd-cfDNA, it is essential to understand the biological variation of this biomarker in stable healthy recipients. This report establishes the biological variation and clinical reference intervals of dd-cfDNA in renal transplant recipients by using an analytically validated assay that has a CV of 6.8%.

Methods: We sampled venous blood at patient surveillance visits (typically at posttransplant months 1–4, 6, 9, and 12) in a 14-center observational study. Patients with stable renal allograft function spanning ≥3 serial visits were selected. We used AlloSure®, a targeted next-generation sequencing-based approach, to measure dd-cfDNA in the plasma and computed the intraindividual CV (CV_I) and interindividual CV (CV_G), the index of individuality (II), and reference change value (RCV).

Results: Of 93 patients, 61% were men, 56% were Caucasian, mean age was 49 years, and 63% were deceased donor kidney recipients. Of 380 blood samples, the dd-cfDNA median value was 0.21% (interquartile range 0.12%–0.39%) and the 97.5th percentile was 1.20%. In 18 patients with an average of 4.1 tests, the CV_I was 21%, CV_G was 37%, II was 0.57, and RCV was 61%.

Conclusions: In a renal transplant recipient, a dd-cfDNA level above 1.2% is out of range and potentially abnormal. A serial increase of up to 61% in level of dd-cfDNA in a patient may be attributable to biological variation.

Clinicaltrials.gov Identifier: NCT02424227

IMPACT STATEMENT

Previous studies have demonstrated that donor-derived cell-free DNA (dd-cfDNA) found in circulating blood of transplant recipients may serve as a noninvasive biomarker of allograft rejection. To better interpret the clinical meaning of dd-cfDNA, it is essential to understand the normal biological variation of this biomarker in stable healthy renal transplant recipients. This report establishes the biological variation and clinical reference intervals of dd-cfDNA in renal transplant recipients, using an analytically validated assay. This information is complementary to other reports that characterize how this biomarker may be used to discriminate pathological allograft rejection from no rejection status.

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The measurement of donor-derived cell-free DNA (dd-cfDNA)[18] in the circulating blood of transplant recipients has shown promise for clinical monitoring of tissue injury in heart, lung, liver, and kidney allografts (*1*). Observations of increased levels of dd-cfDNA during acute rejection and correlation to severity of rejection have indicated the potential utility of dd-cfDNA as an early noninvasive indicator of allograft injury (*2–8*). Among the various strategies to measure dd-cfDNA, we have demonstrated the analytical validity of a targeted next-generation sequencing assay that uses 266 single-nucleotide polymorphisms (SNPs) to accurately quantify dd-cfDNA in the plasma of transplant recipients without the need for genotyping either the donor or the recipient (*6*). Here we establish, for the first time, the biological variation and reference intervals for this commercially available test (AlloSure®) in a population of clinically stable renal transplant recipients. The biological variability of the dd-cfDNA assay in a reference renal transplant population is relevant to the clinical interpretation of results in allograft recipients, who may undergo serial monitoring of dd-cfDNA to assess the status of the allograft over time.

MATERIALS AND METHODS

Definition of reference population

The reference population is comprised of renal allograft recipients who demonstrated well-functioning allografts and no clinical suspicion of rejection as defined by stable and acceptable serum creatinine values, no significant proteinuria, and clinical stability without infections, acute cardiovascular changes, or other acute clinical events. Only patients who had stable graft function and blood samples from at least 3 serial surveillance visits ≥14 days posttransplant are included in the analysis. Patients with impaired function or clinical instability [such as (*a*) an increase in serum creatinine ≥0.5 mg/dL since prior testing; (*b*) delayed graft function defined by ongoing need for dialysis in the posttransplant period; (*c*) a clinically indicated visit or renal transplant biopsy for allograft dysfunction; (*d*) any allograft acute rejection event; or (*e*) active urinary tract, cytomegalovirus, or polyomavirus type BK infections] were excluded. Patients with prior organ transplant(s) in situ were also excluded, because dd-cfDNA could be derived from multiple donor allografts. Blood samples collected before 14 days posttransplant were excluded because dd-cfDNA may be elevated in the first 2 weeks posttransplant (*5*).

This reference population was selected from the cohort of patients enrolled in the DART observational study (Circulating Donor-Derived Cell-free DNA in Blood for Diagnosing Acute Rejection in Kidney Transplant Recipients; Clinical Trials Identifier: NCT02424227) (*9*). The DART study was designed to provide samples and clinical data to enable clinical validation of the performance of the

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[18] **Nonstandard abbreviations:** dd-cfDNA, donor-derived cell-free DNA; SNP, single-nucleotide polymorphism; DART, Circulating Donor-Derived Cell-free DNA in Blood for Diagnosing Acute Rejection in Kidney Transplant Recipients; CV_I, intraindividual CV; CV_G, interindividual CV; CV_A, analytical CV; II, index of individuality; RCV, reference change value; eGFR, estimated glomerular filtration rate; CK-MB, creatine kinase MB isoenzyme; ABMR, antibody-mediated rejection; DSA, donor-specific antibody.

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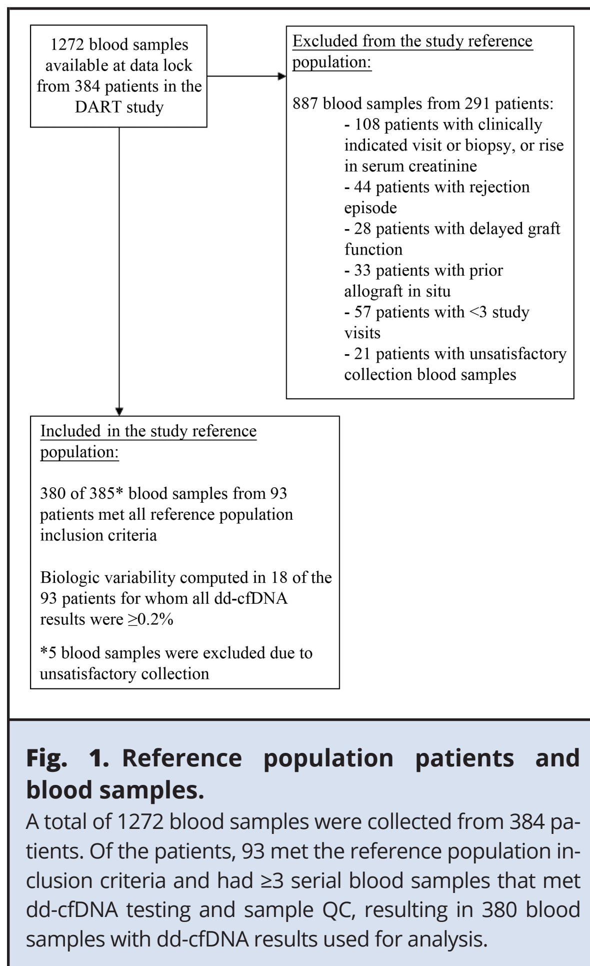

Fig. 1. Reference population patients and blood samples.
A total of 1272 blood samples were collected from 384 patients. Of the patients, 93 met the reference population inclusion criteria and had ≥3 serial blood samples that met dd-cfDNA testing and sample QC, resulting in 380 blood samples with dd-cfDNA results used for analysis.

AlloSure assay of dd-cfDNA, by using histopathological assessment of tissue obtained from a needle biopsy of the allograft kidney as the reference standard, to discriminate acute rejection from nonrejection status. We selected all patients from the whole DART cohort who were qualified to constitute the reference population (Fig. 1).

DART study design

Patients were enrolled in the prospective observational DART study (sponsored by CareDx) at 14 clinical sites from April 2015 to May 2016 (see the Data Supplement that accompanies the online version of this article at http://www.jalm.org/content/vol2/issue3). Of 384 total patients enrolled, 245 had their first study visit at the time of a scheduled post–kidney transplantation clinic appointment, and 139 patients had their first study visits at a time when they were being evaluated for a suspected acute rejection event and associated clinically indicated renal biopsy.

The institutional review board at each site approved the study and all of the patients provided written informed consent. The statistical analysis, data management, and clinical operations coordination were provided by staff employed by the study sponsor.

Blood samples and dd-cfDNA measurement

After transplantation, blood was collected at the time of scheduled surveillance visits at months 1–4, 6, 9, and 12, or at the time of each kidney allograft biopsy, and up to 2 follow-up samples within 8 weeks of the kidney allograft biopsy. Duplicate samples of venous blood were collected from the same venipuncture in Streck Cell-Free DNA BCT® tubes, stored at room temperature, and shipped to the CLIA-certified laboratory at CareDx (Brisbane, CA). On arrival, and within 7 days post-draw, plasma was separated by centrifugation at 1600*g* for 20 min followed by a second centrifugation at 16 000*g* for 10 min and was either stored at −80 °C or cfDNA was extracted immediately using the Circulating Nucleic Acid kit (Qiagen) (*10*, *11*).

We measured dd-cfDNA using AlloSure, a clinical-grade, targeted next-generation sequencing assay described in detail previously (*6*). Briefly, 266 SNPs were selected on the basis of high allele frequency, low error, and minimal linkage. Target genomic regions containing these SNPs are amplified by PCR and sequenced to identify allele frequencies for each SNP. The allele frequencies are used to accurately quantify dd-cfDNA in transplant recipients without the need for separate genotyping of the recipient or the donor (*6*). The assay quantifies the fraction of dd-cfDNA in both unrelated and related donor-recipient pairs. The dd-cfDNA assay is precise across the linear quantifiable

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range (0.2%–16%) with a mean across-run analytical CV of 6.8%. Assay results of the clinical samples in this study were evaluated against established QC criteria described previously (*6*), and only passing results were used for the analysis. Samples that failed QC were repeated at the step where they failed or were repeated using plasma from the duplicate Streck Cell-Free DNA BCT tube collected at the same venipuncture as the first sample. All measurements were performed by staff unaware of the identity of the samples. The final results (% dd-cfDNA) were reported to the database manager, who combined them with the clinical information and transferred the combined data set to the statistical team for analysis.

Statistical analysis

We computed the CV of the dd-cfDNA values within (intraindividual CV [CV_I]) and between (interindividual CV [CV_G]) the reference population patients. The CV_I and CV_G were computed only from the reference patients for whom all samples were greater than the limit of quantification of 0.2%. Because of the nonnormality of the reference dd-cfDNA distribution (Fig. 2), all computations used a robust CV. Robust CV is defined as the (median absolute deviation)/median. The median absolute deviation is the median distance of the dd-cfDNA values away from the median dd-cfDNA value. Robust statistics are preferred for nonnormal distributions to reduce the influence of outlier values. The low analytical variability [analytical CV (CV_A) = 6.8%] has been previously established by performing replicate measurements of reference materials mimicking transplant patients' cfDNA across a wide range of dd-cfDNA levels and replicated in patient samples (*6*). The index of individuality (II) was calculated as the CV_I:CV_G ratio. The reference change value (RCV), defined as the difference that must be exceeded between 2 sequential results for a significant change to occur, incorporated the total variation associated with both results and was calculated as $2^{½} \times 1.96 \times (CV_A^2 + CV_I^2)^{½}$ (*12*).

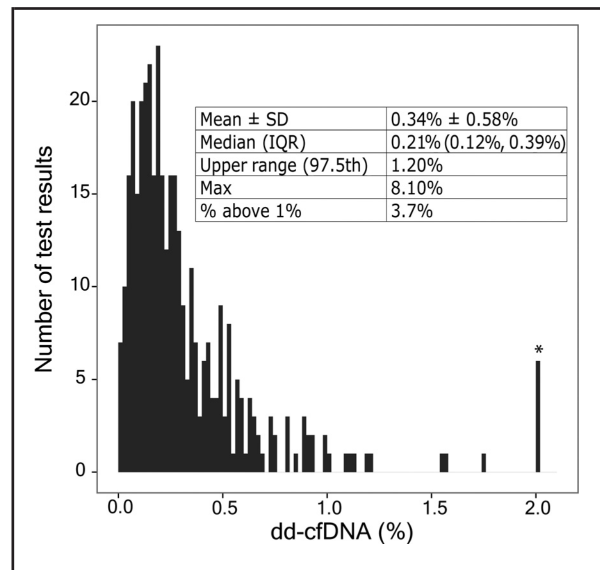

Fig. 2. Distribution of dd-cfDNA in reference population.
Range of dd-cfDNA values and number of test results for each, from the 380 blood samples obtained from the 93 reference population renal transplant patients, are shown. Six results with a value >2.0% are grouped together: 2.6%, 2.6%, 3.0%, 3.3%, 3.9%, and 8.1%.

All analyses were performed with the use of R software, version 3.2.0, 64 bit (Copyright 2015 by The R Foundation for Statistical Computing).

RESULTS

A total of 1272 blood samples were collected from 384 patients for dd-cfDNA measurement after transplantation at months 1–4, 6, 9, and 12, or at the time of kidney allograft biopsy and up to 2 follow-up samples within 8 weeks thereafter (Fig. 1). Of 1272 blood samples, 1137 met the entry requirements for dd-cfDNA testing (e.g., volume, storage and shipping time and temperature) and had clinical information available by the date of data lock for this study; a result was obtained for 1105 of the samples (97%). Ninety-three patients met the criteria for stable graft function (i.e., the reference population inclusion criteria) and had at

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least 3 serial blood samples that met requirements for dd-cfDNA testing and passed sample QC, resulting in 380 blood samples with dd-cfDNA results used for analysis.

The key clinical characteristics of the reference population selected from the DART cohort and the remainder of the DART population were representative of the spectrum of patients found in the entire US renal transplant population (n = 17 878) from the 2015 Organ Procurement and Transplantation Network (OPTN) registry (*13*) (Table 1). As expected, based on inclusion criteria, the reference population had significantly lower baseline serum creatinine and higher estimated glomerular filtration rate (eGFR) than the remainder of DART patients. Additionally, time to enrollment post-transplant was shorter, and prednisone dosing and tacrolimus blood levels were marginally higher in the reference population compared to the remainder of the DART cohort. These differences between the 2 DART subgroups are attributable to a subset of nonreference population DART patients who were enrolled at the time of clinical suspicion of rejection. This subset enrolled at a relatively later time posttransplantation, when their maintenance immunosuppressive drug regimens had been tapered.

The distribution of dd-cfDNA levels for all 380 samples is shown in Fig. 2. The upper limit of the range is depicted up to 2% and includes 6 values above 2%. About 96% of the samples exhibited dd-cfDNA values below 1.0%. The mean level of dd-cfDNA test results was 0.34% (±0.58% SD). The median dd-cfDNA was 0.21%, with the interquartile range of 0.12%–0.39%. The 97.5th percentile was 1.20%. The 6 test results with outlier values of >2.0% of dd-cfDNA are listed in the Fig. 2 legend; the maximum value of these 6 outliers was 8.1%. The median and range of dd-cfDNA from the 380 blood samples obtained from the 93 reference population renal transplant patients are presented in Fig. 3.

The biological variability was calculated for 18 of 93 patients for whom all test results were >0.2% dd-cfDNA, the lower limit of quantification for the assay. Values below 0.2% were not used because the large number of values near 0 would have an undue influence on CV calculations, and the resulting CV would not represent the variability of the test values in the range where clinical decisions may be made: above the lower limit of quantification and near the 97.5th percentile. For comparative purposes, the biological variation for cardiac markers of injury and other common clinical analytes are shown along with those for the dd-cfDNA assay in Table 2 (*14–17*). The CV_I and CV_G for dd-cfDNA were 21% and 37%, respectively. The II was 0.57, and the RCV was 61%. In Fig. 4, the median and range of dd-cfDNA are presented for the subset of 18 patients used for determining the biological variability parameters.

DISCUSSION

This is the first report to define the biological variability and reference intervals for dd-cfDNA in a nominally "normal" renal transplant population (i.e., patients with stable, well-preserved allograft function and no clinically overt evidence of active injury). This information about stable patients is essential and complementary to other study data that describe the levels of this biomarker in patients with active allograft rejection, based on biopsy-based histopathology. There have been limited prior reports of the range and biological variation of dd-cfDNA levels in renal transplant recipients (*2*, *18*). Larger number of samples per patient have been analyzed in studies of heart and lung transplant recipients (*4*, *5*). These studies, however, have not provided analyses of biological variability (e.g., CV_I, CV_G, II, or RCV), and the data were mostly from recipients studied at single transplant centers, by using research-grade assays.

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Table 1. Baseline characteristics of the DART study population.

	DART: all patients	DART: reference population	DART: remainder of other patients	P[a]	Renal transplant patients in the US[b]
Number of patients	384	93	291		17 878
Number of visits	1272	380	892		NA[c]
Number of visits per patient	3.3	4.1	3.1		NA
Race				0.60	
White	187 (49%)	52 (56%)	135 (46%)		8232 (46%)
Black or African-American	133 (35%)	27 (29%)	106 (36%)		5012 (28%)
Hispanic	32 (8%)	8 (9%)	24 (8%)		3119 (17%)
Native Hawaiian or other Pacific Islander	2 (1%)	1 (1%)	1 (0%)		79 (0.4%)
Asian	7 (2%)	1 (1%)	6 (2%)		1141 (6%)
Other	23 (6%)	4 (4%)	19 (7%)		295 (1.7%)
Male sex	220 (57%)	57 (61%)	163 (56%)	0.40	10 877 (61%)
Age at enrollment, years	49 ± 13	49 ± 13	50 ± 13	0.42	NA
Days posttransplant	553 ± 1262	32 ± 17	720 ± 1410	<0.001	NA
Donor type				0.73	
Living unrelated	72 (19%)	16 (17%)	56 (19%)		2972 (17%)
Deceased donor	248 (65%)	59 (63%)	189 (65%)		12 250 (69%)
Living related	63 (16%)	18 (19%)	45 (15%)		2656 (15%)
Sibling	26 (7%)	9 (10%)	17 (6%)		1024 (6%)
Child	13 (3%)	2 (2%)	11 (4%)		744 (4.1%)
Parent	8 (2%)	2 (2%)	6 (2%)		462 (2.6%)
Half sibling	1 (0%)	1 (1%)	0 (0%)		55 (0.3%)
Other biological blood relation	15 (4.1%)	4 (4.3%)	11 (4%)		362 (2%)
Cytomegalovirus serologic status				<0.001	
Donor−/Recipient+	86 (22%)	33 (35%)	53 (18%)		25% (deceased donor), 21% (living donor)
Donor+/Recipient+	125 (33%)	24 (26%)	101 (35%)		43% (deceased donor), 35% (living donor)
Donor−/Recipient−	52 (14%)	16 (17%)	36 (12%)		13% (deceased donor), 24% (living donor)
Donor+/Recipient−	44 (11%)	12 (13%)	32 (11%)		18% (deceased donor), 16% (living donor)
Unknown	77 (20%)	8 (9%)	69 (24%)		[d]
Creatinine, mg/dL	2.2 ± 1.6	1.5 ± 0.5	2.4 ± 1.8	<0.001	NA
eGFR, mL/min	42 ± 20	52 ± 16	39 ± 20	<0.001	NA
Human leukocyte antigen class 1 (A, B) number of mismatches	2.7 ± 1.2	2.8 ± 1.1	2.7 ± 1.3	0.35	[e]

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Table 1. Baseline characteristics of the DART study population. ***(Continued from page 314)***

	DART: all patients	DART: reference population	DART: remainder of other patients	P^a	Renal transplant patients in the US[b]
HLA class 2 (DR) number of mismatches	1.1 ± 0.7	1.2 ± 0.7	1.1 ± 0.7	0.30	[e]
Weight, kg	84 ± 22	85 ± 21	83 ± 22	0.61	NA
Height, cm	172 ± 19	173 ± 11	172 ± 21	0.60	NA
Prednisone dose, mg	16.7 ± 20.3	22.5 ± 28.1	14.8 ± 16.9	0.04	NA
Tacrolimus blood level, ng/mL	8.4 ± 3.9	9.4 ± 3.5	8.1 ± 4.0	0.006	NA

[a] The *P* values are the level of statistical significance in the differences of values found in the DART reference population and the values found in the remainder of DART patients. For continuous covariates, Wilcoxon signed rank test was used to generate the *P* values. For categorical covariates, Fisher exact test was used to generate the *P* values.
[b] Information from the Organ Procurement and Transplantation Network (*13*) and the Scientific Registry of Transplant Recipients 2014 (http://www.srtr.org) (accessed July 2016).
[c] NA, not available.
[d] Donor unknown/recipient positive: 0.2% (deceased donor), 1.9% (living donor), donor unknown/recipient negative: 0.1% (deceased donor), 1.4% (living donor), donor unknown/recipient unknown: 0% (deceased donor), 0.4% (living donor), Scientific Registry of Transplant Recipients 2014 [http://www.srtr.org] [accessed July 2016].
[e] Number of patients with A, B, and DR mismatches: 0 mismatches: 1235; 1 mismatch: 368; 2 mismatches: 1257; 3 mismatches: 2720; 4 mismatches: 4059; 5 mismatches: 4811; 6 mismatches: 2488; unknown mismatches: 160. Scientific Registry of Transplant Recipients 2014 (http://www.srtr.org) (accessed July 2016).

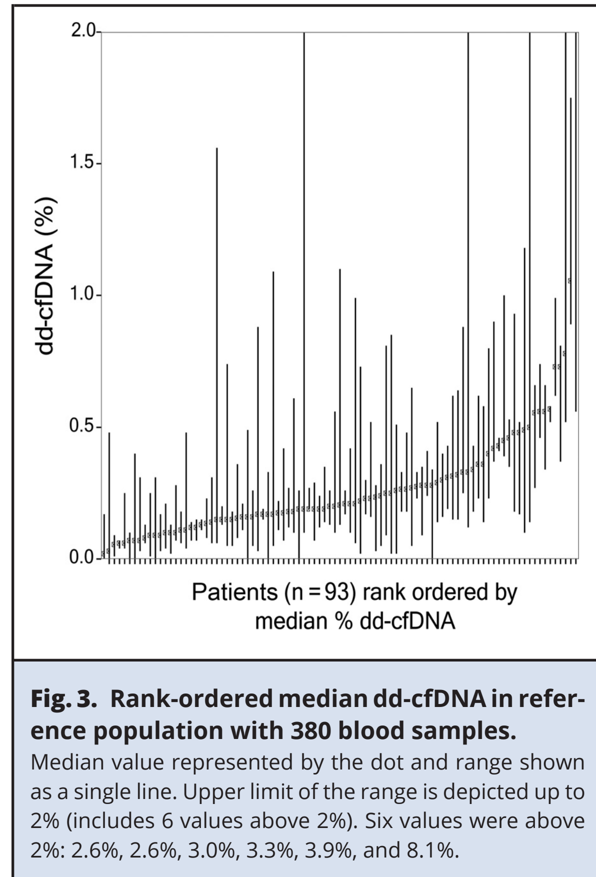

Fig. 3. Rank-ordered median dd-cfDNA in reference population with 380 blood samples.
Median value represented by the dot and range shown as a single line. Upper limit of the range is depicted up to 2% (includes 6 values above 2%). Six values were above 2%: 2.6%, 2.6%, 3.0%, 3.3%, 3.9%, and 8.1%.

The demographics of the reference population in this study are representative of the spectrum of patients who received renal transplants in the US in 2015 (Table 1). The proportions of women, various races, and allografts from living donors are well aligned with the general population of renal transplant recipients in the US. The 93 patients and 380 samples included in this reference population had no clinical evidence of rejection or other acute renal injuries and had an average of 4 serial measurements of dd-cfDNA levels, collected at 4–8 weekly intervals.

The results of this study establish that a cutoff of 1.0% dd-cfDNA delineates the 96th percentile of test results and cutoff of 1.20% delineates the 97.5th percentile cutoff in the "normal" renal transplant population. Outlier values are likely to be associated with acute rejection or other graft injury

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Table 2. Biological variation for dd-cfDNA, cardiac injury biomarkers, and other commonly measured analytes.

Biomarker, typical value[a]	CV_A, %	CV_I, %	CV_G, %	II	RCV, %	RCV, absolute[a]	Reference
dd-cfDNA, 1%	6.8	21	37	0.57	61	0.61%	This study
CK-MB, 174 IU/L	14	22	42	0.52	72.2	125 IU/L	Ross and Fraser (*16*)
Cardiac troponin I, 27 ng/L, short-term measurements	8.3	9.7	57	0.21	46 (log-normal increase)	12.4 ng/L	Wu et al. (*17*)
Creatinine, 100 μmol/L (1.1 mg/dL)	2.3	6.0	14.7	0.4	17.9	18 μmol/L (0.2 mg/dL)	Omar (*14*)
Hemoglobin A_{1c}, 4%	7.1	4.9	14	0.35	20.3	0.8%	Omar (*14*)
Glucose, 100 mg/dL	4.1	6.1	6.1	1.0	20.5	21 mg/dL	Omar[b] (*14*)
Alanine aminotransferase, 40 IU/L	5.3	25.5	69.7	0.36	72.1	29 IU/L	Fraser and Williams (*15*)

[a] Values shown (e.g., 1% for dd-cfDNA) are typical, but arbitrary, example values and units for each biomarker; these arbitrary values are used to compute the absolute RCVs.
[b] Modified from Omar (*14*).

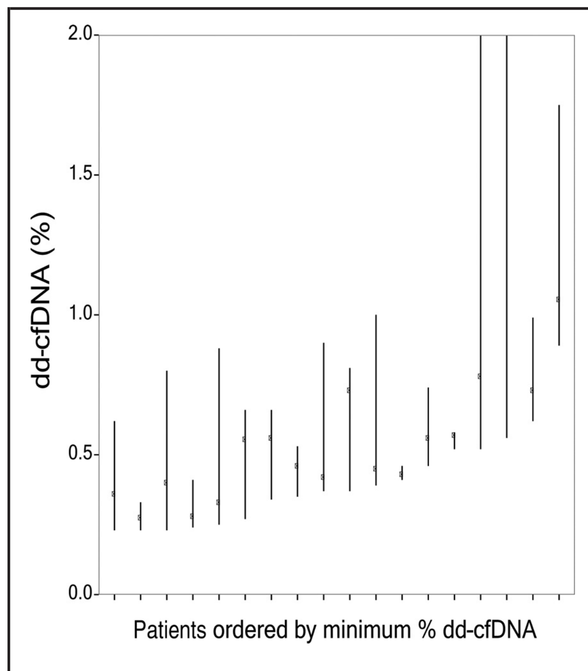

Fig. 4. Biological variability.
Median (dot) and range (line) for 18 patients with all 68 serial values of dd-cfDNA >0.2% (the limit of detection). Patients are rank ordered by minimum % dd-cfDNA. Upper limit of the range is depicted up to 2% (includes 3 values above 2%: 2.6%, 3.0%, and 8.1%).

(*1*, *6*). We previously reported in a pilot study that dd-cfDNA levels were significantly higher (P = 0.029) in 9 renal transplant patients with biopsy-confirmed rejection (mean 4.2%) compared to control samples taken from patients with no acute rejection (mean 0.96%). Longitudinal sample analysis from 15 patients showed increased dd-cfDNA levels after transplant (mean 2.8%) with reduction to levels below 1% by 2 weeks posttransplantation. In 3 cases with clinically treated biopsy-confirmed rejection and 2 serial samples following the rejection treatments, levels of dd-cfDNA decreased after rejection treatment (*19*).

A comparison of the biological variability indices for a number of common analytes provides perspective to the findings for dd-cfDNA in this report. As shown in Table 2, the dd-cfDNA CV_I of 21% and CV_G of 37% are higher than the respective values

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for creatinine (CV_I of 6.0% and CV_G of 14.7%). However, the dd-cfDNA II of 0.57 is marginally higher than the serum creatinine II (0.4) and the IIs of many other common analytes, e.g., cardiac troponin I (0.21), hemoglobin A_{1c} (0.35), and glucose (*1*). All of these analytes have IIs <0.6; therefore, the analyte intraindividual variability is less than the interindividual variability in the respective reference populations. An increase by over 61% from the immediate prior sample (with a quantitative value ≥0.2%, and using a value of 0.2 if the test result is below 0.2%, the lower limit of quantification) is sufficient to establish that an observed change in dd-cfDNA exceeds the biological variability of the test. Multiple test samples can be used to establish a homeostatic set point with variability lower than the biological variability of the test. This would reduce the amount of increase in dd-cfDNA necessary to be considered a significant change. For instance, a homeostatic set point with a CV of 10% can be established with 5 samples from an individual (*12*); this would lead to an RCV of 48% instead of 61%. The analytical precision of our dd-cfDNA assay (CV_A = 6.8%) is moderately less than that of a typical serum creatinine assay (CV_A = 2.3%) or a glucose assay (CV_A = 4.1%) and similar to the analytical precision for cardiac troponin assay (CV_A = 8.3%). The dd-cfDNA RCV = 61% is marginally higher than the RCV for creatinine (18%). However, the dd-cfDNA RCV is marginally lower and hence more favorable than that for creatine kinase MB isoenzyme (CK-MB) (RCV = 72.2%) and alanine aminotransferase (RCV = 72.1%).

Some investigators have recommended that an II of <0.6 should limit the interpretation of an individual's test results relative to a reference population while an II >1.4 is considered optimal (*12*, *20*). However, in Table 2, we observe that few existing common analytes meet the ideal II values. Indeed, reference population–based cutoffs are used for troponin and CK-MB for assisting in diagnosis of acute myocardial infarction, despite their II <0.4. Because the biological

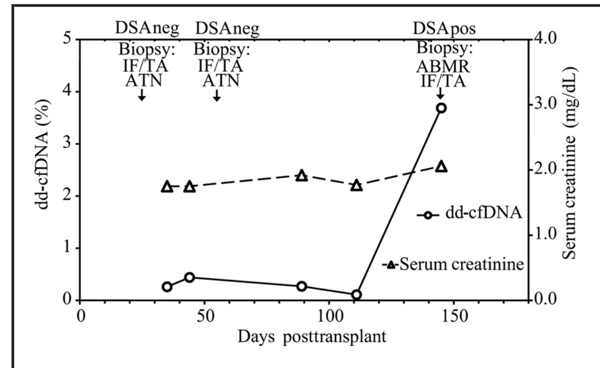

Fig. 5. A significant rise in dd-cfDNA was associated with acute/active ABMR in a renal allograft recipient.
dd-cfDNA level (3.7%) on day 145 posttransplantation significantly increased compared to 0.2% on day 111. Note the actual value on day 111 was below 0.2%, the lower limit of quantification. Serum creatinine rose from 1.77 to 2.06. Allograft biopsies on posttransplant days 30 and 60 (see arrows) revealed mild interstitial fibrosis/tubular atrophy (IF/TA) and focal areas of acute tubular necrosis (ATN). Biopsy on day 145 revealed ABMR. DSAs were negative (neg) at day 30 and 60 and positive (pos) on day 145.

variation in high-sensitivity cardiac troponin I is near the cutoff value for diagnosis of acute myocardial infarction, Wu has recommended that a serial test should be considered if a first test value is near the cutoff, but if a first result is well above the cutoff, one may be relatively confident that it is abnormal. Similarly, we suggest that if a first value of dd-cfDNA in an individual is well above the 1.0% (or 1.2%, for 97.5th percentile) cutoff, it is likely to be abnormal (*12*). Because the RCV is 61% for dd-cfDNA, we interpret this to be the relative change in serially measured values of dd-cfDNA that exceeds the difference attributable to biological variation. If a first dd-cfDNA is, for example 0.7%, a subsequent test result that is more than 61% higher (i.e., approximately 1.13%) may be considered a change that exceeds expected biological variation. Fig. 5 shows an example of a patient with a significant rise in dd-cfDNA that was associated with a

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biopsy-based diagnosis of acute/active antibody-mediated rejection (ABMR). This renal allograft recipient was excluded from the DART reference population cohort because of this event of active rejection. His dd-cfDNA level (3.7%) on day 145 posttransplantation was significantly increased compared to 0.2% on day 111 [1850% increase (3.7/0.2)]. Notably, the actual value on day 111 was below 0.2%, the lower limit of quantification. His serum creatinine rise to 2.06 was marginally significant [17% increase (2.06/1.77)]. Two prior allograft biopsies (performed on posttransplant days 30 and 60) revealed mild interstitial fibrosis/tubular atrophy and focal areas of acute tubular necrosis, while biopsy on day 145 revealed ABMR. Donor-specific antibodies (DSAs), one of the core criteria for the diagnosis of AMBR, were negative at days 30 and 60 and positive on day 145.

In this case, the serial change in dd-cfDNA of 1850% was well over RCV 61% and well over the 97.5th percentile of 1.2% for the reference population with stable allograft function. In contrast, the serum creatinine was only marginally increased from its prior measurement. It is plausible that additional interval measurements (e.g., every 2 weeks) of dd-cfDNA could have detected significant increases earlier, and thus the allograft injury eventually diagnosed as ABMR by biopsy-based histopathology (and the presence of DSAs in his plasma) may have been detected earlier.

We observe that dd-cfDNA has a higher II and an RCV similar or lower than that of CK-MB and cardiac troponin I. These cardiac biomarkers for injury represented in Table 2 are for autologous hearts. A high-sensitivity troponin has been retrospectively correlated to biopsy-based acute rejection events in heart transplants (*21*). The biological variability was not computed, but the range of troponin from a reference population (n = 88) with no history of acute rejection was relatively wide: the median troponin was 9.45 ng/L, the 60th percentile was 15 ng/L, and 10 outliers that ranged from 60.9 to 268 ng/L were excluded from the main data analyses (*21*). In contrast, our current study of a renal transplant reference population indicated that dd-cfDNA has a narrower range between its median of 0.21% and 96th percentile cutoff of 1%, and we did not exclude outliers. Thus, the more limited reference interval of dd-cfDNA values shown in this report seems to be more practical for clinical application than the relatively wider reference interval described for troponin in the heart transplant reference population.

As the half-life of cfDNA in the blood is <1 h (*22*), levels of dd-cfDNA have the potential to change within hours or days. The optimal time interval for serial monitoring of dd-cfDNA for surveillance of renal transplant patients remains to be defined, but monthly would be a feasible option for most patients, because that approximates the routine blood testing frequency in many transplant centers. Additionally, this test may be ordered on an ad hoc basis for a clinical suspicion of rejection or injury. As with all laboratory tests, clinical judgment within the patient's overall context is important in the interpretation of dd-cfDNA.

Additional clinical perspective

Patients with end-stage renal disease who receive a kidney transplant enjoy prospects of excellent allograft function and 1-year survivals. Acute T cell–mediated rejection rates have been significantly reduced, but antibody-mediated rejection appears to be an increasingly recognized problem particularly many months and years after transplant, so that substantial improvement in long-term survival still remains an unmet need (*23*). The loss of allograft function over time may be attributed to a variety of potentially avoidable and/or treatable causes of injuries. Thus, ongoing monitoring of kidney transplant function is a standard part of posttransplantation surveillance to evaluate if the transplant is stable or if there is evidence for dysfunction or injury. Acute rejection, infections, immunosuppression-related toxicity, or recurrent primary renal disease are sources of

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allograft injury that may not be detected in a timely manner by current standards of patient monitoring (*24*). While scheduled surveillance needle biopsy evaluation for renal allograft rejection is used by some, it has not been widely adopted because it is logistically challenging, inconvenient for patients, and optimal timing and frequency are not established; moreover, the benefits vs costs remain controversial. Serum creatinine is useful to estimate the glomerular filtration rate of the kidney, but it is not sensitive or specific. Significant irreversible injury may occur to the allograft before changes are reflected by an increase in serum creatinine (*25*). Urinalysis for proteinuria is also used for monitoring, but it likewise is neither sensitive nor specific for renal allograft dysfunction. The dd-cfDNA biomarker, in contrast to creatinine, is thought to be a measure of ongoing cell injury specifically in the allograft, and the magnitude of increase of this biomarker may be proportional to the severity of injury. Thus, as a biomarker for kidney injury, dd-cfDNA is akin to cardiac CK-MB, or cardiac myocyte-specific protein troponin, which have been established as quantitative biomarkers of acute heart injury such as myocardial infarction (*17*). As such, the dd-cfDNA assay may represent a new dimension for specific surveillance of renal allograft recipients for rejection and other injuries. Because the dd-cfDNA assay is practical to repeat monthly (or more often) in concert with standard-of-care blood testing frequency, the biomarker levels could also be useful to guide the short- and long-term tapering or maintenance of immunosuppression medications, and/or gauge clinical response to treatment of a rejection episode.

Limitations of this study

The number of patients suitable for the characterization of biological variability was limited (n = 18) because the rest of the 75 reference patients had 1 or more test values that were below the limit of detection. However, we do not find publications that have larger sample sizes than 18 for estimating biological variability of biomarkers (e.g., *17*). By design, the dd-cfDNA in the assay was measured as a fraction of total cfDNA. Thus, it is possible that perturbations to the turnover/death rate of cells originating from the recipient's tissues (that are unrelated to acute rejection or other direct injuries to the renal allograft) could confound the results and subsequently the interpretation of the % dd-cfDNA. Characterization of effects of miscellaneous other acute medical conditions is outside the scope of the current study, which identified patients who were stable and not having acute systemic inflammatory disorders such as sepsis. The very low levels of dd-cfDNA found were quantified in relation to the large amounts of cfDNA from the recipient; this method (ratio) has been used in the majority of published studies of dd-cfDNA, and reproducibly has been associated with rejection across organ types (*2*–*8*). Although determining levels of dd-cfDNA may not eliminate the need for biopsy to aid in the confirmation of a specific histopathology, its results could increase the prebiopsy probability of detecting injury, thereby making biopsy a more effective diagnostic tool.

In summary, this report sets essential foundation for interpretation of dd-cfDNA values as a new dimension in clinical monitoring of the health or injury status of the renal transplant allograft. The results reported here establish cutoffs for defining outlier abnormal values and the amount of change observed in serial measurements that may be attributed to biological variation vs changes that are likely to indicate the altered clinical status of the allograft (*14*).

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Additional Content on this Topic

Digital Droplet PCR for Rapid Quantification of Donor DNA in the Circulation of Transplant Recipients as a Potential Universal Biomarker of Graft Injury
Julia Beck, Sarah Bierau, Stefan Balzer et al. Clin Chem 2013;59:1732–41

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

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