Validation and clinical outcome in assessing donor-derived cell-free DNA monitoring insights of kidney allografts with longitudinal surveillance (ADimirAL) study.

**Cohort**
- 1094 patients
- 7 transplant centers
- Single Kidney Adult Recipients
- 90% deceased donor
- Multiorgan and pregnancy excluded
- 3 yrs. post transplant dd-cfDNA surveillance
- Median of 6 results per patient

**Methods**
- Post transplant events
- Centralized biopsy reporting
- Center Standard of Care protocols
- 3 years of outcomes surveillance

**Outcomes**
- **eGFR Decline**
  - Persistently elevated dd-cfDNA (>1 result above 0.5%) predicted a > 25% decline in eGFR over 3 years
- **Temporal Relationship**
  - dd-cfDNA values ≥ 0.5% were associated with a nearly 3-fold increase in the risk of development of de novo donor specific antibody (DSA)
- **Subclinical Rejection**
  - Significant elevations in dd-cfDNA during rejection ahead of changes in serum creatinine

**CONCLUSION:** The ADMIRAL study demonstrates a broad utility of dd-cfDNA as a leading indicator ahead of clinical presentations of allograft injury, formation of dnDSA, eGFR decline and subclinical rejection.
Title Page

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Validation and clinical outcome in assessing donor-derived cell-free DNA monitoring insights of kidney allografts with longitudinal surveillance (ADMIRAL) study.

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Running headline: dd-cfDNA longitudinal surveillance outcome
Abstract

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 (inter quartile 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.

Data Statement: Raw data is available from the study investigators upon request.

Keywords: donor-derived cell free DNA, kidney transplant, biomarker, rejection surveillance, allograft injury, allograft quiescence
Introduction

The deployment of nucleic acid-based non-invasive 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 (DSA). 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 non-invasive 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, ClinicalTrials.gov ID NCT04566055), is a large, multicenter, observational cohort study of kidney transplant (KT) recipients monitored with dd-cfDNA for up to three years. The purpose of this study was to validate clinical trial data 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 non-immune 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 (dnDSA).

2. Methods

2.1 Study Population

1092 adult KT recipients across seven transplant centers were monitored with AlloSure dd-cfDNA (CareDx Inc., Brisbane, CA) as part of their standard of care. Data was collected between June 2016 and January 2020. An IRB waiver of informed consent was obtained, 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, donor specific antibodies) 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, this includes 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.

2.2 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 (CLIA)-certified laboratory at CareDx, Inc. (Brisbane, CA). Details of the standardized specimen processing and analytical methods to determine the percentage of dd-cfDNA (AlloSure®) have been published. The targeted next-generation sequencing assay employs highly polymorphic single nucleotide polymorphisms (SNPs) to quantify dd-cfDNA without need for separate genotyping of the recipient or the donor.

2.3. Diagnosis of graft dysfunction and biopsy-defined rejection

Results of protocol surveillance and for-cause renal transplant biopsies were captured. Indications for ‘for-cause’ biopsy included change in creatinine, worsening proteinuria, and/or development of dnDSA. 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, 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 and the TCMR group did not include borderline. A detailed breakdown of the biopsy findings is provided in Supplementary Table S3.

Other concomitant pathologic diagnoses, such as calcineurin inhibitor (CNI) toxicity, glomerulopathy, or acute tubular injury/acute tubular necrosis 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, dnDSA 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.

2.4 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, NPV, PPV). 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 two-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 (Supplementary Table S4 for model variables). Potential confounding factors were evaluated to ensure interpretation was robust. Statistical analysis was performed in R [Core Team (2020) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/].

Patients and samples were included into non-mutually 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 questions has been outlined in this figure, including the total biopsies taken and the breakdown of results used for analysis.

2.4.1 dd-cfDNA and eGFR Analyses

Renal function was determined by eGFR calculated using the MDRD 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 more than one eGFR measurement was available each month the average was taken. Wong et al, provided an update to the analytical variation and intra-patient variation of AlloSure dd-cfDNA.\[^{12}\] This was used to calculate the serial delta/RCV between dd-cfDNA results associated with pathology using the methods outlined by Lund et al.\[^{13}\]

Analytical variation (CVa) was defined as 2.7%, intra patient variation (CVi) = 61% and the index of individuality = 0.23%.\[^{12}\] K-means Clustering (KM),\[^{14}\] was used, distinct clusters representing timepoints allowed the formation of time horizons from 0 to 3-years post-transplant. The machine learning algorithm, partitioned data into monthly clusters, pre-determined by minimizing sum of squared distance using key features such as ethnicity, sex, age at transplant, evidence of BK infection, dd-cfDNA score, presence of
DSA, allograft rejection and creatinine. Intra-cluster 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: 0-4 months, 4-12 months, and 12-36 months. More information is provided in *Supplementary Methods*.

### 2.4.2 dd-cfDNA and dnDSA Analyses

The relationship between dd-cfDNA and development of dnDSA was assessed in patients with paired dd-cfDNA and HLA DSA testing (both tests drawn at the same time). All patients started with a no identified DSA and normal AlloSure (<0.2%). 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 (MFI) of >500 was agreed to be positive, for both HLA class 1 and class 2, and was used for this analysis.[^15] Freedom from dnDSA was assessed using Kaplan-Meier analysis and once patients developed DSA 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-cfDNA with the development of dnDSA (*Supplementary Table S5 for model variables*).

### 2.4.3 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, UTI, proteinuria, allograft rejection or recurrent focal segmental glomerulosclerosis (FSGS), as confirmed by paired biopsy ≤30 days after dd-cfDNA measurement.

3. Results

The demographic characteristics of the 1,092 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 comprised of a numerically higher percentage of African American recipients (28% vs 24%; p=0.78) and fewer re-transplant candidates (8% vs 13%; p=0.16). There was also a higher proportion of deceased donor recipients in this study compared with the UNOS registry (94% vs. 68%; p=0.04).

3.1 Association of dd-cfDNA level and acute rejection

The analytic sample included 5,873 dd-cfDNA measurements from 1,092 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. 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. 16% of local biopsies were rescored by the central pathologist.

There was no statistically significant difference in the median creatinine in patients with a No Rejection biopsy (1.38 mg/dL; 95% CI 1.26-1.67 mg/dL) and patients with Banff defined Rejection (1.57 mg/dL; 95% CI 1.1-2.2 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% (95% CI 0.22-0.38%) which was significantly lower than the median dd-cfDNA in patients with biopsies demonstrating defined cellular or humoral rejection (1.6%; 95% CI 1.1-3.7%), 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 75 biopsies (40 for-cause, 35 surveillance) from 67 patients. Among these patients, the median dd-cfDNA was 1.8%, p<0.001, compared to non-rejection patients. TCMR was diagnosed in 38 biopsies (28 for-cause, 10 surveillance) from 34 patients. Patients with TCMR had a median dd-cfDNA value of 0.7%, p<0.001, compared to non-rejection patients. The median dd-cfDNA in patients with Borderline TCMR (t1 i1: Banff scores) was 0.23% (CI 0.12-0.76). More information on performance and distributions of rejection groups is provided in Supplementary Figure S2, Figure S3, Figure 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%; QR: 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 non-rejection biopsies, p<0.001. See Supplementary Figure S5 for cumulative distributions.
dd-cfDNA discriminates between 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 of 1.89 (95% CI 1.78-2.1).

**3.2 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 renal function between month 12 and month 36 demonstrated a correlation between the elevation of dd-cfDNA and subsequent decline in renal 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 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.

**3.3 Relationship between dd-cfDNA level and identification of dnDSA**

961 patients with paired dd-cfDNA and HLA DSA results had no pre-existing DSAs. The median calculated panel reactivity antibody (cPRA) was 37% (IQR 11-77%). The median number of paired DSA and dd-cfDNA samples per patient was 5 (IQR 3-9). Of these patients, dnDSA were found in 44 (4.6%) patients, 19 with class I and 25 with class II, 9 of whom also had histologic evidence of allograft rejection. dd-cfDNA above 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 dnDSA (HR 1.19, p =0.004). Serial examination of dd-cfDNA values in patients who developed dnDSA, demonstrated a median 121% (IQR 69%-183%) increase in dd-cfDNA from prior dd-
cfDNA result, which occurred a median of 91 days (IQR 30-125 days) preceding detection of dnDSA. Furthermore, dd-cfDNA remained elevated in all cases with measurable dnDSA.

3.4 Association of dd-cfDNA elevation and graft injury

A composite state of “graft injury” defined as one or more of 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 (FSGS) 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 “Quiescent” category. Shown in Figure 5, the median dd-cfDNA level in the Quiescent (non-injury) 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 of 1.48 mg/dL (95% CI 1.1-2.71mg/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 PPV of 77.5% and NPV of 71.6% for graft injury. In addition to the absolute value, the delta/RCV 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).

4. 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 dnDSA. Most importantly, low dd-cfDNA predicted “allograft quiescence,” which can 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 non-alloimmune 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 which 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 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 0.23%, with wide Confidence Intervals, 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/RCV 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 graft injury. This finding has previously been shown by Stites et al.\textsuperscript{[4]} While a measured level above 0.5% and/or the increase of 149% from baseline does not definitively prove injury, these changes suggest patients should have intensive surveillance, further diagnostic study, and/or potential intervention. 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 \textit{The Circulating Donor-Derived Cell-Free DNA in Blood for Diagnosing Acute Rejection in Kidney Transplant Recipients (DART) study (ClinicalTrials.gov Identifier: NCT02424227)}.\textsuperscript{[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 at least 0.24% (IQR: 0.19%-0.39%). In other studies, Anand et al. demonstrated that an increase in dd-cfDNA of at least 141% was associated with abnormal pathology, supporting the 149% threshold reported here.\textsuperscript{[17]} These data suggest that routine post-transplant surveillance with dd-cfDNA, which utilizes 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.\textsuperscript{[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, broadly “allograft quiescence”. dd-cfDNA less than 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 dnDSA has been correlated with decreased allograft survival, even in the absence of clinically evident ABMR. \[20\] However, while many patients develop dnDSA, not all dnDSA results in significant allograft injury. In a recent prospective, multicenter study of 123 patients who were biopsied after the development of dnDSA 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 dnDSA. 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, identified 87 kidney transplant patients with 90 clinically indicated biopsies along with paired dd-cfDNA and DSA testing.\[24\] In patients with dnDSA with ABMR, the average dd-cfDNA was 2.9% compared with 0.34% in patients with dnDSA without ABMR, and 0.29% in patients without dnDSA. 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 which could complement dnDSA monitoring, by identifying clinical and subclinical ABMR in kidney transplant patients. 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.\textsuperscript{[26]} Furthermore, the temporal observation between dd-cfDNA elevations and dnDSA warrants further investigation to assess both the etiology of dnDSA formation\textsuperscript{[26]} and the potential for therapeutic intervention. Huang et al, previously demonstrated histologic features of ABMR in patients with elevations in dd-cfDNA that did not have any appreciable HLA antibodies.\textsuperscript{[27]} In addition, non-HLA transplantation immunity revealed by lymphocytotoxic antibodies has been well published with Crespo et al showing the importance of AT1R in patients with ABMR DSA negative patients.\textsuperscript{[28, 29]} Thus, the utility of dd-cfDNA in the assessment of non-HLA DSA needs to be considered and, although not performed in this analysis, is planned from patients with stored serum. With the pathogenicity of non-HLA DSA still being determined, the use of dd-cfDNA in its assessment, may be a useful tool for future studies.\textsuperscript{[30]}

Both Clayton et al and Faddoul et al have reported that a decline in eGFR is superior to other surrogate measures of long-term for kidney transplant outcomes.\textsuperscript{[31, 32]} 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.\textsuperscript{[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 regards 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 up to 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 upon 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 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 dnDSA, eGFR decline and subclinical rejection. Additional interventional studies\textsuperscript{[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 dnDSA.
Disclosures

Lihong Bu declares she has no conflict of interest; drafted or revised the manuscript, role in results analysis

Gaurav Gupta serves on the scientific advisory board of CareDx; has received honoraria/grant support from Alexion, CareDx, Mallinckrodt, Natera, Veloxis, Gilead, NIH/NIDDK, Mendez Foundation; drafted or revised the manuscript, acquired data, role in results analysis

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Irfan Moinuddin declares that he has no conflict of interest; acquired data

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David A. Axelrod is a member of the CareDx National Scientific Advisory Board; consultant for CareDx and Talaria; drafted or revised the manuscript, role in results analysis

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**Figures:**

**Figure 1:** Consort flow diagram showing the total data and how it was used to perform all the analyses in the study.

**Figure 2:** Box and Whisker plot showing the median dd-cfDNA and creatinine levels observed in patients with and without allograft rejection. Panel A show the ROC analysis for dd-cfDNA, AUROC 0.798, with a median of 0.23% seen in no rejection patients and 1.6% in allograft rejection patients, p<0.0001. Panel B shows the ROC analysis, for creatinine, AUROC 0.492, with a median creatinine of 1.38mg/dL in no rejection patients versus 1.57mg/dL in rejection patients, p=0.096.

**Figure 3:** 1092 patients’ eGFR trends over the first 36 months post-transplant with a significant decline between 12 and 36 months (Spearman coefficient of -0.84).

**Figure 4:** Free from DSA model, with cox proportional hazard showing risk of dnDSA. 44 events were observed from 961 patients, with 153 patients starting with dd-cfDNA levels ≥0.5% compared to 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). Rejection patients were not included in the analysis or other events leading to censoring (death, loss to follow up, etc.).

**Figure 5:** Box and whisker plot showing the median dd-cfDNA and creatinine levels considering a total of 647 patients, with 467 patients developed allograft injury and 180 patients clinically stable with immune quiescence (IQ). Panel A show the ROC analysis for dd-cfDNA, AUROC 0.727, with a median of 0.21% seen in non-injury patients and 0.51% in injury patients. P<0.0001. Panel B show the ROC analysis for creatinine, AUROC 0.572, with a median creatinine of 1.32 mg/dL in non-injury patients versus 1.48mg/dL in injury patients p=0.08.
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Supplementary Material

Supplementary Table S1: Data variables collected: Donor (D) and Recipient (R) Variables

Supplementary Table S2: Center standard of care protocols

Supplementary Table S3: Biopsy Banff 2019 lesion scores used in the Rejection Analysis.

Supplementary Table S4: Univariate and Multivariate Regression Model for Allograft Rejection

Supplementary Table S5: Univariate and Multivariate Regression Model for dnDSA

Supplementary Figure S1: Histogram for the number of days between dd-cfDNA sampling and biopsy

Supplementary Figure S2: Distributions of dd-cfDNA in No Rejection, ABMR and TCMR groups

Supplementary Figure S3: Performance of dd-cfDNA in allograft rejection

Supplementary Figure S4: Distribution of RCV results for stable patients and those with rejection

Supplementary Figure S5: Cumulative distribution of dd-cfDNA levels in biopsies

Supplementary Methods: K-means Clustering (KM) methods
Table 1: Demographics of the ADMIRAL cohort compared to UNOS 2020/21 published data

| Variable              | ADMIRAL | UNOS 2020/21 | 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 Tx (years)     |         |              |                        |
| Mean                  | 49.5    | 46.7         | 0.16                   |
| Min-Max Range         | 17-84   | 0-96         | 0.22                   |
| Re-transplant         | 8%      | 13%          | 0.16                   |
| Weight (kg)           | 84      | 77           | 0.34                   |
| Height (cm)           | 170     | 168          | 0.46                   |
| Median eGFR           | 69m/min/1.73m² | 73 m/min/1.73m² | 0.52 |
| Serum Creatinine      | 1.52 mg/dL | 1.63 mg/dL | 0.12 |
| cPRA       | Mean  | >80%  | Not Available | N/A |
|-----------|-------|-------|---------------|-----|
|           | Mean  | 34%   | Not Available | N/A |
| Range     | 1-96% | Not Available | N/A |
| Median number of AlloSure tests per patient | 6 | Unknown | N/A |

**Donor Variables**

| Donor age (years) | Mean  | 40.7 | 37.7 | 0.92 |
|-------------------|-------|------|------|------|
| Range             | 0-72  | 0-88 | 0.75 |
| Donor Sex         | Female| 45%  | 46%  | 0.86 |
|                   | Male  | 55%  | 54%  | 0.67 |
| Donor relation    | Unrelated | 94% | 67% | 0.03 |
| Deceased Donor    |       | 94%  | 68%  | 0.04 |
Table 2: Demographics of the ADMIRAL rejection cohort compared to the no rejection cohort

| Variable                  | 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 Tx (years)         | Mean                   | 50.7                | 45.8     | 0.69     |
|                           | Min-Max Range          | 22-78               | 12-75    | N/A      |
| Re-Transplant             |                        | 14%                 | 25%      | 0.88     |
| Weight (kg)               | Mean                   | 80.2                | 84       | 0.67     |
| eGFR (ml/min/1.73m^2)     | Median                 | 53.3                | 47       | 0.41     |
| Serum Creatinine (mg/dL)  | Median                 | 1.37                | 1.55     | 0.34     |
| Number of AlloSure Test Per Patient | Median | 6 | 6 | 0.99 |
| CPRA                      | Mean                   | 34%                 | 41%      | 0.78     |
|                           | Range                  | 1-96%               | 3-100%   | N/A      |
| Donor Variables           |                        |                     |          |          |
| Donor Age (years)         | Mean                   | 42.8                | 40.4     | 0.62     |
|                           | Range                  | 22-55               | 19-49    | N/A      |
| Donor Sex                 | Female                 | 20%                 | 80%      | 0.03     |
|                           | Male                   | 28%                 | 72%      | 0.04     |
| Donor Relation            | Unrelated              | 69%                 | 67%      | 0.73     |
| Deceased Donor            | DCD                    | 53%                 | 47%      | 0.51     |
|                           | DBD                    | 73%                 | 27%      | 0.03     |
Table 3: Performance characteristics of AlloSure dd-cfDNA to discriminate allograft rejection. Non-rejection pathologies were excluded. This data compares 113 rejection biopsies vs 106 no rejection biopsies

| Diagnosis with % Threshold | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|----------------------------|----------------|----------------|---------|---------|
| All Rejection (0.5)        | 80             | 59             | 54      | 89      |
| All Rejection (1.0)        | 64             | 73             | 59      | 87      |
| ABMR (0.5)                 | 79             | 58             | 52      | 88      |
| ABMR (1.0)                 | 65             | 73             | 58      | 86      |
| TCMR (0.5)                 | 90             | 50             | 23      | 99      |
| TCMR (1.0)                 | 60             | 63             | 21      | 87      |
Table 4: Consideration of dd-cfDNA as a molecular marker of injury where the absence of injury is identified as quiescence. This is a comparison of 167 patients with injury against 180 patients who were quiescent.

| 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    |
Table 1: Demographics of the ADMIRAL cohort compared to UNOS 2020/21 published data

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|                          | >80%  | 34%   |                       |     |
|                          |       | 16%   |                       |     |
|                          | Range | 1-96% | Not Available         | N/A |
| Median number of AlloSure tests per patient | 6     | Unknown |                     | N/A |
|                          |       |       |                       |     |
| **Donor Variables**      |       |       |                       |     |
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| **Race**                        |                        |                     |          |
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| Caucasian                       | 62%                    | 38%                 | 0.05     |
| Hispanic                        | 59%                    | 41%                 | 0.41     |
| Other                           | 0                      | 100%                | 0.01     |
| **Age at Tx (years)**           |                        |                     |          |
| Mean                            | 50.7                   | 45.8                | 0.69     |
| Min-Max Range                   | 22-78                  | 12-75               | N/A      |
| **Re-Transplant**               |                        |                     |          |
|                                | 14%                    | 25%                 | 0.88     |
| **Weight (kg)**                 |                        |                     |          |
| Mean                            | 80.2                   | 84                  | 0.67     |
| Height (cm)                     |                        |                     |          |
| Mean                            | 168.2                  | 169.7               | 0.89     |
| **eGFR (ml/min/1.73m²)**        |                        |                     |          |
| Median                          | 53.3                   | 47                  | 0.41     |
| **Serum Creatinine (mg/dL)**    |                        |                     |          |
| Median                          | 1.37                   | 1.55                | 0.34     |
| **Number of AlloSure Test Per Patient** |                     |                     |          |
| Median                          | 6                      | 6                   | 0.99     |
| **CPRA**                        |                        |                     |          |
| Mean                            | 34%                    | 41%                 | 0.78     |
| Range                           | 1-96%                  | 3-100%              | N/A      |
| **Donor Variables**             |                        |                     |          |
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| 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    |
Figure 2:

A. dd-cfDNA discriminate rejection

B. Creatinine discriminate rejection

A. dd-cfDNA

B. Creatinine

P<0.0001

P = 0.096
Figure 3:

*AS = dd-cfDNA (AlloSure)
**Figure 4:**

The Kaplan-Meier survival curve shows the percentage of patients free from dnDSA events post 1st AlloSure test. The x-axis represents days post 1st AlloSure test, while the y-axis indicates the percentage of patients.

| Days post 1st dd-cfDNA | 0-200 | 200-400 | 400-600 | 600-800 | 800-1000 | >1000 |
|------------------------|-------|---------|---------|---------|----------|-------|
| **Low (<0.5)**         | 808   | 801     | 797     | 796     | 796      | 796   |
| (Censored Event of dnDSA) | 7     | 4       | 1       | 1       | 1        |       |
| **High (≥0.5)**        | 153   | 139     | 123     | 122     | 121      | 121   |
| (Censored Event pf dnDSA) | 14    | 16      | 1       | 1       | 1        |       |
Figure 5:

(A) Allosure dd-cfDNA discriminates injury with an AUC of 0.727.

(B) Creatinine discriminates injury with an AUC of 0.575.

(A) Box plots showing significant differences in dd-cfDNA levels between IQ and injury groups (P<0.0001).

(B) Box plots showing a trend in creatinine levels between IQ and injury groups (P = 0.08).