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Early Experience Using Donor-derived Cell-free DNA for Surveillance of Rejection Following Simultaneous Pancreas and Kidney Transplantation

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Background. Allograft biopsy is the gold standard for diagnosing graft rejection following simultaneous pancreas and kidney (SPK) transplant. Intraoperative biopsies are technically challenging and can be burdensome to patients and the healthcare system. Donor-derived cell-free DNA (dd-cfDNA) is well-studied in kidney transplant recipients; however, it has not yet been studied in the SPK population. **Methods.** We hypothesized that dd-cfDNA could be utilized for rejection surveillance following SPK transplant. We prospectively collected dd-cfDNA in 46 SPK patients at a single institution. **Results.** There were 10 rejection events, 5 of which were confirmed with biopsy. The other 5 were treated based on dd-cfDNA and clinical data alone with favorable outcomes. Among all patients who did not have rejection, 97% had dd-cfDNA <0.5%. Dd-cfDNA may also help differentiate rejection from graft injury (ie, pancreatitis) with median values in rejection 2.25%, injury 0.36%, and quiescence 0.18% ($P=0.0006$). **Conclusions.** Similar to kidneys, dd-cfDNA shows promise for rejection surveillance in SPK transplant recipients.

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INTRODUCTION

Allograft biopsy is the gold standard for diagnosing allograft rejection following simultaneous pancreas and kidney transplant (SPK).¹ However, biopsies may not be ideal means for rejection surveillance, as biopsy procedures have risks, and they can be burdensome to patients and the healthcare system.² A less invasive method for surveillance is measuring donor-derived cell-free DNA (dd-cfDNA). Levels of circulating dd-cfDNA can be quantified with a

peripheral blood sample, and can therefore be obtained within patients' routine laboratory evaluations.

In 2017, dd-cfDNA became commercially available and Medicare reimbursable for detecting kidney allograft rejection.³ This occurred in response to the results of the Circulating Donor-derived Cell-free DNA in blood for Diagnosing Active Rejection in Kidney Transplant Recipients trial.⁴ Since then, using dd-cfDNA to detect rejection has become an acceptable practice, although the specifics are still being studied.⁵⁻⁹ In kidney transplantation, a dd-cfDNA cutoff of 0.5% is typically used to differentiate normal from allograft injury.¹⁰ Relative change values can also be utilized—increases of <61% of dd-cfDNA may be because of normal variation, but greater increases should raise suspicion of rejection.¹¹

The role of dd-cfDNA following kidney transplantation has become better defined over time; however, there is a paucity of data regarding dd-cfDNA following SPK transplantation. Little is known about the kinetics of dd-cfDNA or its association with acute rejection in this population. Therefore, the goal of this study was to understand the trends in dd-cfDNA, as they relate to quiescence, rejection, or allograft injury following SPK transplantation.

MATERIALS AND METHODS

Study Design

This was a prospective cohort study that followed all SPK recipients from a single center. Patients who were transplanted before initiation of the study were observed from November 2018 to May 2021. Patients who were transplanted during the

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study period were observed from the time of transplant until May 2021. Dd-cfDNA values were measured using AlloSure (CareDx, Inc, Brisbane, CA). Samples were collected at 2 wk following transplant, monthly for the first year, then every 3 mo for the next 2 y, and then every 6 mo thereafter. The study was approved by the institutional review board and was registered on ClinicalTrials.gov (Identifier: NCT04130685). All patients provided informed consent before data collection.

Allograft biopsies were performed for 1 of 2 indications: elevated levels of circulating dd-cfDNA, or elevated lipase or creatinine. When lipase was elevated, a graft pancreas biopsy was attempted. When creatinine was elevated, a graft kidney biopsy was attempted. If creatinine and lipase were both elevated, biopsy of both grafts was attempted. Any time a pancreas biopsy was considered not to be feasible, a kidney biopsy was attempted as a surrogate. If a biopsy was indicated, but a patient was considered to be high-risk for biopsy-related complications at that time, then biopsy was not attempted and management was based on clinical judgment.

Definitions

Biopsy-proven Rejection

Events were classified as biopsy-proven rejection in patients who underwent a biopsy of the allograft kidney or pancreas and were diagnosed with rejection based on histologic review by an attending pathologist. Kidney rejection was based on kidney allograft biopsy using the 2015 Banff Criteria. Pancreas rejection was evaluated by endoscopic biopsy of donor duodenum until 2019. After this date, directed biopsy of pancreas allograft was performed by interventional radiology. This change was made in response to our own experience, wherein donor duodenum was difficult to consistently reach endoscopically despite a proximal anastomosis. This change was further justified by findings published by Nordheim et al,¹² which demonstrated only 9% sensitivity for detecting pancreas rejection with donor duodenal biopsy as a surrogate.

Clinically Diagnosed Rejection

Some patients were not subjected to allograft biopsy because of the increased risk of biopsy-related complications. If these patients had a convincing constellation of clinical findings, including sustained elevations in dd-cfDNA, rising or de novo donor-specific antibodies (DSA), or sustained elevations of lipase or creatinine, they were treated presumptively for rejection. These events were classified as clinically diagnosed rejection.

All patients with clinically diagnosed rejection were treated with steroids for presumed acute cellular rejection (ACR). Patients with de novo DSA or rising DSA titers received treatment for antibody-mediated rejection (AMR) in addition to ACR treatment.

Injury

Events were designated as allograft injury, where a tissue biopsy led to any pathologic diagnosis other than rejection.

Quiescence

Patients were classified as quiescent if, for the entirety of their participation in the study, they did not have events meeting criteria for injury, clinically diagnosed rejection, and biopsy-proven rejection. Patients who underwent biopsies that were histologically normal, and did not otherwise meet criteria for the above designations are in the quiescent category.

Baseline

Baseline dd-cfDNA values were determined by evaluating each patient's trends over time. For patients transplanted during the study period, baseline was defined as the lowest point reached following initial equilibration after transplantation. For patients who enrolled in the study >6 mo after transplantation, baselines were determined as an average of their lowest 3 consecutive values. All values were determined by consensus among authors.

Statistical Methods

This was a prospective, observational study designed to compare dd-cfDNA values between different groups of patients (ie, quiescent, injury, rejection). The sample size had at least 80% power to identify differences in dd-cfDNA between rejection/injury versus other biopsy diagnoses using Wilcoxon rank sum test when detecting mean difference at 1.5 SD. dd-cfDNA, lipase, creatinine, measurements in rejection/injury, and quiescence groups were compared using Wilcoxon rank sum test.

Multivariate analyses were done using logistic regression models for rejection/injury adjusting for age, lipase, DSA, and dd-cfDNA at time of biopsy. Variables including creatinine, BK, and GAD were considered in model selection and excluded because of multicollinearity and goodness of fit. 95% confidence intervals (CIs) for odds rate were calculated. A longitudinal multivariate analysis of dd-cfDNA association with other clinical function was done using generalized linear models adjusting for random effect of each patient. Variables including creatinine, BK, DSA, lipase, and GAD65 were all considered in model for significance test. All analyses were done in Python (version 3.9.7).

For consistency, all dd-cfDNA for quiescent patients were taken from the most recent follow-up. Dd-cfDNA for injury or rejection patients were taken from the time of the event. If a patient experienced >1 event, only their first event was considered.

RESULTS

Forty-eight patients were enrolled in the study. One patient dropped out because of pregnancy, another was excluded because of developing graft-versus-host disease.¹³ Forty-six patients were included in the data analysis. Median age was 53.2. Patient characteristics are shown in Table 1. A total of 423 dd-cfDNA values were included, with a median of 8 values per patient (interquartile range [IQR], 6–11.75). Median length of follow-up within the study population was 2.0 y (IQR, 1.6–2.2 y). Median time from transplant was 48.9 mo (IQR, 25.1–64.9 mo). Twelve patients were followed from the time of their transplant.

Across all patients, the median baseline dd-cfDNA value was 0.18% (IQR, 0.15%–0.22%). Median time to baseline following transplant was 86 d (IQR, 81–96 d). For those patients who were treated for rejection, a complete return to baseline was seen after a median of 6.0 mo, although a downward trend was typically seen within 2 wk following successful treatment.

There were 19 biopsy procedures. Six biopsies demonstrated graft injury (median dd-cfDNA 0.29%) and 5 demonstrated biopsy-confirmed rejection (median dd-cfDNA 2.4%). All pathologic diagnoses and their associated laboratory

TABLE 1.
Baseline characteristics

	All patients	Rejection/injury	Quiescence	P
	N=46	N=13	N=33	
Median age (IQR)	53 (39–57)	53.7 (38.3–56.7)	53.2 (40.0–57.7)	0.91
Sex (%)				0.70
Male	35 (76)	9	26	
Female	11 (24)	4	7	
Race (%)				0.76
Non-Hispanic White	7 (15.2)	2	5	
Black	23 (50)	8	15	
Hispanic	11 (23.9)	2	9	
Asian	3 (6.5)	1	2	
Indian	2 (4.3)	0	2	
Transplant y (%)				0.12
2012–2015	15	4	11	
2016–2018	19	8	11	
2019–2021	12	1	11	

IQR, interquartile range.

values, can be seen in Table 2. Biopsies with normal tissue histology (median dd-cfDNA 0.36%) are shown in Table 3.

There were 5 episodes of clinically diagnosed rejection, which are charted temporally in Figure 1. The median dd-cfDNA among all rejection events (biopsy confirmed and clinically diagnosed) was 2.25%. Conversely, among all patients who did not have rejection, 97% had dd-cfDNA <0.5%.

Thirty-three patients were classified as quiescent. There were 233 total dd-cfDNA measurements from quiescent patients; the median was 0.18% (IQR, 0.15%–0.35%). Median creatinine was 1.10 mg/dL (IQR, 0.94–1.28), and median lipase was 25 U/L (IQR, 18–39).

Comparing quiescent patients to combined rejection and injury, the median dd-cfDNA was 1.6% in rejection/injury group versus 0.18% in quiescent group ($P=0.0002$). Further subdividing injury from rejection, the median values were rejection 2.25%, injury 0.36%, quiescent 0.18% ($P=0.0006$) (Figure 2).

Logistic regression showed that elevated dd-cfDNA was associated with higher probability of rejection (odds ratio = 9.5; 95% CI, 1.5–58; $P=0.0151$). Younger age at transplant was

also associated with rejection (odds ratio = 0.93; 95% CI, 0.9–0.97; $P=0.0004$).

Patient Highlights

A Patient with Allograft Injury

Approximately 4 y after transplant, patient 14 experienced 2 episodes of acute allograft pancreatitis. She presented to the emergency department with right lower quadrant pain and nausea, and a lipase of 719 U/L (previously 47 U/L). Lipase peaked at 1104 U/L, but dd-cfDNA during this time was 0.35%. Magnetic resonance cholangiopancreatography demonstrated an edematous allograft pancreas with minimal fluid, consistent with allograft pancreatitis, the native pancreas was atrophic, otherwise unremarkable. She was admitted to the hospital for intravenous fluids and supportive care. After her pain resolved and lipase normalized, she was discharged, and transitioned from azathioprine to mycophenolate. However, the patient presented to the emergency department 2 mo later with similar symptoms. On her second presentation, her lipase was elevated at 526 U/L and computed tomography (CT) scan showed edematous pancreas allograft with stranding, the native pancreas was unremarkable; however, dd-cfDNA was 0.40%. At this point, she underwent a CT-guided pancreas graft biopsy, which confirmed the diagnosis of acute pancreatitis and revealed no evidence of rejection. She was once again treated with supportive care and discharged after 3 d. It has been more than a year since her last episode of acute allograft pancreatitis, with no recurrence and no etiology was identified. Her most recent laboratory values include lipase 12 U/L and dd-cfDNA 0.32%.

Patients With Biopsy-confirmed Rejection

Patient 16 was transplanted before the initiation of this study. After enrollment, dd-cfDNA was checked 6 times over 2 y; each value below the detectable limit. During this time, his maximum creatinine was 1.32 and maximum lipase was 50 U/L. Two years after enrollment, the dd-cfDNA increased to 2.0%. This was rechecked 1 mo later and had risen to 2.4%. He underwent kidney graft biopsy, which demonstrated C4d-positive AMR. After treatment with steroids, intravenous immunoglobulin (IVIG), and plasmapheresis, dd-cfDNA began to decrease, with his most recent value 1.2% (2 mo after treatment).

TABLE 2.
Rejection/injury events and associated laboratory values

Patient	Event	Pathologic diagnosis	dd-cfDNA (%)	Creatinine	Lipase	Time from transplant
				mg/dL	U/L	
16	Rejection	Kidney: antibody mediated, C4d positive	2.4	1.06	49	5 y
3	Rejection	Kidney: antibody mediated, C4d positive	1.6	1.01	21	5 y
3	Rejection	Kidney: antibody mediated, C4d negative Pancreas: antibody mediated, C4d positive	3.2	1.10	22	7 y
31	Rejection	Pancreas: antibody mediated, C4d positive with moderate acute cellular rejection	3.7	1.40	782	2 y
31	Rejection	Pancreas: acute cellular rejection, C4d negative	1.2	1.60	96	3 y
19	Injury	Kidney: focal segmental glomerulosclerosis/thrombotic microangiopathy	0.36	1.70	30	3 y
19	Injury	Kidney: allergic/drug-induced nephritis	0.17	1.86	42	3 y
23	Injury	Kidney: interstitial fibrosis and tubular atrophy	<0.19	1.66	23	3 y
33	Injury	Kidney: thrombotic microangiopathy/vacuolization	0.22	1.65	25	6 mo
41	Injury	Kidney: isometric tubular vacuolization	2.0	1.66	714	19 d
14	Injury	Pancreas: pancreatitis	0.40	0.97	526	4.5 y

Biopsy and laboratory data from 5 rejection events and 6 injury events among 8 unique patients. dd-cfDNA, donor-derived cell-free DNA.

TABLE 3.
Normal biopsy results

Patient	Biopsy site	Indication for biopsy	dd-cfDNA (%)	Creatinine mg/dL	Lipase U/L
3	Kidney	Persistently elevated dd-cfDNA in a patient with prior episodes of biopsy-confirmed rejection	2.8	1.00	26
20	Kidney	Elevated creatinine	0.46	1.48	48
20	Pancreas	Elevated lipase	0.40	1.03	82
20	Kidney and Pancreas	Elevated lipase	0.37	1.22	256
26	Pancreas	Elevated lipase	0.12	1.24	127
36	Kidney	Elevated creatinine and DSA	0.25	1.84	17
38	Kidney	Elevated creatinine	0.17	1.65	22
43	Kidney	Elevated creatinine, de novo DSA	0.35	1.56	20

Data from patients who had normal tissue biopsies.

dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibody.

Patient 3 was transplanted 7 y before initiation of the study, and had a history of antibody-mediated rejection. Upon enrollment, the patient demonstrated persistent elevations in dd-cfDNA. His first measurement was 1.6%, at which time a kidney allograft biopsy revealed C4d-positive AMR. This was treated with solumedrol, IVIG, and Rituximab. Following treatment, his dd-cfDNA increased to 2.2%, then 2.8%. At this point, another kidney allograft biopsy was obtained; however, this demonstrated a normal kidney. One year later, dd-cfDNA had increased to 4.0%. This time both kidney and pancreas allografts were biopsied, each demonstrating AMR. He was treated with plasmapheresis, IVIG, and rituximab. Three months after treatment, dd-cfDNA decreased to 2.7%; however, it increased again to 4.2% then 5.1%. Notably, the patient's creatinine and lipase have been within normal limits for the duration of the study, with maximum creatinine 1.2 mg/dL and maximum lipase 40 U/L.

A Patient With Clinically Diagnosed Rejection

Five years after transplant, patient 8 demonstrated a baseline dd-cfDNA of <0.3%. However, she developed an acute increase in dd-cfDNA to 1.2% in the context of a slowly rising creatinine (up to 1.49) and a rising lipase (up to 100 U/L).

Clinically Diagnosed Rejection

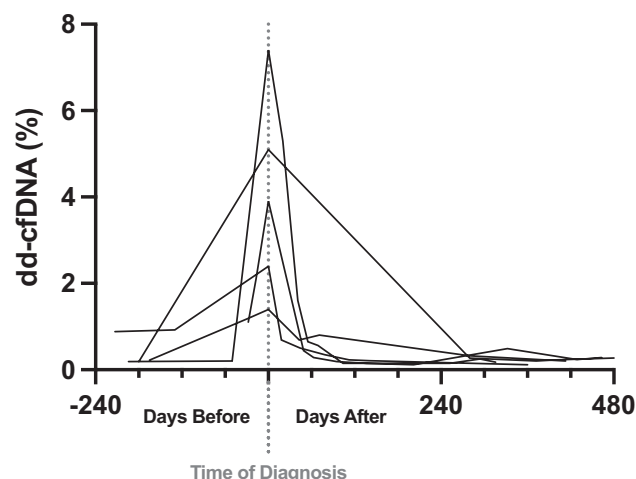


FIGURE 1. dd-cfDNA over time in patients with clinically diagnosed rejection. Dotted vertical line is "time 0", the time at which a diagnosis of rejection was made and treatment was initiated. dd-cfDNA, donor-derived cell-free DNA.

Subsequent dd-cfDNA was 1.4%. This patient was treated with solumedrol for presumed ACR, and her dd-cfDNA slowly decreased and eventually normalized within the next 6 mo.

One year after transplant, patient 28's dd-cfDNA increased to 7.4% from a baseline of 0.2%. The patient had elevated DSA, and lipase had also increased to >1000 U/L. An endoscopic biopsy of graft duodenum was attempted but could not be completed because of technical difficulty. Therefore, the patient was empirically treated with solumedrol as well as IVIG and plasmapheresis for presumed mixed AMR/ACR. Lipase and dd-cfDNA slowly decreased and had normalized within 3 mo.

Quiescent Patients

Patient 42 was considered to be quiescent. Two weeks after transplant, dd-cfDNA was 0.84%. His dd-cfDNA peaked 7 wk posttransplant to 3.1%, then steadily decreased to 0.35% by 3 mo. From 6 mo until the end of the study, dd-cfDNA remained <0.12%.

Patient 43 was also quiescent. Her peak was 8.6% at 20 d posttransplant, which steadily decreased to 1.3% at 3 mo. By 4 mo, dd-cfDNA was 0.41%, and it remained <0.5% for the duration of the study.

Patient 35 showed similar trends following transplant, with a peak dd-cfDNA of 2.7% at 37 d, followed by a nadir of 0.27%. However, this patient developed a transient elevation to 1.3% at 7 mo. Creatinine range remained steady between 0.92 and 1.09 mg/dL, lipase was steady between 27 and 30 U/L, and DSA demonstrated stable, low-level titers. Rejection was considered unlikely, so without initiating treatment, dd-cfDNA was rechecked and had decreased to 0.43% within a month. Thus, this patient was classified as quiescent.

To further demonstrate the dynamics of dd-cfDNA in quiescence, Figure 3 shows dd-cfDNA graphed over time for the quiescent patients who were transplanted during the study period.

DISCUSSION

This study followed 46 patients after SPK transplantation. Numerous studies have evaluated the role of dd-cfDNA following solid organ transplantation; however, few studies have included SPK patients. To our knowledge, this is the first and largest sample of prospectively collected dd-cfDNA data in the SPK population. Other strengths of this study include its racial heterogeneity and the inclusion of patients during all timeframes following transplantation.

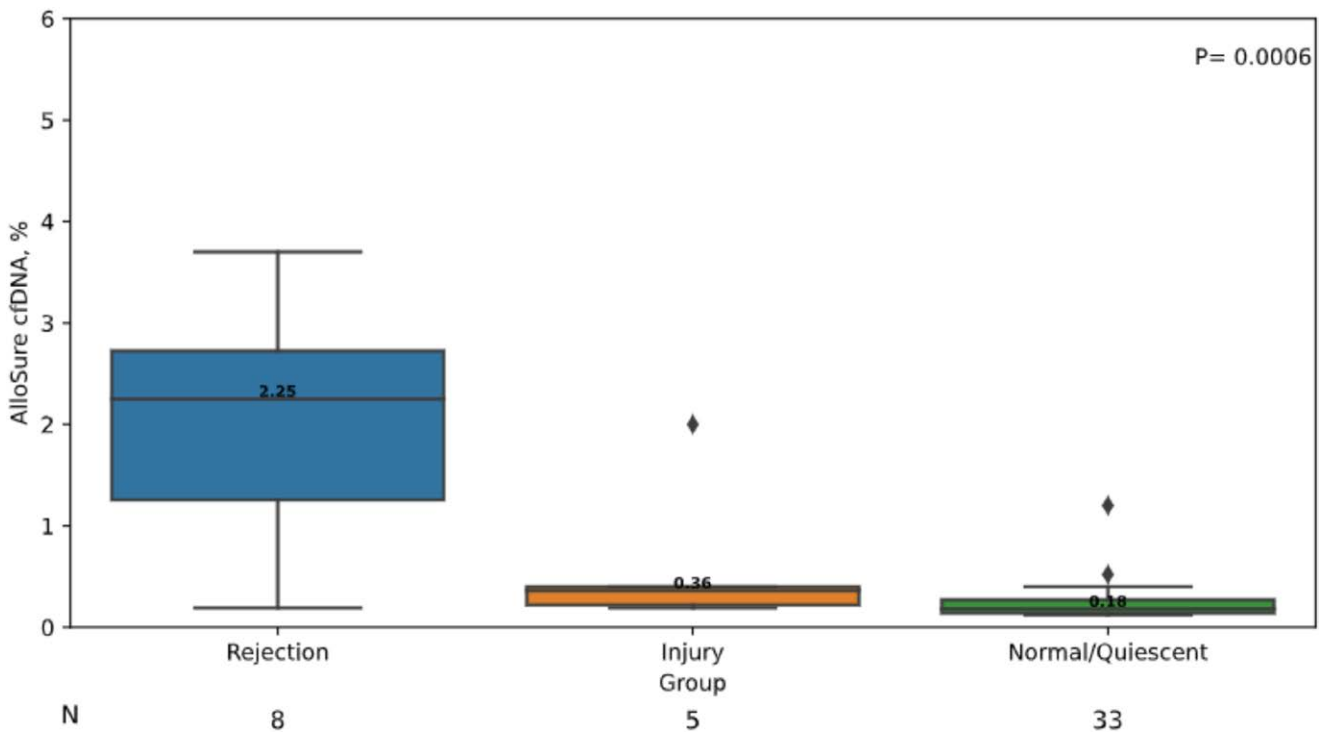


FIGURE 2. Median dd-cfDNA (%) in rejection, injury, and quiescence; includes 1 value per patient. dd-cfDNA, donor-derived cell-free DNA.

Perhaps the most notable finding in this study is the difference in dd-cfDNA values between patients with rejection versus those with injury or quiescence (2.25% in rejection versus 0.36% in injury versus 0.18% in quiescence). It is also striking that 97% of patients without rejection had a dd-cfDNA <0.5%. These findings are consistent with published data in the kidney transplantation population. In particular, the normal range of quiescent SPK patients appears similar to quiescent kidney patients. This is likely because of the relatively small addition of pancreas allograft derived dd-cfDNA, as the average pancreas weighs only 87 g.¹⁴

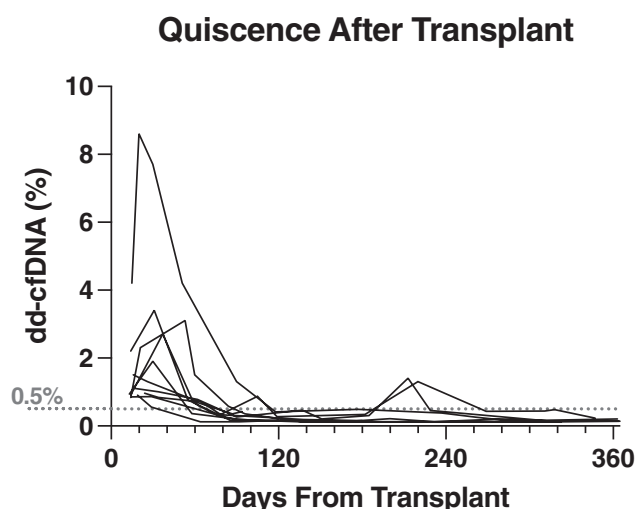


FIGURE 3. Dd-cfDNA over time in quiescent patients following transplant. dd-cfDNA over time, beginning 14 d posttransplant. The dotted horizontal line indicates dd-cfDNA 0.5%. dd-cfDNA, donor-derived cell-free DNA.

Biopsy-confirmed rejection of all types was associated with a median dd-cfDNA of 2.4%. Values at the time of confirmed rejection ranged from 1.2% to 2.7%, with acute cellular rejection of the pancreas being the lowest at 1.2%. These findings are consistent with previously published literature among kidney transplant patients.⁵

The differences in dd-cfDNA between acute rejection versus allograft injury are also worth noting. The difference in dd-cfDNA between rejection and injury is particularly striking in the case of pancreatitis, where extreme elevations of lipase were seen without substantial elevations in dd-cfDNA. Although direct pairwise comparison between rejection and injury did not reach statistical significance ($P=0.1$), there may be potential for differentiation between the 2 with larger sample sizes in the future.

dd-cfDNA values should be interpreted in the context of other clinical and laboratory findings. As seen in our results, multiple patients had acute elevations in dd-cfDNA; however, some were treated for rejection, whereas others were simply rechecked later. For example, an increase in dd-cfDNA with de novo or rising DSA may be more concerning than an increase in dd-cfDNA alone. Therefore, until additional data is published, dd-cfDNA should be used with caution and interpreted within overall clinical context, ideally by those with experience in SPK transplantation. However, based on our current experience, we would suggest that dd-cfDNA be checked at 1, 3, 6, 9, and 12 mo posttransplant for routine surveillance in the low to medium immunological risk SPK recipients; and monthly for the first 12 mo in the high immunological risk or during significant immunosuppression changes. A first check of dd-cfDNA at 2 wk postrejection treatment has also been found useful, because a downward trend can indicate successful treatment, which usually precedes recovery of creatinine or lipase if previously elevated.

Limitations of this study include inconsistent collection of some important variables, such as BK Virus or DSA, which

precluded additional conclusions and should be studied in the future. Another major limitation is sample size. Although 46 patients could be considered a large cohort in the SPK population, the number of biopsy-confirmed rejection events was perhaps too small to draw more meaningful conclusions.

The methods used to diagnose rejection were not perfectly consistent and should be considered a limitation as well. The most obvious inconsistency is that some patients were diagnosed with a biopsy, whereas others were diagnosed clinically. We would have preferred every diagnosis of rejection to be confirmed by allograft pancreas biopsy; however, this was not always feasible. In some cases, the best interest of individual patients (on anticoagulants for example) took priority over the research protocol. In other patients, biopsies were attempted but could not be completed because of the lack of a safe window for CT-guided biopsy. Even in those who were able to undergo biopsy, the findings were not always consistent with clinical impression, and the clinical judgment took precedence. As previously mentioned, our practice changed from duodenal biopsy to pancreas biopsy during the study period. Additionally, pathologic analysis of allograft pancreas biopsies has significant room for improvement. As an example, 1 patient was diagnosed as ACR in the pancreas; however, weeks later, his diagnosis was changed to a mixed rejection after C4d tests returned positive. So although our difficulty obtaining pancreas biopsies should be viewed as a limitation, it also illustrates the challenges with biopsy of the pancreas allograft, which should be the focus of future research and quality improvement projects at pancreas transplant centers.

One final limitation to consider is the cost associated with dd-cfDNA. Although it is reimbursable by Medicare in certain populations, the cost of frequent dd-cfDNA measurements can be taxing on the healthcare system. However, pancreas with or without kidney allograft biopsy procedures are expensive as well, and they come with significantly higher risk to the patient. Therefore, further investigation is justified to identify ideal frequency and patient population in whom dd-cfDNA may be a sensitive marker for allograft surveillance and rule out rejection or graft injury.

In conclusion, the use of dd-cfDNA for surveillance and diagnosis of acute rejection in SPK patients shows promise. However, there is much more work to be done. Additional

studies should be pursued, particularly multicentre collaborations, which would increase sample sizes and statistical power of this relatively small cohort of patients worldwide.

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