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Donor-derived Cell-free DNA Complements De Novo Class II DSA in Detecting Late Alloimmune Injury Post Kidney Transplantation

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Background. We sought to evaluate the association between de novo donor-specific antibodies (dnDSAs) class and their mean fluorescence intensity (MFI) with donor-derived cell-free DNA (dd-cfDNA), aiming to further clarify the biomarker utility of these noninvasive tests in relation to renal allograft function and histology. **Methods.** The study included kidney transplant recipients (n = 171) who underwent surveillance testing with DSA and dd-cfDNA as part of their clinical care between September 2017 and December 2019 at our center. **Results.** We identified dnDSA in 43 patients (25%) at a median of 4.63 y (IQR, 1.5–7) posttransplant. The presence of DSA with MFI >2500 was associated with a median dd-cfDNA of 0.96% (IQR, 0.26–2.95) significantly higher than in patients with DSA MFI <2500 (0.28%; IQR, 0.19–0.39) or without detectable DSA (0.22%; IQR, 0.17–0.37; $P < 0.001$). Class II dnDSAs were the most prevalent dnDSA (88.3%), the majority with MFI >2500 (82.9%). Patients with DQ-dnDSAs (47.4%) had higher MFI and dd-cfDNA levels than other class II dnDSAs. By comparison, all patients that developed only class I DSAs had MFI <2500 and a low dd-cfDNA. In addition, the serum creatinine was 1.55 ± 0.48 mg/dL in those dnDSA-negative, 1.15 ± 0.37 mg/dL in those with dnDSA MFI <2500, and 1.53 ± 0.66 mg/dL in those with dnDSA MFI >2500 ($P = 0.05$). After multivariate adjustment, an elevated dd-cfDNA was independently associated with the presence of dnDSA with MFI ≥2500. We identified that both dd-cfDNA and dnDSAs were strongly associated with antibody-mediated rejection, whereas for individual Banff histological lesions, DSA MFIs ≥2500 had the strongest association with C4d staining score and dd-cfDNA >1% with microvascular inflammation. **Conclusions.** Our study identifies class II dnDSA as being strongly associated with late alloimmune injury post kidney transplant independent of allograft dysfunction and shows that dd-cfDNA may complement the clinical significance of dnDSAs.

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INTRODUCTION

Despite improvements in short-term allograft survival over the past decades, the alloimmune injury remains a major contributor to late graft loss in kidney transplant recipients.[1,2] The impact of acute rejection episodes to death-censored graft failures varies among studied patient cohorts according to immunological risk of their subjects and timing and duration of posttransplant follow-up. Nonetheless, in relation to late allograft injury, antibody-mediated rejection (AMR) has emerged as the leading immunological cause of renal graft loss.[3-6]

Anti-HLA donor-specific antibodies (DSAs) and, increasingly acknowledged, other non-HLA antibodies are associated with histological phenotypes attributed to antibody-mediated injury.[7,8] De novo DSAs (dnDSAs) occur in 15% to 30% of patients several years posttransplant, are related to episodes of clinical or subclinical rejection, and may outline patient specific immune responses, adequacy of immunosuppressive exposure, or nonadherence.[5]

A wide variability of DSA-associated allograft injury exists, ranging from absence of notable pathological lesions to classical histological changes of AMR.[9] Renal dysfunction is not

an implicit defining feature of DSA presence, particularly when detected as part of a surveillance protocol.[10] Because the signs of allograft dysfunction (elevated serum creatinine or proteinuria) are neither sensitive nor specific for rejection, allograft biopsy has remained the gold-standard method to diagnose alloimmune-mediated injury[11]; however, the use of renal biopsy as a surveillance method is controversial because "protocol" biopsies have a low detection rate of subclinical rejection that may not justify the procedure risk.[12,13] Similarly, "for-cause" biopsies may be biased toward capturing advanced lesions that are resistant to therapy.[14]

Irrespective of histological changes at DSA diagnosis, there is an increasing body of evidence that links the presence of dnDSAs, especially those directed against HLA class II antigens, to poorer long-term graft outcomes.[1,2] Wiebe et al described several clinical and pathological phenotypes among patients with dnDSAs, with a lower 10 y graft survival than patients who did not develop dnDSAs (57% versus 96%).[5]

It is increasingly recognized that antibody-mediated injury may not be clinically evident[5,9]; however, patients with dnDSA and stable renal function were more likely to experience progressive renal dysfunction when their biopsies showed higher scores for microvascular inflammation (glomerulitis and peritubular capillaritis) and C4d deposition compared with patients without significant deterioration in kidney function or those without dnDSAs.[5,9] In addition, DSA-induced severe tubular atrophy and interstitial fibrosis were associated with allograft loss independent of histologic lesions found in AMR.[15] This suggests that DSA-associated allograft injury may not always be detected by histologic assessment, especially in early injury phases, and that additional biomarkers are needed.

In solid organ transplantation, donor DNA fragments are released in recipient circulation as a consequence cell apoptosis or necrosis and can be quantified as the donor-derived fraction of total cell-free DNA (dd-cfDNA).[16] The dd-cfDNA has been proposed as a candidate biomarker of allograft injury that may allow more frequent and quantitative assessments due to its noninvasiveness.[11,14] A recent review outlined several studies reporting consistent association between acute rejection and dd-cfDNA levels.[14] In a pilot validation cohort trial, dd-cfDNA was able to accurately discriminate between patients with acute rejection, especially AMR, and those without rejection, outperforming other measures of graft function, particularly serum creatinine.[17] In addition, dd-cfDNA appears to correlate better with the severity of microvascular inflammation as opposed to tubulointerstitial lesions, the close proximity between microvascular endothelial cells and the circulation being proposed as an explannation.[18]

In this project, we sought to evaluate the association between the development of dnDSAs and their characteristics with dd-cfDNA and histology to gain further insight in the biomarker utility of these noninvasive tests in kidney transplantation.

MATERIALS AND METHODS

Study Design and Population

We studied a cross-sectional cohort of patients who underwent DSA screening and dd-cfDNA testing as part of their standard of care between September 2017 and December 2019 at the University of Washington. Patients with multiorgan or retransplantation were excluded. This study was done with the approval of the institutional review board of the University of Washington (STUDY00009002).

The cohort of patients enrolled in this study followed our institutional standards of DSA monitoring postkidney transplantation at 3, 6, and 12 mo, with anniversary visits or at the time of biopsy for graft dysfunction or proteinuria.

DSA Detection and Categories

DSA screening was performed by Luminex technology (FLEXMAP 3D platform) using single antigen beads (One Lambda, CA). DSAs were reported at a mean fluorescence intensity (MFI) cutoff of 500.

At our center, HLA antibodies >2500 MFI are considered clinically significant from an unacceptable antigen perspective. Based on our previous analyses, DSAs with MFI <2500 had limited clinical significance and, for the purpose of this study, were analyzed separately.

Clinical Assessment and Donor-derived Cell-free DNA Assay

The clinical assessment at the time of dd-cfDNA testing consisted of demographic data (age, gender), transplant characteristics (time from transplant, type of transplant, induction, and maintenance immunosuppression), presence, type, and titer (MFI) of DSAs, renal function (serum creatinine and estimated glomerular filtration rate), proteinuria and renal biopsy indication if applicable, as well as pathological diagnoses.

Measurement of dd-cfDNA was done as described in the DART trial[17] using targeted next-generation sequencing assay. This method utilizes 266 single-nucleotide polymorphisms to quantify the dd-cfDNA without the need to separately genotype the donor and recipient (Allosure test, CareDx Inc).[17] The result was expressed as a fraction of total cell-free DNA, and a threshold of 1% was used for sample classification as positive or negative (quantifiable range of 0.1%–16%). The dd-cfDNA testing was done for cause in the context of graft dysfunction or development of dnDSA or at the time of graft biopsy.

Renal Allograft Biopsy Assessment

Clinically indicated biopsies were performed in cases of renal dysfunction, proteinuria, or elevated DSAs. Pathological diagnoses were made according to the 2017 Banff Kidney Rejection Classification.[19] The patients were stratified on whether they had AMR (acute-active AMR or chronic-active AMR), T cell–mediated rejection, or both. In addition, each individual histologic component of AMR and T cell–mediated rejection were analyzed separately to test their association with dd-cfDNA level. The kidney allograft biopsies were evaluated by a renal pathologist who was unaware of the result of dd-cfDNA level measurement.

Statistical Analysis

Continuous variables were expressed as either mean ± SD or median (25th; 75th percentiles), according to their distribution, and categorical variables were expressed as percentages. Differences between groups were assessed in the case of continuous variables by the Student *t* test, Mann-Whitney test, 1-way ANOVA, or Kruskal-Wallis test, according to their distribution of dependent variables and the level of

independent variables, and in the case of categorical variables by the Pearson $\chi 2$ test or Fisher exact test. Univariate and multivariate logistic regression analyses were performed to identify variables associated with the development of dnDSA with MFI >2500. Additionally, in the subgroup of patients with an allograft biopsy, to identify which variable was the strongest predictor, elevated dd-cfDNA and the presence of DSA with MFI >2500 were chosen as independent variables, whereas outcome variables were represented by rejection type or each individual histologic lesion. The results of logistic regression analysis are depicted as odds ratios (ORs) with the corresponding 95% confidence interval (95% CI).

Statistical analyses were performed using the SPSS program (SPSS version 20, Chicago, IL) and XLSTAT (Addinsoft 2019, XLSTAT statistical and data analysis solution; https://www.xlstat.com; Boston, USA).

RESULTS

Baseline Characteristics of the Study Cohort, DSA Characteristics, and Relationship With dd-cfDNA

Between September 2017 and December 2019, 171 patients were screened for the presence of dnDSAs and had concurrent dd-cfDNA determination. Forty-three patients (25.1%) were found to have dnDSAs at a median 4.63 y (IQR, 1.5–7) posttransplantation. Baseline patients' characteristics are depicted in Table 1. Thirty-two patients have developed dnDSAs with at least 1 DSA with MFI levels >2500. These patients were younger and were further away from transplant than patients with DSA MFI <2500 and those with negative DSAs. The majority of patients that developed class II dnDSAs (82.9%) and all patients with both class I/II dnDSAs had an MFI level >2500 (median, 11 900; IQR, 2800–22 900 and 18 600; IQR, 9500–23 700), whereas all patients with only class I DSAs had an MFI <2500 (median, 2000; IQR, 1500–2150). DSA class and titer were significantly associated with dd-cfDNA level ($P < 0.001$) (Figure 1). Patients with DSA MFI ≥2500 had significantly higher levels of dd-cfDNA (0.96%; IQR, 0.26–2.95) than patients with DSA MFI <2500 (0.28%; IQR, 0.19–0.39) and negative DSAs (0.22%; IQR, 0.17–0.37; $P < 0.001$; Table 1 and Figure 1). Among patients with class II dnDSAs, the majority had DQ-dnDSAs (47.4%), followed by DR-dnDSAs (10.5%) and DP-dnDSAs (7.9%). The remaining patients (34.2%) had class II dnDSAs against multiple antigens. The majority of patients with DQ-dnDSAs had an MFI >2500 (94.4%; median MFI, 19 550; IQR, 7700–23 825) compared with those with DP-dnDSAs (33.3%, 3 patients with an MFI of 1200, 1300, and 2600, respectively) or those with DR-dnDSAs (25%; median MFI, 1950; IQR, 1725–2700; $P = 0.002$ for comparison of median MFI values). Similarly, dd-cfDNA levels were associated with class II DSA specificity, those with DQ-dnDSAs having the highest level (median, 1.2%; IQR, 0.32–3.07) compared with patients with DP-dnDSAs (3 patients with dd-cfDNA of 0.15%, 0.15%, and 2.4%, respectively) or DR-dnDSAs (median, 0.23%; IQR, 0.19–0.34; $P = 0.09$; Figure 1). Additionally, those with class II dnDSAs against multiple antigens had a median dd-cfDNA of 0.93% (IQR, 0.2–3.2).

In multivariate logistic regression analysis, the development of dnDSA with MFI ≥2500 was independently associated with a high dd-cfDNA, irrespective of the cutoff chosen for analysis (0.5% or 1%) (Table 2). Additionally, we did not identify any significant association of dnDSAs with graft function or immunosuppression characteristics (Table 2).

Donor-derived Cell-free DNA and DSA Association With Rejection Type and Banff Elementary Lesions

There were 54 patients who underwent kidney allograft biopsies and had concomitant dd-cfDNA determination. Of those, 51 biopsies were done "for-cause" and 3 as part of surveillance protocols. Most biopsies were triggered by signs of allograft dysfunction (increase of serum creatinine in 68.5% or proteinuria in 40.7% of cases) or development of dnDSAs (37%). Three patients (5.6%) underwent allograft biopsy because of BK viremia.

Nineteen patients had class II DSAs with an MFI level over 2500 and underwent allograft biopsy, of which 84.2% had biopsy evidence of AMR (Table 3). Among patients with AMR (n = 18), 89% had concomitant class II dnDSAs with MFI ≥2500, whereas 1 patient with AMR histological lesions had negative DSAs. There was 1 patient with class II DSA and an MFI level of 800 with dd-cfDNA of 2.2% that prompted allograft biopsy, which confirmed AMR.

There was a consistent association between DSA class, titer, and dd-cfDNA level. Patients with class II DSA MFI ≥2500 had higher dd-cfDNA (median, 2.3%; IQR, 1.3–3.7) than patients with negative DSAs (median, 0.23%; IQR, 0.16–0.4) (Figures 2 and 3A). Similarly, patients with AMR had higher dd-cfDNA levels (median, 2.4%; IQR, 2.05–3.67) than patients without AMR on allograft biopsy (median, 0.22%; IQR, 0.16–0.34) (Figure 3B). Additionally, among those with dnDSAs and AMR on biopsy, the majority (94%) had an elevated dd-cfDNA (>1%) compared with those with dnDSAs and without AMR (1 of 3 patients; $P = 0.04$).

Among patients with class II dnDSAs, DQ-dnDSA were the most prevalent (60%) with a median MFI of 12 050 (IQR, 3350–22 750), whereas 35% of patients had class II dnDSAs against multiple antigens (median MFI, 22 600; IQR, 15 400–31 400). The dd-cfDNA level was significantly higher in patients with DQ-dnDSA (median 2.2% [IQR, 1.62–3.47], all patients having a value >1%) or class II dnDSAs against multiple antigens (median 2.3% [IQR, 0.19–4.1], 57.1% having a level >1%) compared with those that did not develop class II dnDSAs (Figure 4). Additionally, 91.7% of those with DQ-dnDSAs and 71.4% of those with class II dnDSAs against multiple antigens had evidence of alloimmune-mediated allograft injury. One patient had isolated DP-dnDSA with MFI 4200 with dd-cfDNA of 2.4% and AMR, whereas there were no patients with isolated DR-dnDSAs.

We then evaluated whether a dd-cfDNA level >1% or presence of DSA with MFI >2500 was a more robust predictor of rejection type or histological lesions. In univariate logistic regression analysis, when using as outcome variables rejection type and Banff elementary lesions, we identified a stronger association between a high dd-cfDNA level and glomerulitis/peritubular capillaritis individual scores or a score of microvascular inflammation (mvi = g + ptc), whereas presence of DSA with MFI >2500 was a more important predictor of AMR and of the intensity of C4d staining, with similar association of these 2 predictors with the presence of transplant glomerulopathy (Table 4). There was no association of either dd-cfDNA or DSAs with tubulointerstitial or chronic lesions on allograft biopsy.

TABLE 1.

Baseline characteristics of the study cohort

Variable	Negative DSAs	DSA MFI <2500	DSA MFI ≥2500	*P*
Number of patients	128	11	32	
Demographic data				
Age (y)	53 ± 15	60 ± 12	46 ± 13	0.016
Gender (n, % males)	75 (58.6)	4 (36.4)	18 (56.2)	0.36
Race, n (%)				
White	78 (60.9)	5 (45.5)	14 (43.8)	0.036
Hispanic	8 (6.3)	0 (0)	9 (28.1)	
Asian	22 (17.2)	2 (18.2)	3 (9.4)	
Black/African American	13 (10.2)	3 (27.3)	4 (12.5)	
Native Hawaiian or Other Pacific Islander/American Indian/Alaska Native	7 (5.5)	1 (9.1)	2 (6.2)	
Time posttransplant to dd-cfDNA determination (y)	0.72 (IQR, 0.25–2.66)	2.11 (IQR, 0.55–6.73)	4.95 (IQR, 2.02–7.15)	<0.001
Time posttransplant, n (%)				
<6 mo	54 (42.2)	2 (18.2)	4 (12.5)	<0.001
6–12 mo	20 (15.6)	2 (18.2)	1 (3.1)	
1–5 y	34 (26.6)	3 (27.3)	11 (34.4)	
5–10 y	9 (7)	3 (27.3)	13 (40.6)	
>10 y	11 (8.6)	1 (9.1)	3 (9.4)	
Type of Tx, n (%)				
Deceased donor	99 (77.3)	6 (54.5)	19 (59.4)	0.049
Living (unrelated/related) donor	29 (22.7)	5 (45.5)	13 (40.6)	
Immunosuppression characteristics				
Induction immunosuppression, n (%)				
Thymoglobulin	101 (78.9)	8 (72.7)	23 (71.8)	0.97
Basiliximab	15 (11.7)	1 (9.09)	3 (9.37)	
Maintenance immunosuppression, n (%)				
Tacrolimus	122 (95.3)	10 (90.9)	31 (96.9)	0.036
Cyclosporine	2 (1.6)	0 (0)	0 (0)	
Sirolimus	0 (0)	1 (9.1)	0 (0)	
Belatacept	2 (1.6)	0 (0)	0 (0)	
Mycophenolic acid	106 (82.8)	9 (81.8)	29 (90.6)	0.22
Azathioprine	3 (2.3)	0 (0)	0 (0)	
Leflunomide	1 (0.8)	1 (9.1)	0 (0)	
Prednisone	125 (97.7)	11 (100)	32 (100)	0.59
Immunosuppression dosage/level at induction and at dd-cfDNA measurement				
Thymoglobulin (total dose, mg)	204 ± 118	173 ± 138	185 ± 134	0.4
FK level (ng/mL)	7.37 ± 2.71	7.81 ± 2.32	6.83 ± 3.35	0.8
Mycophenolic acid (mg/d)	720 (IQR, 360–720)	720 (IQR, 360–720)	720 (IQR, 540–720)	0.3
Laboratory data				
Serum creatinine at dd-cfDNA measurement (mg/dL)	1.55 ± 0.48	1.15 ± 0.37	1.53 ± 0.66	0.05
Serum creatinine at last follow-up (mg/dL)	1.56 ± 0.55	1.22 ± 0.4	1.7 ± 1.11	0.04
eGFR at dd-cfDNA measurement (mL/min/1.73m^2)	49 ± 20	63 ± 19	56 ± 26	0.05
eGFR at last follow-up (mL/min/1.73m^2)	50 ± 20	59 ± 19	57 ± 29	0.15
Urine protein/creatinine at dd-cfDNA measurement	0.2 (IQR, 0.1–0.39)	0.1 (IQR, 0.1–0.26)	0.19 (IQR, 0.1–0.97)	0.34
Urine protein/creatinine at last follow-up	0.2 (IQR, 0.1–0.4)	0.1 (IQR, 0.1–0.5)	0.2 (IQR, 0.1–0.53)	0.46
Calculated panel reactive antibody				
<20%	107 (83.6%)	8 (72.7%)	25 (78.1%)	0.74
20%–50%	6 (4.7%)	1 (9.1%)	1 (3.1%)	
>50%	15 (11.7%)	2 (18.2%)	6 (18.8%)	
dd-cfDNA and dnDSAs characteristics				
dd-cfDNA level (%)	0.22 (IQR, 0.17–0.37)	0.28 (IQR, 0.19–0.39)	0.96 (IQR, 0.26–2.95)	<0.001
Patients with dd-cfDNA >1% (n, %)	14 (10.9)	2 (18.2)	16 (50)	<0.001
Patients with dd-cfDNA >0.5% (n, %)	22 (17.2)	2 (18.2)	20 (62.5)	<0.001
Patients with multiple types of DSA (n,%)	0 (0)	1 (9.1)	14 (43.8)	<0.001

dd-cfDNA, donor-derived cell-free DNA; dnDSA, de novo donor-specific antibody; DSA, donor-specific antibody; eGFR, estimated glomerular filtration rate; MFI, mean fluorescence intensity; Tx, transplant.

DISCUSSION

In this study, we have identified a robust association between dnDSA class and titer with dd-cfDNA level in a cross-sectional cohort of kidney transplant recipients screened for the presence of DSAs. Despite the subclinical presentation in relation to kidney function, we found that there is a very strong association between dd-cfDNA and AMR and the individual histologic elements that define AMR. Our study

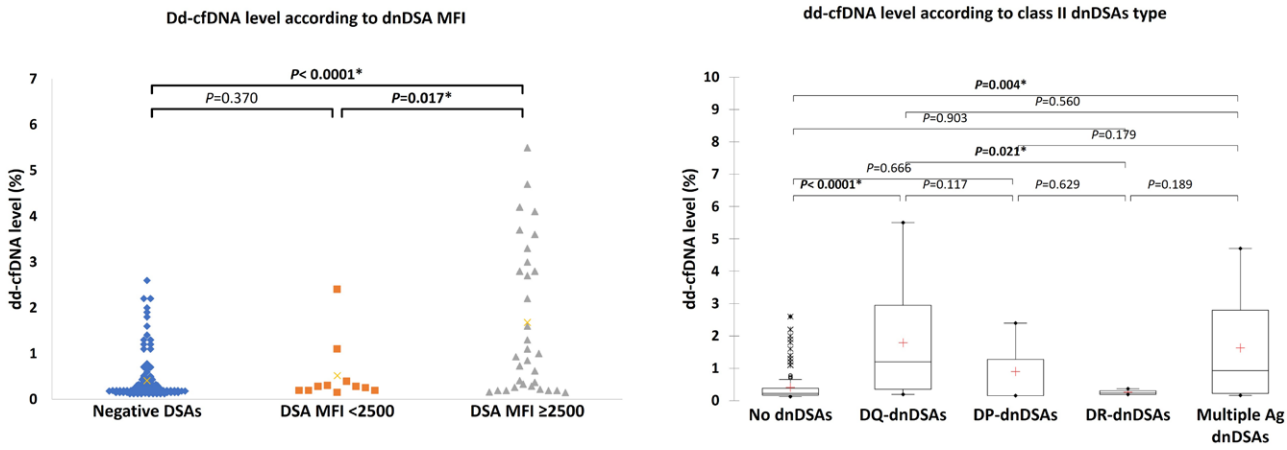

FIGURE 1. Donor-derived cell-free DNA level by presence and titer of DSAs (entire cohort). dd-cfDNA, donor-derived cell-free DNA; dnDSA, de novo donor-specific antibody; DSA, donor-specific antibody; MFI, mean fluorescence intensity.

TABLE 2.

Binary logistic regression analysis regarding variables associated with development of DSAs with titer >2500

	Univariate analysis		Multivariate analysis[a] (model A)		Multivariate analysis[a] (model B)	
Variable	**Odds ratio (95% CI)**	***P***	**Odds ratio (95% CI)**	***P***	**Odds ratio (95% CI)**	***P***
Recipient's age (for each 1 y)	0.96 (0.94-0.99)	0.015	0.97 (0.94-1.00)	0.08	0.97 (0.94-1.007)	0.12
Recipient's gender (female vs male)	1.024 (0.47-2.22)	0.95	0.5 (0.19-1.47)	0.2	0.5 (0.18-1.4)	0.18
Recipient's ethnicity (other vs White)	1.9 (0.87-4.14)	0.1	3.63 (1.32-9.95)	0.01	3.35 (1.23-9.14)	0.018
Type of transplant (deceased vs living)	0.47 (0.21-1.05)	0.06	0.34 (0.12-1.00)	0.05	0.29 (0.1-0.87)	0.027
Time from Tx to dd-cfDNA measurement (for each 1 mo)	1.074 (1.00-1.15)	0.051	–	–	–	–
Serum creatinine (for each 1 mg/dL)	1.066 (0.51-2.2)	0.86	1.49 (0.65-3.39)	0.34	1.32 (0.57-3.05)	0.51
Urine protein/creatinine ratio (for each 1 *g*/g)	1.18 (0.88-1.57)	0.25	–	–	–	–
dd-cfDNA level (≥1% vs <1%)	7.68 (3.23-18.2)	<0.001	11.1 (3.99-30.9)	<0.001	–	–
dd-cfDNA level (≥0.5% vs <0.5%)	7.98 (3.44-18.4)	<0.001	–	–	10.7 (4.09-28.1)	<0.001
Calculated panel reactive antibody (vs <20%)	–	–	–	–	–	–
20%–50%	0.62 (0.07-5.58)	0.7	2.01 (0.2-19.9)	0.55	0.83 (0.06-10.9)	0.88
>50%	1.62 (0.58-4.53)	0.35	4.76 (1.21-18.6)	0.02	4.38 (1.09-17.6)	0.03
Induction IS (ATG vs basiliximab)	1.12 (0.3-4.18)	0.86	–	–	–	–
FK level (for each 1 ng/mL)	0.95 (0.84-1.08)	0.5	–	–	–	–
Mycophenolate dose (for each 1 mg)	1.001 (1.00-1.002)	0.12	–	–	–	–

[a]After multivariate adjustment for age, race, gender, type of transplant, serum creatinine, and calculated panel reactive antibody.
ATG, antithymocyte globulin; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibody; IS, immunosuppression; Tx, transplant.

suggests that dd-cfDNA may be useful as a noninvasive complementary method to potentially refine the clinical impact of the presence of dnDSAs.

Alloimmune-mediated injury remains an important contributor to death-censored graft loss in kidney transplant recipients.[1,4,20] The dnDSAs occur with variable frequency (up to 30%) depending on the patient's immunologic risk and posttransplant follow-up period.[5,21] In our cross-sectional cohort, 43 patients (25.14%) developed dnDSAs after a median of 4.95 y for those with strong dnDSAs (MFI >2500) and 2.11 y posttransplant for those with weak dnDSA (MFI <2500); however, this was not a continuous cohort given that we also included patients that were returning to our center for a 3, 6, 12 mo, or anniversary visit or tested for DSA in the context of allograft dysfunction during the study period. Accordingly, the percentage of patients with dnDSAs is likely higher than the incidence of dnDSA in a continuous cohort, and it is mainly due to the inclusion of patients tested for allograft dysfunction that were more likely to have a positive DSA result.

Given that dnDSAs are associated with poorer allograft survival, there is an unmet need to identify noninvasive biomarkers that allow early and accurate identification of graft injury, particularly as there is no standardized nor effective treatment currently available for patients that developed DSA.[5,14,15] As such, dd-cfDNA emerged as a candidate biomarker in solid organ transplantation.[18]

This is, to our knowledge, the first study that attempted to identify the characteristics of dnDSAs associated with allograft injury by means of measuring dd-cfDNA. We have identified that the cutoff for DSA MFI of 2500 may be clinically significant, as 50% of these patients had a dd-cfDNA level >1%. Of these, the most important contribution to elevated dd-cfDNA levels was attributed to class II DSAs, the majority with MFI >2500 (82.9%). In comparison, class I DSAs had low MFI values and were associated with low dd-cfDNA values. In the subgroup of patients that underwent allograft biopsy, we identified that both dd-cfDNA >1% and DSA MFI >2500 were strongly associated with AMR, with comparable ORs in logistic regression analysis (OR, 128 [95%

TABLE 3.

Baseline patient characteristics according to the presence and titer of DSAs (patients that had undergone allograft biopsy)

Variable	Negative DSAs	DSA MFI ≥2500	*P*
Number of patients	34	19	
Demographic characteristics			
Age (y)	55 ± 17	46 ± 13	0.05
Gender (n, % males)	21 (61.8)	12 (63.2)	>0.99
Race, n (%)			
White	17 (50)	9 (47.4)	>0.99
Other	17 (50)	10 (52.6)	
Time posttransplant to dd-cfDNA measurement (y)	0.55 (IQR, 0.24–1.07)	4.63 (IQR, 2.04–6)	<0.001
Time posttransplant, n (%)			
<6 mo	15 (44.1)	2 (10.5)	<0.001
6–12 mo	8 (23.5)	1 (5.3)	
1–5 y	8 (23.5)	7 (36.8)	
5–10 y	0 (0)	9 (47.4)	
>10 y	3 (8.8)	0 (0)	
Type of Tx, n (%)			
Deceased donor	29 (85.3)	12 (63.2)	0.09
Living donor	5 (14.7)	7 (36.8)	
Immunosuppression characteristics			
Induction immunosuppression, n (%)			
Thymoglobulin	25 (73.5)	16 (84.2)	0.69
Basiliximab	6 (17.6)	2 (10.52)	
Maintenance immunosuppression, n (%)			
Tacrolimus	33 (97.1)	19 (100)	>0.99
Cyclosporine	1 (2.9)	0 (0)	
Mycophenolic acid	30 (88.2)	16 (84.2)	0.69
Prednisone	34 (100)	19 (100)	>0.99
Immunosuppression dosage/level at induction and at dd-cfDNA measurement			
Thymoglobulin (total dose, mg)	248 ± 69	275 ± 84	0.36
FK level (ng/mL)	7.84 ± 2.89	6.2 ± 1.68	0.012
Mycophenolic acid (mg/d)	720 (IQR, 360–720)	720 (IQR, 360–1080)	0.4
Laboratory data			
Serum Creatinine at dd-cfDNA measurement (mg/dL)	1.85 ± 0.62	1.38 ± 0.44	0.003
Serum Creatinine at last follow-up (mg/dL)	1.84 ± 0.75	1.51 ± 0.74	0.015
eGFR at dd-cfDNA measurement (mL/min/1.73m^2)	43 ± 25	59 ± 20	0.02
20–50%	44 ± 23	58 ± 25	0.05
Urine protein/creatinine at dd-cfDNA measurement	0.34 (IQR, 0.2–1.05)	0.2 (IQR, 0.1–1.1)	0.09
Urine protein/creatinine at last follow-up	0.43 (IQR, 0.1–2.2)	0.2 (IQR, 0.1–0.6)	0.19
Calculated panel reactive antibody			
<20%	32 (94.1%)	16 (84.2%)	0.18
20–50%	1 (2.9%)	0 (0%)	
>50%	1 (2.9%)	3 (15.8%)	
dd-cfDNA level (median %, IQR)	0.23 (IQR, 0.16–0.4)	2.3 (IQR, 1.3–3.7)	<0.001
Patients with dd-cfDNA >1% (n, %)	4 (11.8)	16 (84.2)	<0.001
Patients with dd-cfDNA >0.5% (n, %)	7 (20.6)	17 (89.5)	<0.001
dnDSA characteristics			
Patients with dnDSAs, n (%)			
No DSAs	34 (100)	–	–
Class I DSAs	–	–	
Class II DSAs	–	16 (84.2)	
Class I + II DSAs	–	3 (15.8)	
Biospy findings			
Any rejection, n (%)	13 (38.2)	16 (84.2)	0.001
Type of rejection, n (%)			
No rejection	21 (61.8)	3 (15.8)	<0.001
AMR	1 (2.9)[a]	10 (52.6)	
TCMR	12 (35.3)	0 (0)	
AMR + TCMR	0 (0)	6 (31.6)	

Continued next page

TABLE 3. (Continued)

Baseline patient characteristics according to the presence and titer of DSAs (patients that had undergone allograft biopsy)

Variable	Negative DSAs	DSA MFI ≥2500	*P*
Individual lesions, n of pts (%)			
Cd4 staining ≥1	2 (5.9)	12 (63.2)	<0.001
Glomerulitis (g) ≥1	8 (23.5)	13 (68.4)	0.003
Peritubular capillaritis (ptc) ≥1	4 (11.8)	16 (84.2)	<0.001
Microvascular inflammation (g + ptc)			
0–1	31 (91.2%)	5 (26.3%)	<0.001
2–3	3 (8.8%)	5 (26.3%)	
≥4	0 (0%)	9 (47.4%)	
Presence of transplant glomerulopathy	1 (2.9%)	9 (47.4%)	<0.001
Presence of arteritis	2 (5.9%)	2 (10.5%)	0.61
Tubulitis (t) ≥2	8 (23.5%)	2 (10.5%)	0.29
Interstitial inflammation (i) ≥2	2 (5.9%)	3 (15.8%)	0.33
Tubular atrophy (ct) ≥2	4 (11.8%)	2 (10.5%)	>0.99
Interstitial fibrosis (ci) ≥2	5 (14.7%)	2 (10.5%)	>0.99
Arteriosclerosis (cv) ≥2	4 (11.8%)	5 (26.3%)	0.25

[#]This patient is DSA negative with histological evidence alloimmune-mediated injury (C4d0, g1, ptc1).

AMR, antibody-mediated rejection; dd-cfDNA, donor-derived cell-free DNA; dnDSA, de novo donor-specific antibody; DSA, donor-specific antibody; eGFR, estimated glomerular filtration rate; MFI, mean fluorescence intensity; TCMR, T cell–mediated rejection; Tx, transplant.

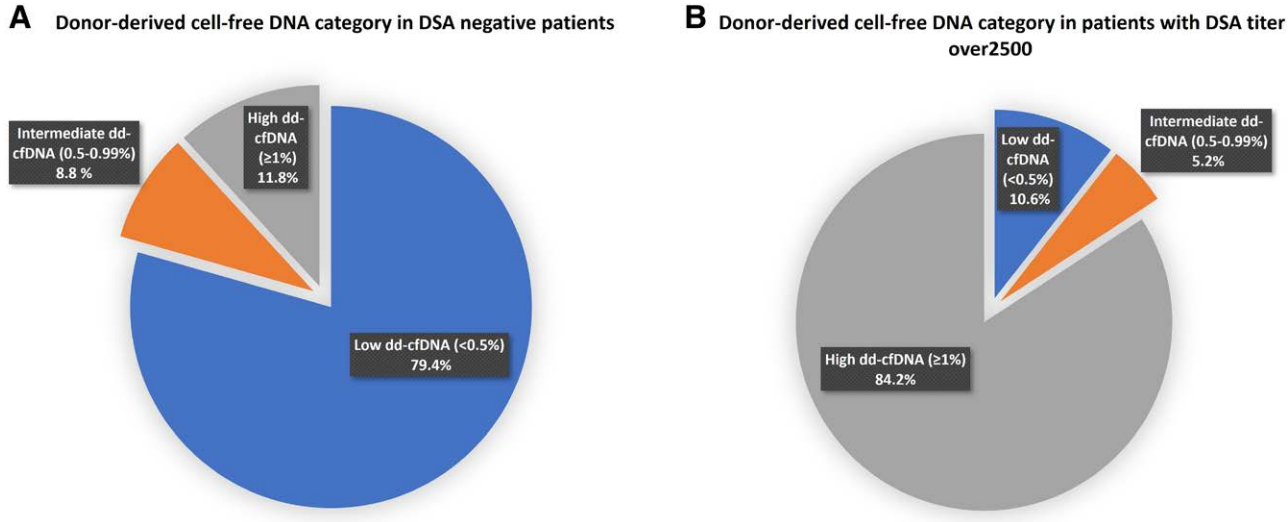

FIGURE 2. Donor-derived cell-free DNA category (low, intermediate, high) by presence and titer of DSAs. dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibody.

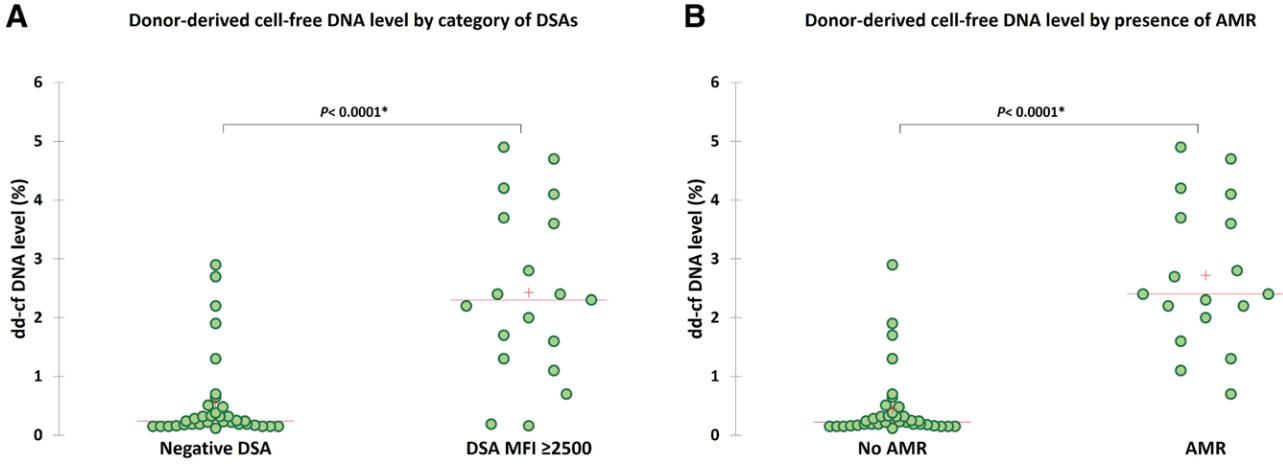

FIGURE 3. Donor-derived cell-free DNA level by presence of DSA and AMR. AMR, antibody-mediated rejection; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibody; MFI, mean fluorescence intensity.

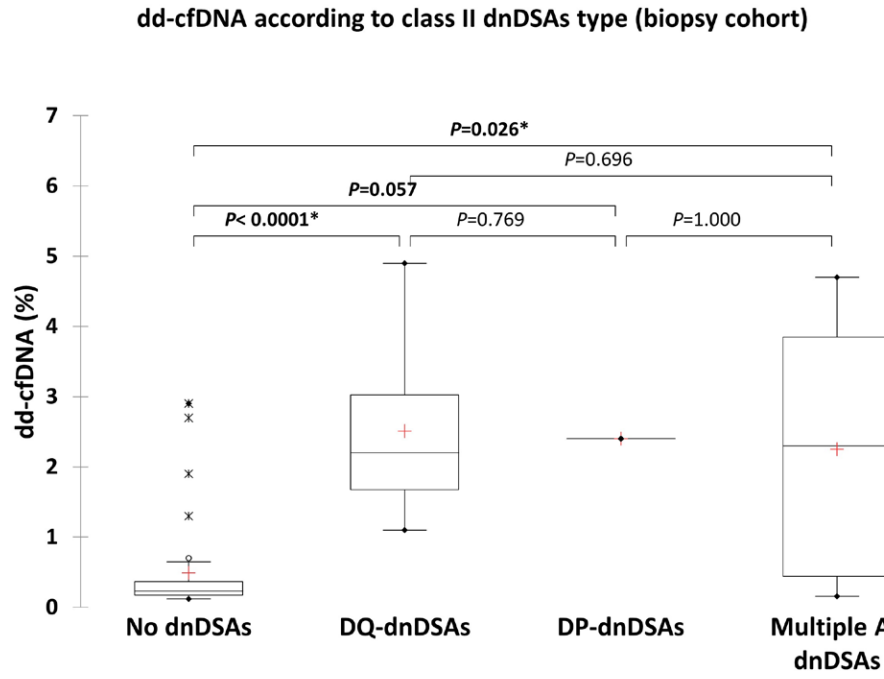

FIGURE 4. Donor-derived cell-free DNA by type of class II dnDSAs (biopsy cohort). dd-cfDNA, donor-derived cell-free DNA; dnDSA, de novo donor-specific antibody.

CI, 13.2-1241] for dd-cfDNA ≥1%; OR, 176 [95% CI, 16.9-1828] for DSA MFI ≥2500); however, when we evaluated the individual Banff elementary lesions, we noted that DSA MFI ≥2500 was more strongly associated with C4d staining score than dd-cfDNA ≥1%, consistent with the presence of C4d-positive AMR.[22] By contrast, a dd-cfDNA >1% was more strongly associated with microvascular inflammation (glomerulitis and peritubular capillaritis score). Additionally, both dd-cfDNA >1% and DSA MFI >2500 had strong association with transplant glomerulopathy (OR, 26.1 [95% CI, 2.97-230] for dd-cfDNA ≥1%; OR, 29.7 [95% CI, 3.34-263] for DSA MFI ≥2500), consistent with the observation that the majority of AMR fit the Banff classification for chronic, active AMR. Our data are supported by several studies over the past years, which have shown that dd-cfDNA identifies with high accuracy AMR, strongly correlates with microvascular inflammation, and may allow real-time monitoring of rejection treatment response.[14,17,18,23-29] It has been hypothesized that dd-cfDNA may not be rejection specific, as other types of graft injury have been associated with elevated levels, such as recurrences of glomerular disorders, graft infection, BK nephropathy, or acute tubular necrosis.[17,26] In our cohort, we found no histological association between elevated dd-cfDNA with nonrejection histological changes (we had 2 patients with BK nephropathy, 1 with pyelonephritis, 1 with acute tubular necrosis, and 4 patients with recurrent glomerular disorders, of which 3 had concomitant T cell–mediated rejection IA, all having a low dd-cfDNA level). Despite the mounting evidence

TABLE 4.

Univariate binary logistic regression analysis regarding variables associated with type of rejection and individual histologic lesions

	dd-cfDNA >1%		DSA titer ≥2500	
Variable	**Odds ratio (95% CI)**	*P*	**Odds ratio (95% CI)**	*P*
Presence of AMR	128 (13.2-1241)	<0.001	176 (16.9-1828)	<0.001
Presence of TCMR	1.53 (0.48-4.9)	0.47	0.84 (0.25-2.79)	0.78
Cd4 staining ≥1	**7.25 (1.85-28.3)**	**0.004**	**27.4 (4.98-151)**	**<0.001**
Glomerulitis (g) ≥1	**13.5 (3.52-51.7)**	**<0.001**	**7.04 (2.01-24.58)**	**0.002**
Peritubular capillaritis (ptc) ≥1	**139 (18.06-1077)**	**<0.001**	**40 (7.95-201)**	**<0.001**
Microvascular inflammation (g + ptc ≥2 vs <2)	**181 (17.4-1879)**	**<0.001**	**21 (4.87-90)**	**<0.001**
Presence of transplant glomerulopathy	**26.1 (2.97-230)**	**0.003**	**29.7 (3.34-263)**	**0.002**
Presence of arteritis	1.72 (0.23-13.3)	0.6	1.88 (0.24-14.56)	0.54
Tubulitis (t) ≥2	0.65 (0.14-2.89)	0.57	0.38 (0.07-2.02)	0.25
Interstitial inflammation (i) ≥2	2.73 (0.41-18)	0.29	3 (0.45-19.8)	0.25
Tubular atrophy (ct) ≥2	0.8 (0.13-4.85)	0.81	0.88 (0.14-5.33)	0.89
Interstitial fibrosis (ci) ≥2	0.62 (0.11-3.55)	0.59	0.68 (0.11-3.91)	0.66
Arteriosclerosis (cv) ≥2	2.41 (0.56-10.35)	0.23	2.67 (0.62-11.53)	0.18

Bold signifies that the P value is statistically significant.
AMR, antibody-mediated rejection; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibody; TCMR, T cell–mediated rejection.

regarding the utility of dd-cfDNA, there are still many questions that need to be addressed regarding the ideal testing frequency, the best assay, the possibility of complementing DSA screening and graft biopsy, and the optimal cutoff levels.[18]

Our findings have multiple pertinent clinical implications. The increasing interest in refining the alloimmune-mediated injury over the past years has led to the recognition and inclusion in the Banff classification of the C4d-negative AMR and non-HLA AMR.[8,22,30] Given the strong association with microvascular inflammation elementary lesions, dd-cfDNA may reflect the presence of endothelial injury, possibly mediated by the presence of DSAs or other types of non-HLA antibodies. This hypothesis may be inferred by the observation that 10.9% of the patients without DSAs included in this study had an elevated (≥1%) dd-cfDNA level. Furthermore, in 1 patient, AMR has been diagnosed in the absence of detectable DSA but with an elevated dd-cfDNA; however, in the absence of an allograft biopsy in all patients, we cannot conclude if in cases of patients without dnDSAs the elevated dd-cfDNA level is due to an alloimmune injury mediated by non-HLA antibodies or other types of graft injuries. A dedicated, ideally prospective, study to better delineate the spectrum of allograft injuries that trigger an elevation in dd-cfDNA level is clearly needed. Nonetheless, dd-cfDNA could be a valuable method for redefining the spectrum of alloimmune-mediated injury, potentially allowing identification of patients with early lesions not currently captured through the classical methods of assessing the graft function.

The excellent allograft function in our cohort (serum creatinine 1.55 ± 0.48 mg/dL in those dnDSA-negative, 1.15 ± 0.37 mg/dL in those with dnDSA MFI <2500, and 1.53 ± 0.66 mg/dL in those with dnDSA MFI >2500; $P = 0.05$) outlines the limitations of routine clinical monitoring (creatinine- and proteinuria-based) given that they fail to detect early significant pathological changes of AMR that may contribute to subclinical graft injury and loss. Additionally, the short half-life, along with the possibility of frequent and quantitative assessments, makes dd-cfDNA a dynamic indicator of allograft health.[31]

Our study has several limitations that need to be acknowledged. First, this is a cross-sectional study where dd-cfDNA was assessed for cause in the presence of dnDSAs or in patients considered for a renal biopsy in the context of allograft dysfunction or proteinuria, purposefully enriching the association between dd-cfDNA and dnDSAs in this context. Additionally, given the observational nature of the study, association does not necessarily imply causality. As such, future prospective designed trials are needed to assess the association between dd-cfDNA and DSAs from a development perspective and to refine their utility as biomarkers for rejection. Third, an allograft biopsy (majority was for cause biopsy) was not available in all patients, which may limit the generalizability of our results. Nonetheless, this is one of the largest cohorts of patients tested concomitantly for dnDSAs and dd-cfDNA. Additionally, the biopsy cohort consisted of a wide range of histological lesions, including nonrejection injury.

In this study, we have shown that, despite preserved renal function, a substantial number of posttransplant patients develop subclinical allograft injury associated with the development of dnDSA. In these patients, dd-cfDNA was may be able to further clarify the clinical significance of dnDSAs in regards to association with AMR and its associated histological lesions. Future prospective studies are warranted using dd-cfDNA in surveillance protocols testing, not only for the potential of earlier identification of allograft injury that may allow more efficient and less aggressive interventions but also for the potential of confirming allograft quiescence as projected by its high negative predictive value.

REFERENCES

1. Loupy A, Lefaucheur C, Vernerey D, et al. Complement-binding anti-HLA antibodies and kidney-allograft survival. *N Engl J Med.* 2013;369:1215–1226.
2. Matas AJ, Fieberg A, Mannon RB, et al. Long-term follow-up of the DeKAF cross-sectional cohort study. *Am J Transplant.* 2019;19:1432–1443.
3. El-Zoghby ZM, Stegall MD, Lager DJ, et al. Identifying specific causes of kidney allograft loss. *Am J Transplant.* 2009;9:527–535.
4. Sellarés J, de Freitas DG, Mengel M, et al. Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. *Am J Transplant.* 2012;12:388–399.
5. Wiebe C, Gibson IW, Blydt-Hansen TD, et al. Evolution and clinical pathologic correlations of de novo donor-specific HLA antibody post kidney transplant. *Am J Transplant.* 2012;12:1157–1167.
6. Clayton PA, McDonald SP, Russ GR, et al. Long-term outcomes after acute rejection in kidney transplant recipients: an ANZDATA analysis. *J Am Soc Nephrol.* 2019;30:1697–1707.
7. Zhang R. Donor-specific antibodies in kidney transplant recipients. *Clin J Am Soc Nephrol.* 2018;13:182–192.
8. Lefaucheur C, Viglietti D, Bouatou Y, et al. Non-HLA agonistic anti-angiotensin II type 1 receptor antibodies induce a distinctive phenotype of antibody-mediated rejection in kidney transplant recipients. *Kidney Int.* 2019;96:189–201.
9. Bertrand D, Gatault P, Jauréguy M, et al. Protocol biopsies in patients with subclinical de novo donor-specific antibodies after kidney transplantation: a multicentric study. *Transplantation.* 2020;104:1726–1737.
10. Parajuli S, Joachim E, Alagusundaramoorthy S, et al. Subclinical antibody-mediated rejection after kidney transplantation: treatment outcomes. *Transplantation.* 2019;103:1722–1729.
11. Singh N, Samant H, Hawxby A, et al. Biomarkers of rejection in kidney transplantation. *Curr Opin Organ Transplant.* 2019;24:103–110.
12. Moulin B, Merville P, Renaudin K, et al. Evaluation of protocol biopsy utility 12 months after renal transplantation: a multicenter observational analysis. *J Transplant.* 2012;2012:781263.
13. Krejí K, Tich T, Al-Gabri S, et al. Protocol biopsy of a transplanted kidney as a tool for monitoring adequacy of immunosuppressive therapy: 10 years of experience from a single transplant center. *Transplant Proc*. 2011;43:1576–1582.
14. Knight SR, Thorne A, Lo Faro ML. Donor-specific cell-free DNA as a biomarker in solid organ transplantation. A systematic review. *Transplantation.* 2019;103:273–283.
15. Gosset C, Viglietti D, Rabant M, et al. Circulating donor-specific anti-HLA antibodies are a major factor in premature and accelerated allograft fibrosis. *Kidney Int.* 2017;92:729–742.
16. Celec P, Vlková B, Lauková L, et al. Cell-free DNA: the role in pathophysiology and as a biomarker in kidney diseases. *Expert Rev Mol Med.* 2018;20:e1.
17. Bloom RD, Bromberg JS, Poggio ED, et al. Cell-free DNA and active rejection in kidney allografts. *J Am Soc Nephrol*. 2017;28:2221–2232.
18. Bloom RD. Using (cell-free) DNA to incriminate rejection as the cause of kidney allograft dysfunction: do we have a verdict? *Am J Transplant.* 2019;19:1609–1610.
19. Roufosse C, Simmonds N, Clahsen-van Groningen M, et al. A 2018 reference guide to the Banff classification of renal allograft pathology. *Transplantation.* 2018;102:1795–1814.
20. Gaston RS, Cecka JM, Kasiske BL, et al. Evidence for antibody-mediated injury as a major determinant of late kidney allograft failure. *Transplantation.* 2010;90:68–74.
21. Everly MJ, Rebellato LM, Haisch CE, et al. Incidence and impact of de novo donor-specific alloantibody in primary renal allografts. *Transplantation.* 2013;95:410–417.
22. Haas M. Evolving criteria for the diagnosis of antibody-mediated rejection in renal allografts. *Curr Opin Nephrol Hypertens.* 2018;27:137–143.
23. Jordan SC, Bunnapradist S, Bromberg JS, et al. Donor-derived cell-free DNA identifies antibody-mediated rejection in donor specific antibody positive kidney transplant recipients. *Transplant Direct.* 2018;4:e379.

24. Oellerich M, Shipkova M, Asendorf T, et al. Absolute quantification of donor-derived cell-free DNA as a marker of rejection and graft injury in kidney transplantation: results from a prospective observational study. *Am J Transplant.* 2019;19:3087–3099.
25. Huang E, Sethi S, Peng A, et al. Early clinical experience using donor-derived cell-free DNA to detect rejection in kidney transplant recipients. *Am J Transplant.* 2019;19:1663–1670.
26. Gielis EM, Ledeganck KJ, Dendooven A, et al. The use of plasma donor-derived, cell-free DNA to monitor acute rejection after kidney transplantation. *Nephrol Dial Transplant.* 2020;35:714–721.
27. Hinojosa RJ, Chaffin K, Gillespie M, et al. Donor-derived cell-free DNA may confirm real-time response to treatment of acute rejection in renal transplant recipients. *Transplantation.* 2019;103:e61.
28. Stites E, Kumar D, Olaitan O, et al. High levels of dd-cfDNA identify patients with TCMR 1A and borderline allograft rejection at elevated risk of graft injury. *Am J Transplant.* 2020;20:2491–2498.
29. Whitlam JB, Ling L, Skene A, et al. Diagnostic application of kidney allograft-derived absolute cell-free DNA levels during transplant dysfunction. *Am J Transplant.* 2019;19:1037–1049.
30. Orandi BJ, Alachkar N, Kraus ES, et al. Presentation and outcomes of C4d-negative antibody-mediated rejection after kidney transplantation. *Am J Transplant.* 2016;16:213–220.
31. Gielis EM, Ledeganck KJ, De Winter BY, et al. Cell-free DNA: an upcoming biomarker in transplantation. *Am J Transplant.* 2015;15:2541–2551.