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Association Between Total Cell Free DNA and SARS-CoV-2 In Kidney Transplant Patients: A Preliminary Study

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ABSTRACT

Background. Kidney transplant (KT) recipients are at high risk for developing severe COVID-19. Lowering immunosuppression levels in KT recipients with COVID-19 encourages native immune responses but can raise the risk of rejection. Donor-derived cell-free DNA (dd-cfDNA), reported as a fraction of total cfDNA, is a proven biomarker for KT rejection. Total cfDNA levels are elevated in patients with COVID-19, which may depress dd-cfDNA fractions, potentially leading to missed rejections.

Methods. A retrospective analysis of 29 KT recipients hospitalized with COVID-19 between April and November 2020 examined total and dd-cfDNA levels. Blood samples were collected after onset of COVID-19, with follow-up samples collected from a subset of patients, when infection had likely subsided.

Results. After COVID-19 diagnosis, the median total cfDNA level was elevated (7.9 multiples of median [MoM]). A significant decrease in total cfDNA levels was observed between the first and second time points (6.2 MoM, 1.0 MoM; $P < .001$). A significant positive association was identified between total cfDNA levels and COVID-19 severity ($P = .02$; $R^2 = .19$). Two patients with biopsy-proven acute cellular rejection had dd-cfDNA fractions below the 1% cutoff for rejection (0.20% and 0.78%), with elevated total cfDNA levels of 7.9 MoM and 41.8 MoM, respectively.

Conclusions. In this preliminary study, total cfDNA levels were elevated in KT patients with COVID-19, subsiding after resolution of infection. High total cfDNA levels may confound dd-cfDNA results, leading to failure to identify rejection. Considering total cfDNA levels is important in interpretation of dd-cfDNA tests for assessment of rejection in KT patients with COVID-19 or other infection.

Data Sharing: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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KIDNEY transplantation (KT) is considered the ideal treatment for patients with end-stage kidney disease and can lead to substantial improvements in patient survival and quality of life [1]. Unfortunately, recipient-mediated allograft damage and failure are common, with most patients experiencing acute kidney injury (AKI) within 2 years of transplant [2,3], an annual allograft failure rates of ~3% to 5% beyond the first year, and a 10-year transplant attrition rate of ~55% [4]. Acute rejection is a predominant cause of kidney allograft failure, most commonly owing to alloimmune-mediated injury [5, 6]. Chronic immunosuppression is the main treatment strategy to help prevent allograft rejection, functionally counteracting the inflammatory and immunologic responses mounted by the recipients [7, 8].

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that causes COVID-19 has brought significant challenges to the treatment and management of KT recipients [9]. Chronic immunosuppression may place transplant recipients at a heightened risk of developing more severe courses of COVID-19 [10], and virus-positive transplant recipients have poorer survival outcomes compared with healthy individuals [11]. Consequently, physicians typically lower immunosuppression in patients with COVID-19, which increases the risk of allograft rejection. Additionally, comorbidities common in KT patients, such as diabetes, obesity, hypertension, and cardiac disease, are also major risk factors for severe COVID-19 symptoms and poor outcomes [11,12].

Compounding this, SARS-CoV-2, itself, reportedly causes kidney damage, including acute kidney injury (AKI) and acute kidney failure (AKF) owing to virally induced multi-organ failure, reduced renal perfusion, and cytokine storm [13, 14]. Kidney damage is found to increase with COVID-19 severity, and AKI and AKF are associated with poor prognosis [15]. In severe SARS-CoV-2 infection, immunosuppressive treatments may help mitigate the cytokine storm and consequential kidney damage during the inflammatory stage of the disease [16, 17]. Stratification of virally infected KT patients into high- and low-risk groups for AKI and AKF could aid in physician decision-making regarding patient management and treatment, including the use, dose, and timing of immunosuppressants.

Circulating, donor-derived cell-free DNA (dd-cfDNA) is now a proven biomarker that can detect AKI and AKF reliably and with high sensitivity [18–20]. Owing to its circulation in the blood, dd-cfDNA can be measured noninvasively and serially through a simple blood test, and is reportedly more accurate than measurement of serum creatinine [21]. Current commercial tests generally report dd-cfDNA as a fraction of total cfDNA, with >1% considered high risk for rejection. Elevated total cfDNA levels associated with COVID-19 and other infections have been hypothesized to depress dd-cfDNA fractions, potentially confounding interpretation of dd-cfDNA test results. Here, we present results of dd-cfDNA testing using a massively-multiplexed polymerase chain reaction (mm-PCR) amplification followed by next-generation sequencing (NGS) measurement of amplicons, in a series of hospitalized KT

patients with COVID-19, examining the effect of COVID-19 infection on the total cfDNA levels and the effect on dd-cfDNA fractions.

PATIENTS AND METHODS

Patients and Samples

A retrospective analysis of dd-cfDNA test results was conducted on blood samples collected from renal allograft patients at Loyola University Medical Center, Maywood, Illinois, and Hospital das Clinicas of University of São Paulo, Brazil who were diagnosed with COVID-19 and had dd-cfDNA testing performed with Prospera (Natera, Inc.) as part of clinical care between April and November 2020. This study received an exemption from the Institutional Review Board review (study ID 213800) from Loyola University Chicago for being considered as secondary research for which consent is not required, and Institutional Review Board approval (CAAE 40606115.6.0000.0068, version 5) from the Research Ethics Committee at Hospital das Clinicas of University of São Paulo. This study was performed in full adherence to the Declaration of Helsinki. The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the Declaration of Istanbul on Organ Trafficking and Transplant Tourism.

Patients had an initial dd-cfDNA test performed shortly after infection, with a subset of patients having a follow-up test after COVID-19 clearance. Demographic, clinical, and outcome data was collected for each patient and de-identified before analysis.

Individuals who were under 18 years of age, had more than 1 organ transplanted, were pregnant, or had a blood transfusion within 2 weeks of enrollment were excluded. The inclusion of samples in the primary analysis was based on availability of adequate plasma to run the dd-cfDNA assay and availability of clinical follow-up.

Analysis of dd-cfDNA Using mm-PCR NGS Assay

Blood samples were processed and analyzed at Natera, Inc.'s Clinical Laboratory Improvement Amendments-Certified and College of American Pathologists-accredited laboratory (San Carlos, California). Laboratory testing was performed as previously described, using massively multiplexed-PCR (mmPCR), targeting over 13,000 single nucleotide polymorphisms [22]. Next generation sequencing, with an average of 10 to 11 million reads per sample, was performed on the Illumina HiSeq 2500 on rapid run. For all patients, both the total cfDNA level (reported in multiples of the median [MoM] and copies/mL) and the dd-cfDNA fraction (analyzed as the percentage of total cfDNA) were measured.

Biopsy samples were analyzed and graded according to the standard practice at each site by their respective pathologists using Banff 2017 classification [23]. AKI was defined as increases in serum creatinine levels (>2.0x baseline), urine output <0.5 mL/kg/h for >12 hours, or “de novo” appearance of proteinuria (≥ 0.3 g/d) [24]. Diagnosis of COVID-19 and its severity was classified based on the 8-point ordinal scale of clinical improvement published by the World Health Organization in February 2020, in which 1 indicates no evidence of infection and 8 indicates mortality [25].

Statistical Analysis

Differences in either total cfDNA levels or dd-cfDNA fractions were assessed between tests performed closest to the onset of COVID-19 symptoms and the follow-up time point (a proxy for baseline levels) using paired *t* tests. To determine if elevated cfDNA levels are

Table 1. Clinical Features of the 29 Hospitalized Kidney Transplant Recipients Infected With COVID-19

Patient	Age	Sex	Time from KT to COVID-19 onset (d)	IS at COVID-19 onset	ISManagement	OtherTreatment	WHOScore	AKI	Ventilation	RRT	Death	Reason for Hospital Admission
1	39	M	703	TacXR/MPS/Pred	MPS dec.	Remdesivir	4	Y	N	N	N	COVID-19
2	52	M	627	Tac/MPS	MPS d/c; Pred start	Remdesivir	8	N	Y, invasive	N	Y	COVID-19
3	26	M	555	Tac/MPS/Pred	MPS d/c		3	Y	N	N	N	COVID-19
4	61	F	556	Tac/MPS/Pred	MPS d/c		3	Y	N	N	N	COVID-19
5	66	F	261	TacXR/MPS	MPS dec and d/c; Pred start	Remdesivir	4	N	N	N	N	COVID-19
6	64	M	155	TacXR/MPS	MPS d/c; Pred start	HCQ	4	Y	N	N	N	COVID-19
7	64	M	165	CsA/MPS	CsA/MPS d/c; Pred start	Tocilizumab	8	Y	Y, invasive	N	Y	COVID-19
8	63	F	98	TacXR/MPS	MPS dec.; Pred Start		3	N	N	N	N	COVID-19
9	50	F	62	TacXR/MPS	MPS dec.; Pred Start		3	Y	N	N	N	COVID-19
10	58	F	6694	Sir/MMF/Pred	MMF d/c; Sir. d/c	Convalescent plasma; Dexamethasone	5	Y	N	Y	N	COVID-19
11	71	F	927	TacXR/Pred	No change	Convalescent plasma, Dex	4	Y	N	N	N	COVID-19
12	57	M	781	Tac/MMF	MMF d/c; Pred Start		3	Y	N	N	N	COVID-19
13	73	M	815	TacXR/MPS	MPS d/c; Pred Start	Convalescent plasma, Dex	5	N	N	N	N	COVID-19
14	33	M	1467	Tac/MPS/Pred	MPS dec. and d/c	Convalescent plasma, Dex, Remdesivir	6	Y	Y, invasive	N	N	COVID-19
15	70	M	1619	Tac/MPS/Pred	Tac/MPS/Pred d/c; Hydrocortisone start		7	Y	Y, invasive	Y	Y	COVID-19
16	43	M	10	Tac/MPS/Pred	Tac/MPS/Pred d/c; Hydrocortisone start	Convalescent plasma; Methylprednisolone pule for possible acute rejection	7	Y	Y, invasive	Y	N	Kidney Transplant
17	54	M	4707	Tac/Aza/Pred	Tac/Aza/Pred d/c	Methylprednisolone start	7	N	Y, invasive	N	Y	COVID-19
18	66	M	1883	CsA/MPS/Pred	MPS d/c		3	N	N	N	N	COVID-19
19	54	M	2318	Tac/MPS/Pred	MPS/Pred d/c	Dex start	5	N	N	N	N	COVID-19
20	45	F	503	Tac/MPS/Pred	MPS d/c		3	Y	N	N	N	COVID-19
21	59	F	5330	belatacept/MMF/Pred	belatacept/MMF/Pred d/c; Hydrocortisone start		7	Y	Y, invasive	Y	N	
22	71	M	3476	Tac/Aza/Pred	Tac/Aza/Pred d/c; Hydrocortisone start		7	Y	Y, invasive	Y	Y	peripheral arterial obstruction, foot gangrene
23	68	M	3781	CsA/MPS/Pred	MPS d/c		5	Y	Y, invasive	Y	N	COVID-19

(continued on next page)

Table 1 (Continued)

Patient	Age	Sex	Time from KT to COVID-19 onset (d)	IS at COVID-19 onset	IS Management	Other Treatment	WHOScore	AKI	Ventilation	RRT	Death	Reason for Hospital Admission
24	45	F	6	Tac/MPS/Pred	Tac/MPS/Pred d/c	methylprednisolone start; ATG for acute rejection	7	Y	Y, invasive	Y	N	Kidney transplant
25	21	M	6	Tac/MPS/Pred	MPS d/c	Ecilizumab for thrombotic microangiopathy	4	Y	N	Y	N	Kidney transplant
26	68	F	2084	Tac/MPS/Pred	Tac/MPS/Pred d/c; Hydrocortisone start		5	Y	Y, invasive	Y	Y	COVID-19
27	57	M	712	Tac/MPS/Pred	MPS d/c		4	N	N	N	N	chronic constrictive pericarditis
28	47	F	868	Tac/MPS/Pred	Tac/MPS/Pred d/c; Hydrocortisone start		7	Y	Y, invasive	Y	Y	COVID-19
29	72	M	6208	CsA/MPS/Pred	MPS d/c		3	N	N	N	N	COVID-19

Aza, azathioprine; CsA, cyclosporine A; COVID-19, coronavirus disease 2019; dec, decreased; Dex, Dexamethasone; d/c, discontinued; F, female; HCC, hydroxychloroquine; M, male; MMF, mycophenolate mofetil; MPS, mycophenolate sodium; N, no; Pred, prednisone; Tac, tacrolimus; TacXR, tacrolimus extended release; Y, yes.

Table 2. Characteristics of the 29 Hospitalized Kidney Transplant Recipients Infected With COVID-19

Cohort Characteristics	N = 29 (%)
Age (median)	58 (21-73)
Women	11 (37.9)
Race	
White	12 (41.4)
Black	6 (20.7)
Asian	1 (3.4)
Hispanic	8 (27.6)
Mixed	2 (6.9)
BMI (median)	27.8 kg/m ²
COVID-19 Severity Score (median)	5
Time analyses (d)	Median (range)
Time from KT to onset of COVID-19	781 (6-6694)
Time from onset of COVID-19 to hospital admission	6 (-13 to 17)
Time between dd-cfDNA tests	71 (27-112)
Time between admission and first dd-cfDNA test	9 (2-65)
Time between onset of COVID-19 and death	29 (20-53)
Outcomes	N (%)
AKI	20 (69.0)
RRT	10 (34.5)
Ventilation	12 (41.4)
Death	7 (24.1)
Donor Type	
Deceased donor	23 (79.3)
Living related	4 (13.8)
Living unrelated	2 (6.9)

AKI, acute kidney injury; BMI, body mass index; dd-cfDNA, donor-derived cell-free DNA; KT, kidney transplant; RRT, renal replacement therapy

attributed to either AKI or renal replacement therapy (RRT), paired *t* tests were performed across time periods and Wilcoxon rank sum tests were performed for intra-time period comparisons. All *t* test and Wilcoxon tests were two-tailed and the cutoff for statistical significance was <.05. Stepwise regressions were used to investigate associations of cfDNA measures (both total and dd-cfDNA) with COVID-19 severity scores (linear) and mortality (logistic regression). In addition to total cfDNA level and dd-cfDNA fraction, potential predictor variables included in these models were age, donor type, and AKI. Donor type and AKI were entered as binary variables. Total cfDNA, dd-cfDNA, and age were entered into models as continuous variables. Variables were entered in models at *P* ≤ .10 and retained at *P* < .15. Body mass index and baseline creatinine were considered for inclusion in analyses but were inestimable in all models. Clinical significance was based on the assessment of the observed effect and the magnitude of the appropriate *P* value.

RESULTS

Clinical Characteristics and Outcomes

The study included 29 KT patients who were diagnosed with COVID-19 between April and November 2020. Six of these patients were admitted to the hospital for other reasons (3 for KT surgery) and contracted COVID-19 nosocomially (Table 1). The median age among the cohort was 58 years (range, 21-73 years), with a median time from transplant to onset of COVID-19 of 781 days (range, 6-6694). The cohort was predominantly

Table 3. Summary of Total cfDNA Findings for Hospitalized Kidney Transplant Recipients Infected With COVID-19

	Paired Tests		
	First Time Point (MoM)	Follow-up Time Point (MoM)	P Value
Full cohort (N = 15) (median, range)	6.2 (0.6-18.6)	1.0 (0.4-7.8)	<i>P</i> = .0009
AKI (n = 9) (median, range)	7.9 (0.6-18.6)	0.9 (0.4-7.8)	<i>P</i> = .01
Non-AKI (n = 6) (median, range)	5.7 (1.1-18.1)	1.1 (0.7-2.4)	<i>P</i> = .06
RRT (n = 2)	7.9 (18.6)	0.6 (3.1)	N/A
Non-RRT (n = 13) (median, range)	5.2 (0.6-18.1)	1.0 (0.4-7.8)	<i>P</i> = .003

AKI, acute kidney injury; MoM, multiples of median; N/A, not applicable; RRT, renal replacement therapy.

white (41.4%) and men (62.1%), with allografts received from deceased donors (79.3%) (Table 2). The median time from onset of COVID-19 symptoms to hospital admission was 5 days, with a range of 17 days before to 13 days after hospital admission.

AKI was diagnosed in 69.0% (20 of 29) of patients. RRT was required for 34.4% (10 of 29) of patients; 3 of these individuals were initiated on RRT before COVID-19 diagnosis owing to delayed graft function after KT. Biopsies were performed on 5 individuals with AKI, which confirmed acute cellular rejection in 2 of these patients and had inconclusive borderline findings in 1 individual who was nonetheless treated for possible acute rejection. Mechanical ventilation was initiated for 41% (12 of 29) of the cohort, of which 58.3% patients (7 of 12) died. The median time from onset of symptoms to death among these 7 patients was 29 days (range, 20-53 days).

Patient Management

At the time of COVID-19 diagnosis, the most common maintenance immunosuppressants among the cohort included mycophenolate mofetil or mycophenolate sodium for 90% (26 of 29) of patients, tacrolimus or tacrolimus extended release for 79% (23 of 29) of patients, and prednisone for 72% (21 of 29) of patients. Lesser common treatments among the cohort included maintenance with belatacept (1 of 29), sirolimus (1 of 29), azathioprine (2 of 29), and cyclosporine A (4 of 29) (Table 1). In the majority of patients, the change in immunosuppression owing to COVID-19 was the decrease or discontinuation of mycophenolate mofetil/mycophenolate sodium and the initiation/increase of steroid treatment (prednisone or hydrocortisone). For treatment of COVID-19, 4 patients received remdesivir; 8 received dexamethasone or methylprednisolone; 5 were administered convalescent plasma; and 1 patient was treated with hydroxychloroquine (Table 1).

Elevated Total cfDNA Levels at Onset of COVID-19

After admission to the hospital, all patients were monitored for allograft rejection using a dd-cfDNA test. For these patients, the median time from the onset of COVID-19 symptoms to the first dd-cfDNA test reading was 14 days (range, 5-72) with 25 tests (86%) being performed within 30 days. Fifteen of the 29 patients (51.7%) had a second follow-up dd-cfDNA test performed, after COVID-19 symptoms had subsided, with a

median time of 71 days between blood draws (range, 27-112), and a median of 94 days from the onset of COVID-19 (range, 64-129). Calculation of the time in days from the onset of COVID-19 to each dd-cfDNA test performed (*n* = 44) indicated minimal overlap between the 2 testing periods. Comparison of total cfDNA levels to a reference median value of 5.60×10^3 cp/mL (1 MoM), as determined from 150 sequential samples processed for dd-cfDNA testing in Natera's Clinical Laboratory Improvement Amendments-certified laboratory, revealed that 21 of 29 (72.4%) of initial total cfDNA readings were ≥ 4 MoM (2.24×10^4 cp/mL), and 14 (48.3%) were ≥ 8 MoM (4.48×10^4 cp/mL); only 1 reading from a follow-up time point was elevated ≥ 4 MoM (Fig 1A). The median total cfDNA level was substantially higher for initial tests (7.9 MoM; 4.44×10^4 cp/mL; *n* = 29), occurring closest to COVID-19 symptom onset compared with the follow-up tests (1.0 MoM; 5.66×10^3 cp/mL; *n* = 15; Fig 1B). For the 15 patients who had 2 tests performed, the reading at the first time point was significantly higher (median: 6.2 MoM; 3.46×10^4 cp/mL; *P* < .001) compared with the follow-up time point (median: 1.0 MoM).

Among results from initial tests, patients who received RRT before the first cfDNA measurement had significantly higher total cfDNA levels (median 17.8 MoM; range, 6.8-53.4; *n* = 7), compared with those who did not receive RRT (median 5.2 MoM; range, 0.6-29.2; *n* = 21) (*P* = .01). Total cfDNA levels were similar in patients with AKI (median 8.1 MoM; range, 0.6-53.4; *n* = 20) and those without AKI (median 6.2 MoM; range, 1.1-29.2; *n* = 9) (*P* = 1.0). We observed similar trends of decreasing cfDNA levels between the initial time-point and the follow-up time-point for individuals who did not receive RRT (*n* = 13; *P* = .003), who experienced AKI (*n* = 9; *P* = .01) and those who did not experience AKI (*n* = 6; *P* = .06). We had an inadequate sample size to assess this change in patients who did receive RRT (*n* = 2; Table 3).

The median dd-cfDNA fraction among the initial test results from the 29 patients was 0.11% (range, 0.01-1.54%) vs 0.32% (range, 0.03-0.98%) for the 15 follow-up tests; this difference was not significant for the 15 individuals with paired test results (*P* = .67; Fig 1C).

Elevated Total cfDNA Levels Obscured Indication of Rejection by dd-cfDNA Testing

Biopsy showed acute cellular rejection (Banff 1B) in 2 individuals in our cohort. Tests from the initial time points

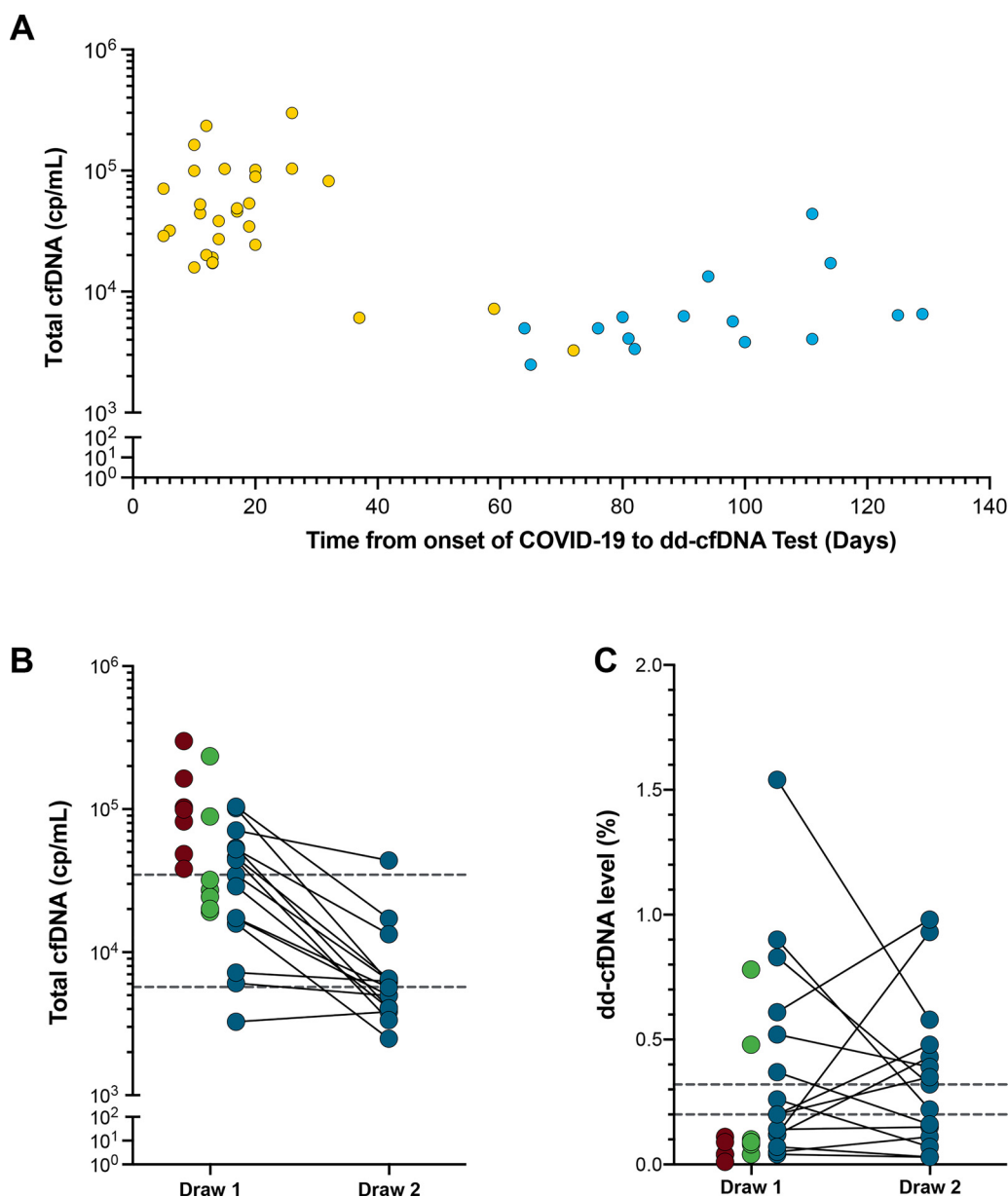


Fig 1. Donor-derived and total cfDNA levels in kidney transplant recipients with COVID-19. **(A)** Total cfDNA levels, represented as cp/mL, were plotted against time in days from onset of COVID-19 symptoms to date of blood draw for dd-cfDNA tests at both the initial time point (yellow) and the follow-up time-point (blue). **(B)** Total cfDNA levels at the initial time point (Draw 1) and the follow-up time point (Draw 2), stratified by patients who had a single draw owing to death (red), or a second draw was unavailable (green), and patients with two draws (blue). Black lines connect paired tests. Grey dotted lines indicate medians for 15 paired values at first draw (3.46×10^4 cp/mL) and second draw (5.66×10^3 cp/mL). **(C)** dd-cfDNA levels at the initial time point and the follow-up time point, stratified as indicated in **(B)**. Black lines connect paired tests. Grey dotted lines indicate medians for 15 paired values at first draw (0.2%) and second draw (0.32%). cfDNA, cell-free DNA; dd-cfDNA, donor-derived cell-free DNA.

indicated dd-cfDNA fractions of 0.2% and 0.78%, accompanied by total cfDNA levels of 7.9 MoM (4.44×10^4 cp/mL) and 41.8 MoM (2.3×10^5 cp/mL), respectively. For the first individual, biopsy-confirmed rejection occurred 10 days after their initial dd-cfDNA test. Total cfDNA levels decreased to 0.60 MoM (3.35×10^3 cp/mL)

accompanied by a dd-cfDNA fraction of 0.48% at the follow-up time-point, 71 days later, after treatment of the rejection with antithymocyte globulin. For the second individual, biopsy-confirmed rejection occurred 54 days after dd-cfDNA testing; follow-up dd-cfDNA testing was not performed for this individual.

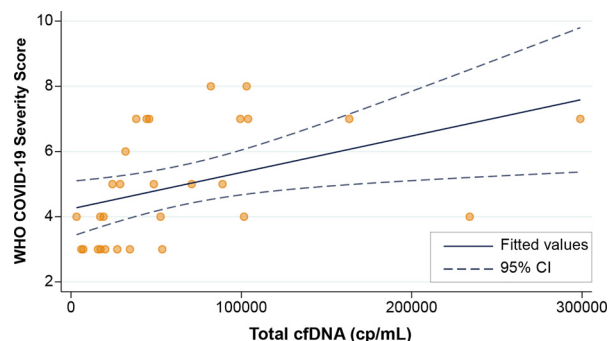


Fig 2. Linear regression for COVID-19 severity. Relationship between total cfDNA (cp/mL) and World Health Organization (WHO) COVID-19 Severity Score (Beta = 1.1×10^{-5} , SE = 4.4×10^{-6} , $P = .02$). cfDNA, cell-free DNA; CI, confidence interval.

Total cfDNA Levels are Associated With COVID-19 Severity and Death

Clinical COVID-19 severity scores in this cohort ranged from 3 (hospitalization with no oxygen therapy) to 8 (mortality), with a median score of 5 [25]. Stepwise regression identified a significant positive association between total cfDNA levels and the COVID-19 severity score ($R^2 = .19$; $P = .02$; Fig 2). No other covariates achieved the $P \leq .10$ level of significance required for inclusion in the model.

Stepwise regression analysis identified total cfDNA and dd-cfDNA fractions as the only predictors of mortality. A clinically relevant trend toward an association between each of these variables with mortality was observed (dd-cfDNA $P = .07$; total cfDNA $P = .11$), but did not reach statistical significance. The probability of death increased with increasing total cfDNA levels (Fig 3A). In contrast, the probability of death increased with decreasing dd-cfDNA fractions, but only for dd-cfDNA values less than 0.25%; above this value, probability of death was estimated to be 0 (Fig 3B).

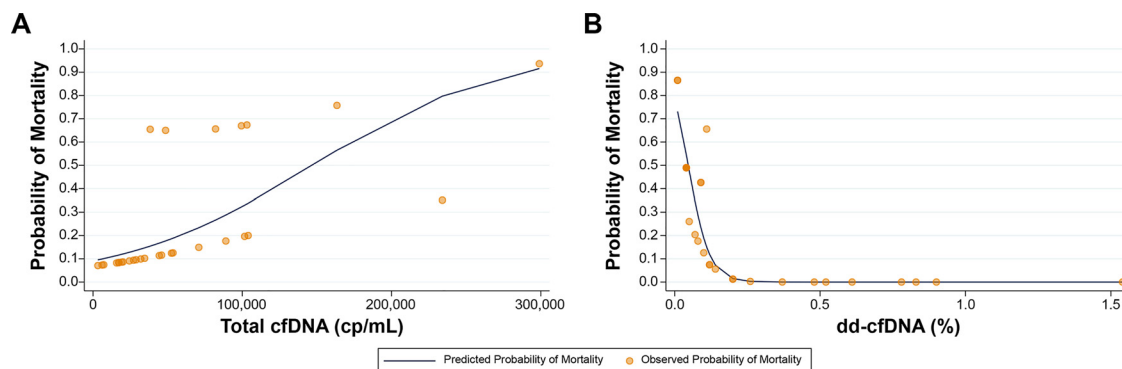


Fig 3. Logistic regression for predicting mortality. **(A)** Relationship between total cfDNA (first measurement; cp/mL) and probability of mortality (stepwise regression results: $P = .11$, Beta = 3.2×10^{-5} , SE = 2.0×10^{-5}). **(B)** Relationship between dd-cfDNA and probability of mortality (stepwise regression results: $P = .07$, Beta = -38.7 , SE = 21.2). cfDNA, cell-free DNA; dd-cfDNA, donor-derived cell-free DNA.

DISCUSSION

SARS-CoV-2 infection is especially dangerous to patients with a KT. First, AKI is a common complication of COVID-19 [9, 13], and second, tapering of immunosuppression to enable immune responses against the virus increases the risk of allograft rejection. dd-cfDNA is an emerging noninvasive marker for monitoring allograft injury and risk of rejection [18–20]. However, elevated total cfDNA levels, which may result from viral infections such as SARS-CoV-2, could lead to misinterpretation of the dd-cfDNA test results. Here, we analyzed total cfDNA levels and dd-cfDNA fractions in 29 hospitalized KT patients with COVID-19, and, for a subset of patients, at a time point approximately 2 months after the initial test once infection subsided. Our preliminary study showed that total cfDNA levels were elevated during COVID-19 infection and significantly decreased after the infection had abated.

Total cfDNA levels were highly elevated in patients at the time of their first test, close to the onset of COVID-19. In this cohort, 75% and 48% of total cfDNA readings from initial tests were elevated above 4 and 8 MoM, respectively, as compared with 4.8% and 1.2%, respectively, in a cohort of unselected KT recipients tested for dd-cfDNA testing during routine care.[26]. This is consistent with literature showing a positive correlation between total cfDNA and viral infection [27–29]. We also observed a significant decrease in total cfDNA levels, with only 1 reading ≥ 4 MoM at the follow-up time point, after patients are expected to have recovered from the COVID-19. Additionally, 14 of the 15 patients for whom 2 tests were performed experienced decreases in their total cfDNA levels between time points. This trend is corroborated by a recent case study wherein a KT recipient with COVID-19 had total cfDNA levels elevated to 57 MoM (3.10×10^5 cp/mL) during infection, with levels declining to 2.9 MoM (1.62×10^4 cp/mL) over the course of 1.5 months, during clearance of the infection [30].

The majority of the samples from this cohort with elevated total cfDNA levels were drawn within 32 days of the onset of COVID-19 symptoms. The median duration of SARS-CoV-2 positivity is approximately 20 days, but can last as long as

53 days, in a general population [31, 32]. Infection can last significantly longer in immunocompromised and organ transplant patients [33–35], as well as in critically ill patients, with ~60% of patients clearing the virus within 30 days [31]. Thus, our data indicate that the elevated cfDNA levels observed at the initial time point occurred during active SARS-CoV-2 infection. Other factors that may have contributed to elevated cfDNA levels include secondary bacterial infections, lung inflammation owing to invasive ventilation, or thrombosis.

In contrast to total cfDNA levels, dd-cfDNA fractions at the first time point were not elevated, and no significant difference in levels was observed between the first and second time points. This is not surprising, as elevations in total cfDNA levels would be expected to depress the proportion of dd-cfDNA. Specifically, in this cohort only 3.4% of patients (1/29) had dd-cfDNA fractions at or above the 1% threshold for indication of allograft injury/rejection, as compared with clinical cohorts in which we previously identified test positivity rates of 32% (37/114) and 47% (48/103) in individuals with surveillance and for-cause biopsies respectively [18].

Total cfDNA levels were higher at the first time point compared with the second in all subcohorts queried, regardless of RRT or AKI status. Although studies have implicated RRT, such as hemodialysis in elevations in cfDNA [36], our findings suggest that neither AKI nor RRT can fully account for the changes observed. While many of these changes in total cfDNA were statistically significant, they are likely not clinically useful.

Two individuals in our cohort with biopsy-confirmed active rejection had substantially elevated total cfDNA. The dd-cfDNA fractions for both individuals were below the standard 1% dd-cfDNA cutoff for high risk for rejection, indicating that interpretation of the dd-cfDNA test results was confounded by the elevated total cfDNA. Studies have suggested that subtle changes in dd-cfDNA levels could be masked when reporting dd-cfDNA fractions owing to fluctuations in total cfDNA. Additionally, measuring dd-cfDNA quantity (cp/mL) independent of total cfDNA is valuable in assessing allograft rejection [37, 38]. We recently reported on a methodology that incorporates both the fraction and quantity of dd-cfDNA for assessment of allograft rejection [39]; this algorithm would have identified high risk for rejection for both of these cases during active infection with SARS-CoV-2 when total cfDNA levels were elevated. Further studies are needed to assess broad use of dd-cfDNA quantity to test for allograft rejection during viral infection.

Our analysis demonstrated a clinically significant correlation between total cfDNA levels and COVID-19 severity. These findings corroborate another study that similarly identified an association between cfDNA concentrations and World Health Organization clinical progression scores in hospitalized patients [10]. Although these findings are scientifically interesting, they are likely not clinically useful.

Limitations to our study include an analysis restricted to only hospitalized KT patients, thus we were unable to evaluate if these trends also are present in allograft recipients with symptomatically mild COVID-19 cases. Additionally, serial dd-cfDNA testing and tracking of COVID-19 severity over the

course of hospitalization in these individuals would enable a more precise understanding of the relationship between progression of COVID-19 and total cfDNA levels. As this is a preliminary study, future studies will be needed to validate the relationship between total cfDNA and dd-cfDNA levels and COVID-19 infection in KT patients.

In summary, the data presented herein indicate an association between elevated total cfDNA and COVID-19 infection and its severity in hospitalized KT patients. Additionally, when using dd-cfDNA testing for monitoring allograft rejection in individuals with COVID-19, consideration of total cfDNA levels along with the dd-cfDNA fraction is important to ensure that cases of rejection are not missed.

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