OPEN

The Measurement of Donor-Specific Cell-Free DNA Identifies Recipients With Biopsy-Proven Acute Rejection Requiring Treatment After Liver Transplantation

Su Kah Goh, MBBS,[1,2] Hongdo Do, PhD,[2,3,4] Adam Testro, MBBS, FRACP, PhD,[5,6] Julie Pavlovic, BN, MNP,[5] Angela Vago, BN, Dip App Sc,[5] Julie Lokan, MBBS, FRCPA,[7] Robert M. Jones, MBChB, FRACS, FRCS (Ed),[1,5,8] Christopher Christophi, MBBS, MD, FRACS,[1,5,8] Alexander Dobrovic, PhD,[1,2,3,4*] and Vijayaragavan Muralidharan, MBBS, PhD, FRACS[1,5,8*]

Background. Assessment of donor-specific cell-free DNA (dscfDNA) in the recipient is emerging as a noninvasive biomarker of organ rejection after transplantation. We previously developed a digital polymerase chain reaction (PCR)-based approach that readily measures dscfDNA within clinically relevant turnaround times. Using this approach, we characterized the dynamics and evaluated the clinical utility of dscfDNA after liver transplantation (LT). **Methods.** Deletion/insertion polymorphisms were used to distinguish donor-specific DNA from recipient-specific DNA. Posttransplant dscfDNA was measured in the plasma of the recipients. In the longitudinal cohort, dscfDNA was serially measured at days 3, 7, 14, 28, and 42 in 20 recipients. In the cross-sectional cohort, dscfDNA was measured in 4 clinically stable recipients (>1-y posttransplant) and 16 recipients (>1-mo posttransplant) who were undergoing liver biopsies. **Results.** Recipients who underwent LT without complications demonstrated an exponential decline in dscfDNA. Median levels at days 3, 7, 14, 28, and 42 were 1936, 1015, 247, 90, and 66 copies/mL, respectively. dscfDNA was higher in recipients with treated biopsy-proven acute rejection (tBPAR) when compared to those without. The area under the receiver operator characteristic curve of dscfDNA was higher than that of routine liver function tests for tBPAR (dscfDNA: 98.8% with 95% confidence interval, 95.8%-100%; alanine aminotransferase: 85.7%; alkaline phosphatase: 66.4%; gamma-glutamyl transferase: 80.1%; and bilirubin: 35.4%). **Conclusions.** dscfDNA as measured by probe-free droplet digital PCR methodology was reflective of organ health after LT. Our findings demonstrate the potential utility of dscfDNA as a diagnostic tool of tBPAR.

(*Transplantation Direct* 2019;5: e462; doi: 10.1097/TXD.0000000000000902. Published online 21 June, 2019.)

Received 23 November 2018. Revision received 15 March 2019.

Accepted 28 March 2019.

[1] Department of Surgery, The University of Melbourne, Austin Health, Heidelberg, VIC, Australia.

[2] Translational Genomics and Epigenomics Laboratory, Olivia Newton-John Cancer Research Institute, Heidelberg, VIC, Australia.

[3] School of Cancer Medicine, La Trobe University, Heidelberg, VIC, Australia.

[4] Department of Clinical Pathology, The University of Melbourne, Parkville, VIC, Australia.

[5] Liver Transplant Unit, Austin Health, Heidelberg, VIC, Australia.

[6] Department of Medicine, The University of Melbourne, Austin Health, Heidelberg, VIC, Australia.

[7] Department of Anatomical Pathology, Austin Health, Heidelberg, VIC, Australia.

[8] Hepato-Pancreato-Biliary & Transplant Surgery Unit, Austin Health, Heidelberg, VIC, Australia.

**Joint senior author.*

This study was supported by Austin Medical Research Foundation, Austin Health; Research Scholarship, Australian and New Zealand Hepatic, Pancreatic & Biliary Association; Foundation of Surgery, Royal Australasian College of Surgeons; Victorian Liver Transplantation Unit, Austin Health; Department of Surgery, The University of Melbourne, Austin Health; and Operational Infrastructure Support Program Funding of the Victorian State Government.

S.K.G., A.D., and H.D. are co-inventors of the patent application PCT/AU2017/050657 (pending). This patent application describes the methodology that was utilized in this study to quantify donor-specific cell-free DNA. The other authors declare no conflicts of interests.

S.K.G., H.D., A.T., J.P., A.V., R.M.J., C.C., A.D., and V.M. participated in research design. S.K.G., A.D., and V.M. participated in writing of the paper. S.K.G., H.D., A.T., J.P., and A.V. participated in performing the research. S.K.G., H.D., J.P., A.V., R.M.J., C.C., A.D., and V.M. participated in data analysis.

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantationdirect.com).

Correspondence: Vijayaragavan Muralidharan, MBBS, PhD, FRACS, Department of Surgery, The University of Melbourne, Austin Health, Level 8 Lance Townsend Building, 145 Studley Rd, Heidelberg, VIC 3084, Australia. (muv@unimelb.edu.au) or Alexander Dobrovic, PhD, Translational Genomics and Epigenomics Laboratory, Olivia Newton-John Cancer Research Institute, 145 Studley Rd, Heidelberg, Victoria, Australia 3084. (alex.dobrovic@onjcri.org.au)

ISSN: 2373-8731

DOI: 10.1097/TXD.0000000000000902

Despite significant improvements in outcomes after liver transplantation (LT), organ rejection remains a common complication. Up to 30% of recipients will experience an episode of acute rejection within the first 12 months.[1,2] Furthermore, the occurrence of acute rejection may have significant impact on both mortality and morbidity.[1]

Contemporary diagnosis of acute rejection is based on the clinician's acumen in assessing the constellation of clinical signs and investigations. However, many of the investigations including serum liver biochemistry and radiological imaging are not specific for acute rejection.[3,4] Pathological states such as cholestasis, infection, and vascular thrombosis can also result in abnormalities for the above investigations. Liver biopsies are thus performed to establish a definitive diagnosis. However, liver biopsies are invasive and associated with the risks of pain, bleeding, and bile leak, as well as sampling issues.[5,6]

Reliable, rapidly performed, minimally invasive methods in diagnosing acute rejection will improve the surveillance of graft health. The measurement of cytokines, chemokines, and small molecules has been described, but all of these have varying degrees of specificity for acute rejection. As such, these tests have not gained widespread adoption by clinicians.[7,8]

Following solid-organ transplantation, cellular turnover in the donor organ continuously sheds cell-free DNA into the recipient circulation as donor-specific cell-free DNA (dscfDNA).[9] Cellular injury during episodes of acute rejection increases dscfDNA levels in the recipient circulation, a consistent finding of previous dscfDNA studies in heart,[10] lung,[11] kidney,[12] and liver[13] transplantation. As an emergent biomarker, dscfDNA may thus address the clinical need for a rapid blood test to diagnose acute rejection after transplantation.

Detecting dscfDNA relies on identifying genetic differences between the donor and recipient. The use of the Y chromosome to distinguish donor-specific DNA from recipient-specific DNA limits the detection of donor-specific DNA in female recipients who are transplanted with male donor organs.[9,14-16]

Normal genetic variants, such as single-nucleotide polymorphisms, deletion/insertion polymorphisms (DIP), and structural variations,[17,18] are potential donor-specific markers. The use of a panel of such polymorphisms chosen for high heterozygosity enables the differentiation of donor-specific DNA from recipient-specific DNA of all transplant recipients (except genetically identical twins).[19-27]

Modern techniques for measuring dscfDNA mostly utilize next-generation sequencing (NGS) or droplet digital PCR (ddPCR) platforms.[28] The analysis of cell-free DNA by ddPCR is superior compared with conventional PCR platforms.[29] Most importantly, the straightforward ddPCR workflow can be rapidly completed.[25] NGS workflows, on the other hand, are expensive and require complex sample handling steps for library preparation and sequencing. Results from NGS can take between 3 and 28 days to turn around.[20-22]

We previously reported a DIP-based probe-free ddPCR methodology to measure dscfDNA that circumvented many of the described limitations.[25] Allelic breakpoints of DIPs were exploited for amplification specificity using primers that hybridized across the breakpoints. Unlike prior ddPCR methodologies,[13,23,30] our methodology harnessed the reproducibility and precision of ddPCR without the need for fluorescent probes to detect dscfDNA. Such design translates to more economical assays that are better suited for implementation in diagnostic laboratories.

Using our novel methodology, we aimed to characterize the dynamics of dscfDNA after LT and assess the utility of dscfDNA in diagnosing acute rejection after LT.

MATERIALS AND METHODS

Ethics

Ethical approval for this study was obtained from the Austin Health Human Research Ethics Committee (reference number: HREC/15/Austin/142) and Donate Life Australia (project number: 2015#04). Ethical approval for the use of control normal blood samples was obtained from the Australian Red Cross Blood Service (agreement number: 15-06VIC-07).

Cohort A: Longitudinal Monitoring of dscfDNA After LT

The longitudinal cohort was prospectively recruited at the Liver Transplant Unit of Victoria at Austin Health between June 2015 and Dec 2016 (Figure 1). Twenty recipients who underwent LT (under the donation after brain death pathway) were recruited. Informed consent was obtained upon recruitment.

LT was performed according to the institutional protocols. The management of the recipients undergoing LT was directed by a team of experienced transplant clinicians. Routine immunosuppression comprised a tapering regime of steroids, an antimetabolite (azathioprine or mycophenolate mofetil), and a calcineurin inhibitor (tacrolimus or cyclosporine).

Liver biopsies were performed following a clinical need to do so, as protocol biopsies are not performed at our institution. Blood tests such as full blood examination, urea electrolytes and creatinine, liver function tests (LFTs), and serum tacrolimus levels were performed on a regular basis to monitor the clinical course. Recipients were closely followed up with routine blood tests at outpatients following discharge, and blood tests were performed upon their outpatient visits.

In addition to the routine blood tests, an additional 15 mL of blood was sampled for dscfDNA analysis 24 hours before transplantation and on posttransplant days 3, 7, 14, 28, and 42. Fifteen milliliters of blood from the corresponding organ donor was also sampled before the organ procurement procedure by the organ procurement team.

In this specific cohort, the recipients were categorized into 3 groups: (1) uneventful, (2) treated biopsy-proven acute rejection (tBPAR), or (3) cholestasis. The uneventful group comprised recipients who underwent LT without complications. In this group, each recipient demonstrated typical and expected improvements in LFTs. As our institution does not perform protocol biopsies, recipients in this group did not undergo a liver biopsy after LT.

Recipients with persistent or atypical elevations in LFTs all underwent liver biopsies to determine a cause for the abnormality, and the recipients were classified into 2 further groups. The tBPAR group comprised recipients with acute cellular rejection (confirmed by a transplant pathologist) that was successfully treated by the modification of their immunosuppression therapy (pulsed steroids or modification of maintenance therapy). The cholestasis group comprised recipients with abnormal cholestatic LFTs and histopathology findings that did not require antirejection therapy.

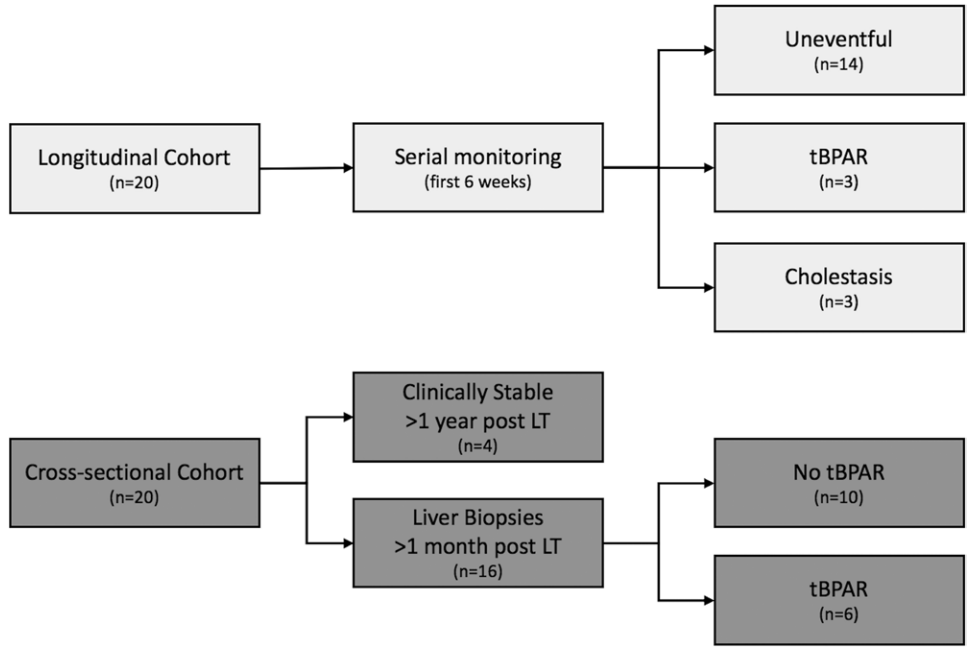

FIGURE 1. Outline of the study comprising a longitudinal cohort of 20 recipients (cohort A) and a cross-sectional cohort of 20 recipients (cohort B). LT, liver transplantation; tBPAR, treated biopsy-proven acute rejection.

Cohort B: Cross-sectional Evaluation of Diagnostic Accuracy of dscfDNA for tBPAR

The cross-sectional cohort was recruited at the Liver Transplant Unit of Victoria between June 2016 and July 2017 (Figure 1). Four clinically stable recipients (>1-y post-LT) who did not develop any posttransplant complications with normal LFTs were identified by the transplant clinicians from the Liver Transplant Unit of Victoria database. The 4 recipients were recruited, and 15 mL of blood was drawn for dscfDNA analysis.

Sixteen recipients (>1-mo posttransplant) who were undergoing liver biopsies to investigate the cause for deteriorating LFTs (unexplained rising transaminases and cholestatic parameters) were recruited. A sample of blood (15 mL) was drawn from each recipient up to 3 hours before the liver biopsy procedure. In this cohort, the primary outcome was defined as tBPAR. Therefore, only the recipients who were treated (by pulsed steroids or adjustment of maintenance immunosuppression) for the episode of biopsy-proven acute rejection (acute cellular rejection confirmed by a pathologist) were classified as a positive event.

Archived pretransplant serum samples of the recipients and their corresponding organ donors were retrieved from −80°C storage for genotyping purposes (detailed below).

Quantification of dscfDNA

Blood processing and quantification of dscfDNA were performed according to our previously published protocols.[25,31] A schematic of the methodology is summarized in **Figure S1, SDC,** http://links.lww.com/TXD/A212.

All blood samples designated for dscfDNA analysis were collected using VACUETTE potassium-EDTA blood collection tubes (Greiner Bio-One International). The samples were processed within 3 hours of collection. Each blood sample was initially centrifuged at 800*g* for 10 minutes. The plasma fraction of the blood sample was transferred into a collection tube for a second centrifugation step at 1600*g* for 10 minutes. Subsequently, the plasma was aspirated and transferred into cryovials for storage at −80°C.

In the first step, each recipient and corresponding donor were genotyped using a panel of 9 DIPs. DNA was first extracted from the pretransplant buffy-coat (cohort A: organ donor and recipients) and pretransplant serum (cohort B: organ donor and recipients) using the QIAamp DNA Mini Kit (Qiagen) and QIAamp Circulating Nucleic Acid Kit (Qiagen), respectively.

Genotyping was subsequently performed by high-resolution melting analysis. A DIP was considered informative if an allele (deletion or insertion) was present in the donor and absent in the recipient. This step was performed only once for each recipient and organ donor pair.

The second step measured dscfDNA. DNA was first extracted from 4 mL of plasma derived from recipient blood samples using the QIAamp Circulating Nucleic Acid Kit (Qiagen). Probe-free assays that only amplified the respective informative donor-specific alleles were selected according to the genotyping information obtained in the first step. dscfDNA was measured using the plasma DNA extracted from the recipient blood samples on the Bio-Rad QX200 ddPCR platform (Bio-Rad Laboratories).

dscfDNA levels for each blood sample were calculated and presented as copies/mL of recipient plasma or copies/mL. All dscfDNA measurements were analyzed in batches. The dscfDNA results did not influence the management of the recipients.

Statistical Analysis

Means and SDs were presented for normally distributed continuous variables. Median and interquartile ranges were presented for nonnormally distributed continuous variables. Where appropriate, the Student *t* test and ANOVA were used for parametric testing, and the Mann-Whitney *U* test and Kruskal-Wallis test were used for nonparametric analysis.

Diagnostic sensitivity and specificity were determined using receiver operating characteristics analysis. The area under the curve was used to compare the diagnostic accuracy of dscfDNA and LFTs. The Youden index was used to calculate optimal cutoffs. Statistical analyses were performed using SPSS Statistics v24 for MAC (IBM Corporation).

RESULTS

Cohort A: Longitudinal Monitoring of dscfDNA After LT

The average recipient age was 57.5 ± 8.6 years. The majority of the recipients were males. The common causes for LT are outlined in Table 1. The average model for end-stage liver disease (MELD) score for this cohort of recipients was 18 ± 7. Forty percent had an underlying hepatocellular carcinoma before transplantation. The average organ donor age was 48 ± 15 years. There were more female than male organ donors. The causes of death before organ donation are listed in Table 1. Three of the 20 recipients received a split LT (3/4 of the donor liver). The average donor risk index of the organ donor was 1.6 ± 0.3.

The average age of transplantation of the recipient was 55.5 ± 8.6 years. The average operative time was 423 ± 71 minutes. The cold and warm ischemic times were 360 ± 84 and 43 ± 6 minutes, respectively. The average maximum alanine aminotransferase (ALT) was 1243 ± 1091 U/L. The average length of stay (LOS) was 15 ± 8 days, and the intensive care unit (ICU) LOS was 3 ± 2 days.

There were no significant differences between the clinical variables of the 3 subgroups. In this cohort, dscfDNA was analyzed on a total of 119 blood samples (derived from the 20 recipients across 6 time points). Due to logistical reasons, one of the recipients in the uneventful subgroup missed an episode of blood sampling at day 28.

TABLE 1.

Clinical variables of the longitudinal cohort (cohort A)

	Overall cohort (n = 20)	Subgroup: uneventful (n = 14)	Subgroup: cholestasis (n = 3)	Subgroup: tBPAR (n = 3)
	Mean or count (SD or %)	Mean or count (SD or %)	Mean or count (SD or %)	Mean or count (SD or %)
Recipient demographics				
Recipient age (y)	57.5 (8.6)	57.4 (8.1)	64.5 (4.6)	50.6 (10.6)
Recipient sex				
Female	7 (35%)	4 (29%)	0 (0%)	3 (100%)
Male	13 (65%)	10 (71%)	3 (100%)	0 (0%)
Cause				
Alcohol	2 (10%)	2 (14%)	0 (0%)	0 (0%)
Cryptogenic	2 (10%)	2 (14%)	0 (0%)	0 (0%)
Others[a]	5 (25%)	2 (14%)	2 (67%)	1 (33%)
PSC or PBC	4 (20%)	2 (14%)	0 (0%)	2 (67%)
Viral hepatitis	7 (35%)	6 (43%)	1 (33%)	0 (0%)
MELD score	18 (7)	19 (7)	19 (8)	16 (6)
Underlying HCC				
No	12 (60%)	8 (57%)	1 (33%)	3 (100%)
Yes	8 (40%)	6 (43%)	2 (67%)	0 (0%)
Donor demographics				
Donor age (y)	48 (15)	47 (16)	44 (10)	55 (19)
Donor sex				
Female	12 (60%)	8 (57%)	1 (33%)	3 (100%)
Male	8 (40%)	6 (43%)	2 (67%)	0 (0%)
Donor cause of death				
Cerebral	8 (40%)	6 (43%)	0 (0%)	2 (67%)
Hypoxia	9 (45%)	6 (43%)	3 (100%)	0 (0%)
Trauma	3 (15%)	2 (14%)	0 (0%)	1 (33%)
Split transplantation				
No	17 (85%)	11 (79%)	3 (100%)	3 (100%)
Yes	3 (15%)	3 (21%)	0 (0%)	0 (0%)
Donor risk index	1.6 (0.3)	1.6 (0.3)	1.4 (0.4)	1.5 (0.4)
LT characteristics				
Recipient age at LT (y)	55.5 (8.6)	55.5 (8)	62.6 (4.6)	48.7 (11.2)
Operative time (min)	423 (71)	426 (77)	433 (58)	397 (70)
Warm ischemic time (min)	43 (6)	45 (6)	42 (3)	38 (4)
Cold ischemic time (min)	360 (84)	352 (78)	448 (99)	314 (47)
Maximum ALT (U/L)	1232 (1091)	1054 (423)	2060 (2841)	1238 (902)
Hospital length of stay (d)	15 (8)	13 (6)	17 (5)	22 (14)
ICU length of stay (d)	3 (2)	3 (1)	4 (2)	4 (3)

ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; ICU, intensive care unit; LT, liver transplantation; MELD, model for end-stage liver disease; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; SD, standard deviation; tBPAR, treated biopsy-proven acute rejection.

[a]Others include α1-antitrypsin deficiency (n = 1), hepatocellular carcinoma (n = 1), autoimmune hepatitis (n = 1), common variable immune deficiency (n = 1), and nonalcoholic steatohepatitis (n = 1).

Exponential Decline of dscfDNA After LT Reflected Uneventful Recovery

All 14 recipients who underwent LT without any complications (uneventful subgroup) demonstrated stereotypic decline in dscfDNA. dscfDNA levels were higher in the earlier phases (days 3 and 7) after LT compared with the later phases (days 14, 28, and 42) (Figure 2).

Median dscfDNA at days 3 and 7 was 1936 copies/mL (interquartile range [IQR], 1067–2658 copies/mL) and 1015 copies/mL (IQR, 363–1125 copies/mL), respectively. dscfDNA continued declining at day 14 with a median dscfDNA of 247 copies/mL (IQR, 153–358 copies/mL). dscfDNA plateaued at low median levels of 90 copies/mL (IQR, 67–241 copies/mL) at day 28 and 66 copies/mL (IQR, 50–105 copies/mL) at day 42.

dscfDNA Was Elevated in tBPAR and Was Independent of Cholestasis

Three recipients in the longitudinal cohort developed biopsy-proven acute rejection that required treatment (clinical progress summarized in Table 2). dscfDNA was higher, compared with the median of the uneventful subgroup, in all 3 of the recipients on the day when the episode of biopsy-proven acute rejection was diagnosed and subsequently treated (Figure 3). Following treatment, dscfDNA decreased toward the median of the uneventful subgroup.

Three other recipients in the longitudinal cohort were diagnosed with cholestatic pathologies that were associated with notably abnormal cholestatic LFTs. Two were conservatively managed, and one required surgical reconstruction of the biliary anastomosis (Table 2). dscfDNA in all 3 of these recipients was within the median of the uneventful group (Figure 4).

Overall, the recipients with tBPAR demonstrated distinctively higher dscfDNA compared with the uneventful subgroup and cholestasis subgroup (**Figure S2, SDC,** http://links.lww.com/TXD/A212, and **Table S1, SDC,** http://links.lww.com/TXD/A212).

Notably, LFTs showed greater overlap between the subgroups (**Figure S2, SDC,** http://links.lww.com/TXD/A212). ALT and bilirubin did not differentiate the 3 different subgroups (**Table S1, SDC,** http://links.lww.com/TXD/A212). Alkaline phosphatase (ALP; at days 7, 14, and 28) and gamma-glutamyl transferase (GGT; at days 7 and 14) differentiated the uneventful subgroup from recipients with cholestasis but did not differentiate between recipients with cholestasis or tBPAR (**Table S1, SDC,** http://links.lww.com/TXD/A212).

Cohort B: Cross-sectional Evaluation of Diagnostic Accuracy of dscfDNA for tBPAR

The average recipient age was 55.3 ± 12 years (Table 3). The causes leading to LT were similar to that of cohort A. The MELD score was 22 ± 7. Almost 50% of the cohort had an underlying hepatocellular carcinoma before transplantation. The average organ donor age was 42 ± 17 years. Eighteen of the recipients received grafts from donation after brain death organ donors, and 2 of the recipients received grafts from organ donors after circulatory death. One of the recipients received a split LT. The average donor risk index was 1.5 ± 0.4.

The average age of transplantation of the recipient was 50.1 ± 11.6 years. The average operative time was 477 ± 118 minutes. The cold and warm ischemic times were 398 ± 99 and 42 ± 7 minutes, respectively. The average maximum ALT was 1266 ± 1963 U/L. The average LOS was 15 ± 8 days, and the ICU LOS was 3 ± 2 days. The duration from LT to biopsy was 1641 ± 1357 days.

No significant differences were identified among the clinical variables of the 3 subgroups. In this cohort, dscfDNA was analyzed on 20 blood samples (1 sample/recipient) (Figure 5).

dscfDNA Identified tBPAR With Superior Performance Compared With LFTs

Posttransplant recipients may present at the outpatient clinic with abnormal LFTs that require further investigations. These include imaging, endoscopy, or liver biopsies. We assessed the value of dscfDNA in discriminating recipients

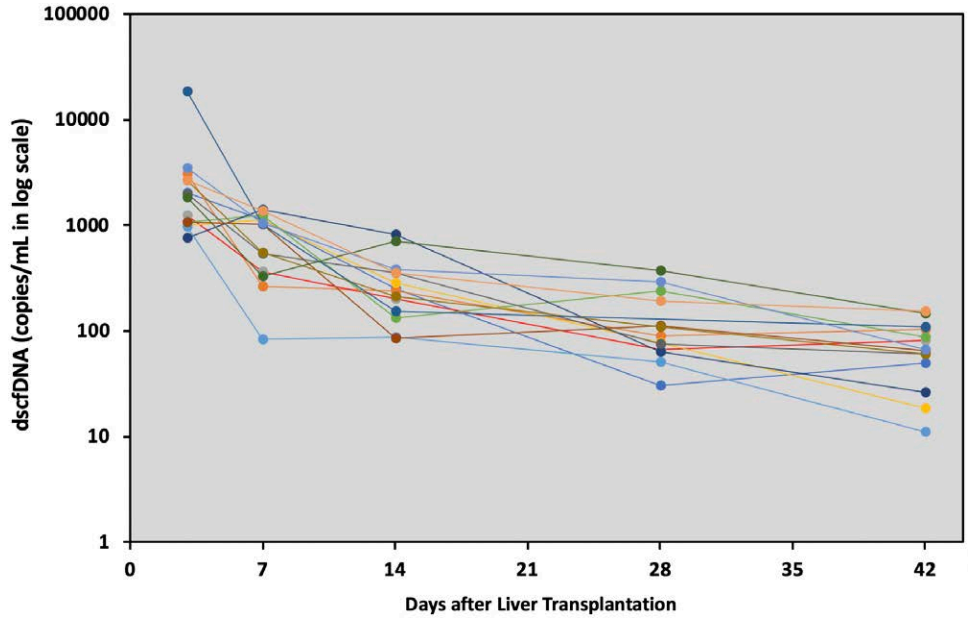

FIGURE 2. Donor-specific cell-free DNA (dscfDNA) dynamics over time of the 14 recipients who underwent liver transplantation without any complications. A decline in dscfDNA over time was indicative of successful implantation.

TABLE 2.

Serial monitoring of dscfDNA of the longitudinal cohort (cohort A)

		dscfDNA (copies/mL) after LT					
		Day 3	Day 7	Day 14	Day 28	Day 42	Comments
tBPAR (n = 3)	Recipient 1	1826	4266	952	692	447	tBPAR: diagnosed on day 7 after LT and treated by adjustment of maintenance immunosuppression
	Recipient 2	3819	2088	1821	651	513	tBPAR: diagnosed on day 9 after LT and treated by adjustment of maintenance immunosuppression
	Recipient 3	14 839	8404	10 769	341	97	tBPAR: diagnosed on day 14 after LT and treated by adjustment of maintenance immunosuppression
Cholestasis (n = 3)	Recipient 1	7238	1122	517	53	59	Cholestasis relating to ischemic/reperfusion injury: diagnosed on day 9 and conservatively managed
	Recipient 2	10 277	847	274	509	122	Cholestasis relating to anastomotic stricture: diagnosed on day 14 followed by surgical reconstruction
	Recipient 3	602	569	135	39	84	Cholestasis relating to nonspecific biliary injury: diagnosed on day 12 and conservatively managed
Uneventful (n = 14)	25th percentile	1067	363	153	67	50	
	Median	1936	1015	247	90	66	
	75th percentile	2658	1125	358	241	105	

dscfDNA, donor-specific cell-free DNA; LT, liver transplantation; tBPAR, treated biopsy-proven acute rejection.

with abnormal LFTs and tBPAR (n = 6) from the recipients with abnormal LFTs and without tBPAR (n = 10) in this context. The findings showed that higher dscfDNA was significantly associated with tBPAR (**Figure S3, SDC,** http://links.lww.com/TXD/A212). On the other hand, ALT, ALP, GGT, and bilirubin were not indicative of tBPAR (**Figure S3, SDC,** http://links.lww.com/TXD/A212).

Among the recipients who underwent liver biopsies, dscfDNA accurately identified recipients with tBPAR and recipients without tBPAR (Table 4; **Figure S4, SDC,** http://links.lww.com/TXD/A212). The diagnostic performance based on the area under the curve of dscfDNA (96.7%; confidence interval [CI], 88.5%-100%) was superior to ALT (64.2%; CI, 31.5-96.8), ALP (48.3%; CI, 15.0%-81.6%), GGT (55.8%; CI, 24.9%-86.8%), and bilirubin (38.3%; CI, 9.5%-67.1%). At a threshold of 898 copies/mL, dscfDNA had a clinical sensitivity of 83.3% (CI, 35.9%-99.6%) and clinical specificity of 100% (87.7%–100%).

The analysis was extended to determine the value of dscfDNA in discriminating recipients with tBPAR (n = 6) from a group which included both patients without tBPAR and those who were clinically well (n = 28 total) with normal LFTs. dscfDNA, ALT, and GGT were significantly higher in recipients with tBPAR, when compared with those recipients without tBPAR and those who were clinically well (**Figure S3, SDC,** http://links.lww.com/TXD/A212).

Receiver operating characteristics analysis demonstrated that dscfDNA was superior to LFTs in discriminating recipients with tBPAR from those without tBPAR and those who were clinically well (Table 5; **Figure S5, SDC,** http://links.lww.com/TXD/A212). At a threshold of 898 copies/mL, dscfDNA had a clinical sensitivity of 83.3% (CI, 35.9%-99.6%) and clinical specificity of 100% (87.7%–100%).

Applying the dscfDNA Threshold for Surveillance of tBPAR Early After LT

The threshold of 898 copies/mL was applied to the longitudinal cohort (cohort A). Due to the high median dscfDNA of the uneventful subgroup at days 3 (1936 copies/mL) and 7 (1015 copies/mL), the diagnostic performance is of limited value. The threshold was most reliable from day 14 onward. Notably, from day 14 onward, all 3 of the recipients with cholestasis had dscfDNA that were below the tBPAR threshold of 898 copies/mL (Table 2).

At day 14, all 3 of the recipients with tBPAR had dscfDNA levels that were above the threshold (Table 2). For recipients 1 and 2, dscfDNA was measured during the decay phase after the treatment for the episode of acute rejection was instituted. Recipient 1 was diagnosed with tBPAR on day 7. One week after the treatment, the dscfDNA was 952 copies/mL. Although the dscfDNA was marginally above the threshold, the levels continued to decline below the threshold at subsequent time points after successful treatment. Recipient 2 was diagnosed with tBPAR on day 9. Five days after the treatment, the dscfDNA remained at markedly elevated levels of 1821 copies/mL. The levels continued to decline below the threshold after successful treatment. Recipient 3 was diagnosed with tBPAR on day 14. For this recipient, dscfDNA was markedly elevated at 10 769 copies/mL and then declined below the threshold after successful treatment at subsequent time points.

DISCUSSION

There is a clinical need for accurate blood-based tests to diagnose acute rejection after solid-organ transplantation. Several large studies have established the clinical utility of dscfDNA for acute rejection after heart, lung, and kidney transplantation.[10-12,24,32,33] Reports pertaining to the role of dscfDNA in LT remain limited. At the time of writing, only a few small studies (≤17 recipients)[16,19,25,34] and one large prospective study (115 recipients) have been published to date[13].

In this study, we demonstrated that dscfDNA was indicative of acute rejection after LT. Uncomplicated clinical progress was associated with a stereotypic decrease in dscfDNA (Figure 2). Furthermore, serial monitoring of dscfDNA identified the recipients with tBPAR and facilitated the assessment of response to antirejection therapy (Figure 3). Importantly, we also showed that dscfDNA was superior to LFTs in identifying recipients with tBPAR (Tables 4 and 5).

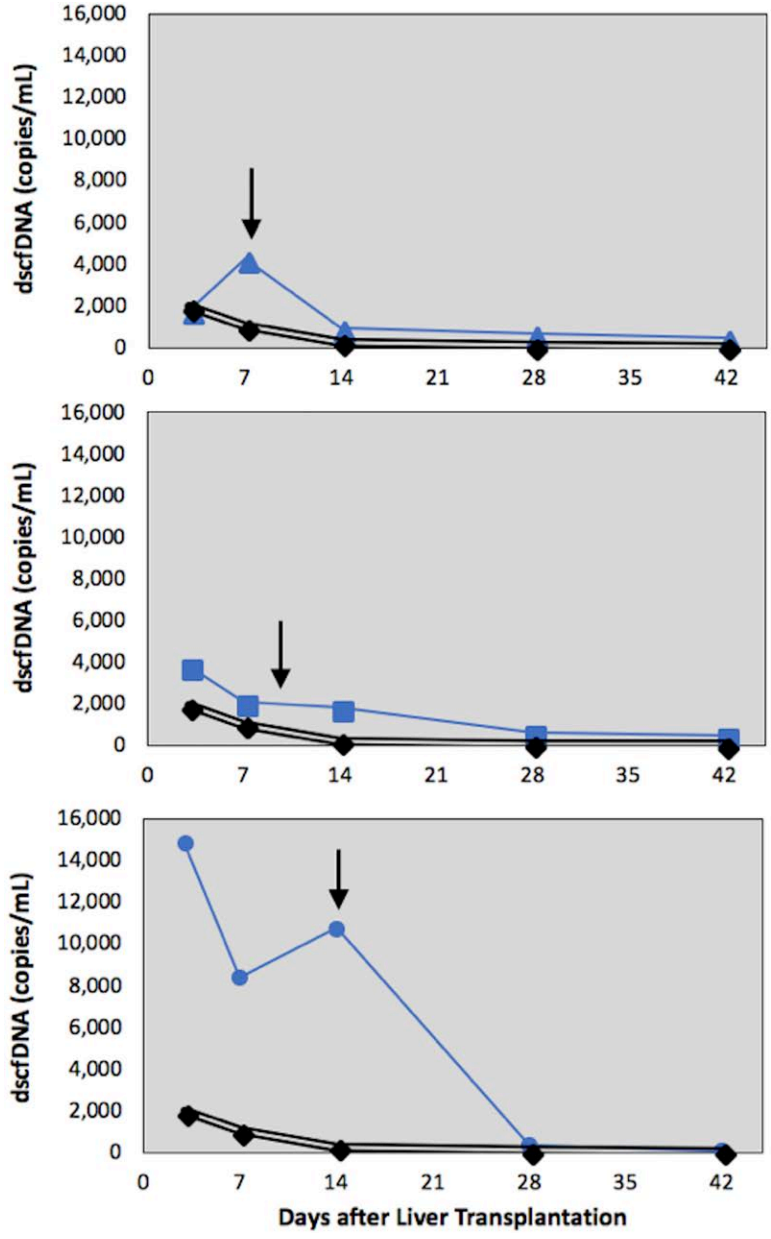

FIGURE 3. Donor-specific cell-free DNA (dscfDNA) was higher in the 3 recipients with treated biopsy-proven acute rejection when compared with the median dscfDNA of recipients who underwent liver transplantation without any complications. Improving dscfDNA reflects the successful treatment of acute rejection. The arrow with solid line denotes the day when the episode of biopsy-proven acute rejection was diagnosed and treated. Recipient 1: ▲, recipient 2: ■, recipient 3: ●and median of the uneventful recipients: ♦.

Our findings were consistent with previous dscfDNA studies in LT[13,16,19,25,34] and independently affirmed that dscfDNA is of clinical value for acute rejection after LT. There were, however, 2 key aspects of our study that differed to the other published studies.

First, the more clinically relevant endpoint of tBPAR was adopted. Mild histological rejection is often not treated, and maintenance immunosuppression is not modified.[35,36] Many recipients with mild rejection will improve spontaneously without adverse clinical outcomes. To evaluate the value of dscfDNA in identifying clinically relevant episodes of acute rejection that required treatment, the endpoint of tBPAR (a standard in high-quality LT trials[37-39]) was thus employed in our study.

Second, unlike majority of the other published methodologies,[19,20,22] our ddPCR methodology measured dscfDNA by absolute quantification (ie, copies/mL of dscfDNA) as compared to relative abundance (ie, percentage of dscfDNA: donor-specific DNA divided by the sum of donor-specific and recipient-specific DNA).

Measurement by relative abundance internally controls for sample processing variables (ie, DNA extraction yields). The disadvantage in doing so is that numerous factors such as exercise,[40] infection,[16] poor collection techniques,[41] and poor sample processing[42] increase recipient-specific DNA. These factors can confound relative abundance measurements and hence reduce the clinical sensitivity of detecting an event. Absolute quantification of donor alleles was thus adopted in this study to maximize clinical sensitivity. Attributed to this difference, direct comparison of healthy dscfDNA threshold (ie, 10% with the study by Schütz et al[13]) was not possible.

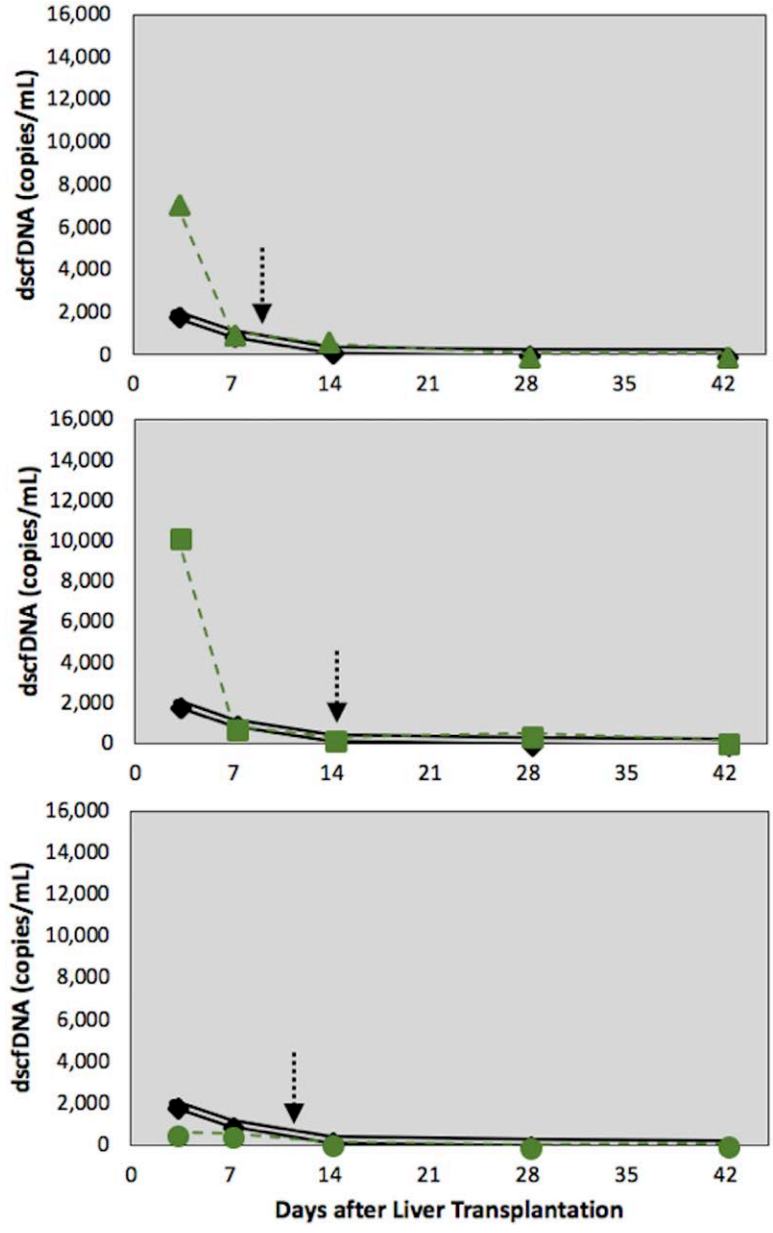

FIGURE 4. Donor-specific cell-free DNA (dscfDNA) of the 3 recipients in the cholestasis subgroup was similar to the median dscfDNA of the recipients who underwent liver transplantation without any complications. Arrow with broken line denotes the day when the recipient underwent a liver biopsy, and the diagnosis of cholestasis was made. Recipient 1: ▲, recipient 2: ■, recipient 3: ●and median of the uneventful recipients: ♦.

Despite the disparities in study endpoint and assay methodologies, our results supported the notion that the performance of dscfDNA is superior to LFTs in diagnosing recipients with acute rejection. This finding is readily explained by the unique and inherent physiology of dscfDNA.[11,13] Because each cell has 2 haploid copies of the genome, the death of a cell derived from the donor organ will release 2 haploid copies of the donor-specific genome into the blood circulation. The amount of donor-specific DNA that is quantifiable in the plasma directly correlates to the degree of cell death that occurs during acute rejection.

On the other hand, LFTs are intrinsically different. The levels of bilirubin, ALP, GGT, and ALP are highly dependent on complex cellular interactions of transcriptional activity, translational processes, biochemical modifications, clearance, membrane permeability, and enzymatic leakage. These factors hence limit both the sensitivity and specificity of LFTs.

On the basis of our reported findings, we considered that dscfDNA may improve several aspects of clinical management after LT. First and foremost, dscfDNA could be used as a liquid biopsy for the surveillance of tBPAR in recipients after LT. In recipients with dscfDNA that surpass the threshold (ie, >898 copies/mL), antirejection therapy may be instituted. The size of our study precluded the formal assessment of the relationship between dscfDNA, rejection severity, and the type of antirejection therapy that was required to treat the rejection episode. Larger multicenter studies are necessary to further evaluate and refine the diagnostic and treatment thresholds of dscfDNA.

Second, dscfDNA could be used to guide investigational decisions early (ie, within the first 2 wk) after LT. Numerous perioperative variable such as donor organ quality, ischemic reperfusion injury, ischemic times, and transfusion of blood products could increase dscfDNA.[43,44] These factors

TABLE 3.

Clinical variables of the cross-sectional cohort (cohort B)

	Overall cohort (n = 20)	Subgroup: stable (n = 4)	Subgroup: No tBPAR (n = 10)	Subgroup: tBPAR (n = 6)
	Mean or count (SD or %)	Mean or count (SD or %)	Mean or count (SD or %)	Mean or count (SD or %)
Recipient demographics				
Recipient age (y)	55.3 (12)	59.8 (7.9)	53.6 (13.2)	55.1 (13.3)
Recipient sex				
Female	6 (30%)	2 (50%)	2 (20%)	2 (33%)
Male	14 (70%)	2 (50%)	8 (80%)	4 (67%)
Cause				
Cryptogenic	2 (10%)	0 (0%)	2 (20%)	0 (0%)
NASH	2 (10%)	0 (0%)	0 (0%)	2 (33%)
Others[a]	7 (35%)	3 (75%)	3 (30%)	1 (17%)
PSC or PBC	5 (25%)	0 (0%)	3 (30%)	2 (33%)
Viral hepatitis	4 (20%)	1 (25%)	2 (20%)	1 (17%)
MELD score	22 (7)	17 (6)	25 (6)	19 (4)
Underlying HCC				
No	11 (55%)	1 (25%)	7 (70%)	3 (50%)
Yes	9 (45%)	3 (75%)	3 (30%)	3 (50%)
Donor demographics				
Donor age (y)	42 (17)	50 (21)	38 (15)	43 (17)
Donor sex				
Female	12 (60%)	3 (75%)	6 (60%)	3 (50%)
Male	8 (40%)	1 (25%)	4 (40%)	3 (50%)
Donor cause of death				
Cerebral	10 (50%)	2 (50%)	5 (50%)	3 (50%)
Hypoxia	5 (25%)	2 (50%)	2 (20%)	1 (17%)
Trauma	5 (25%)	0 (0%)	3 (30%)	2 (33%)
Donation pathway				
DBD	18 (90%)	3 (75%)	9 (90%)	6 (100%)
DCD	2 (10%)	1 (25%)	1 (10%)	0 (0%)
Split LT				
No	19 (95%)	4 (100%)	9 (90%)	6 (100%)
Yes	1 (5%)	0 (0%)	1 (10%)	0 (0%)
Donor risk index	1.5 (0.4)	1.7 (0.4)	1.4 (0.4)	1.5 (0.4)
LT characteristics				
Recipient age at LT (y)	50.1 (11.6)	56.2 (6.6)	47.8 (12.8)	49.9 (12.3)
Operative time (min)	477 (118)	367 (37)	488 (119)	531 (113)
Warm ischemic time (min)	42 (7)	43 (10)	41 (7)	44 (7)
Cold ischemic time (min)	398 (99)	306 (53)	427 (81)	412 (121)
Maximum ALT (U/L)	1266 (1963)	2910 (4349)	748 (452)	1034 (413)
Others				
Biopsy, days from LT (d)	1641 (1347)	–	1782 (1194)	1406 (1664)

ALT, alanine aminotransferase; DBD, donation after brain death; DCD, donation after circulatory death; HCC, hepatocellular carcinoma; LT, liver transplantation; MELD, model for end-stage liver disease; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; SD, standard deviation; tBPAR, treated biopsy-proven acute rejection.
[a]Others include autoimmune hepatitis (n = 2), hemochromatosis (n = 2), oriental cholangiopathy (n = 1), alcoholic cirrhosis (n = 1), and Budd-Chiari syndrome (n = 1).

compromise and limit the clinical utility of dscfDNA to diagnose tBPAR. However, we considered that the serial monitoring of dscfDNA within the first 2 weeks after LT may be useful to determine the choice of further diagnostic tests when investigating recipients with abnormal cholestatic LFTs (a common finding early after LT).

For instance, the clinician may opt to perform a liver biopsy to confirm acute rejection in a recipient with abnormal cholestatic LFTs and abnormal dscfDNA (ie, twice the median of the uneventful subgroup). In a separate clinical scenario, the clinician may elect to watch and wait and not perform a liver biopsy in a recipient with abnormal cholestatic LFTs and normal dscfDNA. The analysis of dscfDNA could, hence, be used to determine the group of recipients who may benefit the most from undergoing tissue biopsies.

Third, serial monitoring of dscfDNA could complement LFTs in monitoring antirejection therapy response following the diagnosis and treatment of acute rejection. Consistent with studies in both liver and other types of solid-organ transplantation,[10,11,13] dscfDNA normalized with successful treatment of acute rejection (Figure 3). Serial monitoring of dscfDNA has particular relevance in those patients where LFTs are slow to normalize after commencement of treatment of acute rejection. It may obviate the need for repeat liver biopsies to assess response. Owing to the intrinsic characteristics of dscfDNA, close monitoring of dscfDNA in conjunction to LFTs may offer valuable information pertaining to both graft integrity and treatment responses.

We recognize that our study has several limitations. The sample size is small, and this precluded formal multivariate

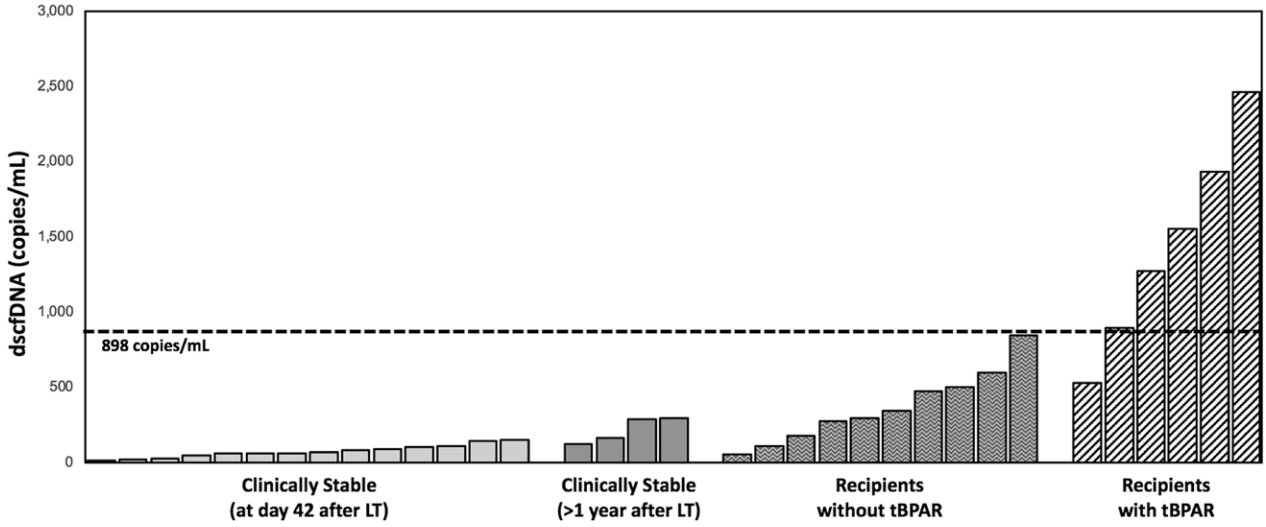

FIGURE 5. Cross-sectional analysis of donor-specific cell-free DNA (dscfDNA) of 14 clinically stable recipients at day 42 after liver transplant (LT) from cohort A (light gray), 4 clinically stable recipients at least 1 y after LT from cohort B (dark gray), 10 recipients without treated biopsy-proven acute rejection (tBPAR) from cohort B (zig-zag pattern), and 6 recipients with tBPAR from cohort B (stripe pattern). Dotted line represents the optimal threshold for discriminating recipients with tBPAR from those without tBPAR or those who were clinically stable.

TABLE 4.

ROC analysis of the recipients with tBPAR (n = 6) when compared with those without tBPAR (n = 10)

	ROC analysis				Youden index				
	AUC (%)	SE (%)	*P* value	95% CI (%)	Optimal cutoff	Sensitivity (%)	95% CI (%)	Specificity (%)	95% CI (%)
dscfDNA	96.7	4.2	0.002	88.5-100.0	898	83.3	35.9-99.6	100.0	87.7-100.0
ALT	64.2	16.6	0.357	31.5-96.8	117	66.7	22.3-95.7	80.0	44.4-97.5
ALP	48.3	17.0	0.914	15.0-81.6	388	33.3	4.3-77.7	90.0	55.5-99.7
GGT	55.8	15.8	0.704	24.9-86.8	485	33.3	4.3-77.7	90.0	55.5-99.7
Bilirubin	38.3	14.7	0.448	9.5-67.1	8	83.3	35.9-99.6	20.0	2.5-55.6

95% CI, 95% confidence interval; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AUC, area under the curve; dscfDNA, donor-specific cell-free DNA; GGT, gamma-glutamyl transferase; ROC, receiver operating characteristic; tBPAR, treated biopsy-proven acute rejection.

TABLE 5.

ROC analysis of the recipients with tBPAR (n = 6) when compared with those without tBPAR and those who were clinically well (n = 28)

	ROC analysis				Youden index				
	AUC (%)	SE (%)	*P* value	95% CI (%)	Optimal cutoff	Sensitivity (%)	95% CI (%)	Specificity (%)	95% CI (%)
dscfDNA	98.8	1.5	0.000	95.8-100	898	83.3	35.9-99.6	100.0	87.7-100.0
ALT	85.7	7.7	0.007	70.7-100	117	66.7	22.3-95.7	92.9	76.5-99.1
ALP	66.4	14.6	0.214	37.8-95.0	150	66.7	22.3-95.7	71.4	51.3-86.8
GGT	80.1	8.7	0.023	63.1-97.1	135	83.3	35.9-99.6	71.4	51.3-86.8
Bilirubin	35.4	12.7	0.268	10.6-60.3	22	16.7	0.4-64.1	89.3	71.8-97.7

95% CI, 95% confidence interval; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AUC, area under the curve; dscfDNA, donor-specific cell-free DNA; GGT, gamma-glutamyl transferase; ROC, receiver operating characteristic; tBPAR, treated biopsy-proven acute rejection.

analyses. Nevertheless, we considered that this sample size was sufficient to demonstrate the clinical utility of dscfDNA and also test the feasibility of our novel methodology to effectively measure dscfDNA within a clinically relevant turnaround time. Although only 40 recipients were presented, this is the second largest dscfDNA study in LT at the time of writing to reinforce the clinical value of dscfDNA in LT.

Importantly, protocol biopsies were not performed in our study to correlate dscfDNA with histopathological outcomes. While protocol biopsies may identify recipients with subclinical rejection, the risks (ie, pain, bleeding, infection) outweighed the benefits (ie, identifying clinically significant outcomes that require treatment). Similar to mild rejection, subclinical rejection may spontaneously improve without further treatment, and this suggests that, if truly present, the episode of rejection would have negligible impact on clinical outcomes. The use of biopsy-proven acute rejection requiring treatment (tBPAR) as an endpoint, as discussed above, was considered to address this limitation.

In conclusion, our findings support the recent study showing that dscfDNA provides an independent clinical value for sensitive detection of acute rejection after LT.[13] Herein, we used a highly feasible and readily performed probe-free ddPCR methodology with clinically relevant turnaround

times to measure dscfDNA in 2 separate cohorts of recipients after LT. Despite differences in endpoint and assay platforms, our findings provide more evidence supporting the value of dscfDNA for acute rejection in LT.

ACKNOWLEDGMENTS

We acknowledge Boris Wong, Fang Shen, Tom Witkowski, Ashan Musafer, and Ramyar Molania for their technical assistance and the staff of the Liver Transplantation Unit of Victoria and DonateLife for their continuous support of this project.

REFERENCES

1. Levitsky J, Goldberg D, Smith AR, et al. Acute rejection increases risk of graft failure and death in recent liver transplant recipients. *Clin Gastroenterol Hepatol.* 2017;15:584.e2–593.e2.
2. Agopian VG, Petrowsky H, Kaldas FM, et al. The evolution of liver transplantation during 3 decades: analysis of 5347 consecutive liver transplants at a single center. *Ann Surg.* 2013;258:409–421.
3. Hickman PE, Potter JM, Pesce AJ. Clinical chemistry and post-liver-transplant monitoring. *Clin Chem.* 1997;43(8 Pt 2):1546–1554.
4. Girometti R, Como G, Bazzocchi M, et al. Post-operative imaging in liver transplantation: state-of-the-art and future perspectives. *World J Gastroenterol.* 2014;20:6180–6200.
5. Grant A, Neuberger J. Guidelines on the use of liver biopsy in clinical practice. British Society of Gastroenterology. *Gut*. 1999;45(Suppl 4):IV1–IV11.
6. Regev A, Berho M, Jeffers LJ, et al. Sampling error and intraobserver variation in liver biopsy in patients with chronic HCV infection. *Am J Gastroenterol.* 2002;97:2614–2618.
7. Sood S, Testro AG. Immune monitoring post liver transplant. *World J Transplant.* 2014;4:30–39.
8. Verhelst XP, Troisi RI, Colle I, et al. Biomarkers for the diagnosis of acute cellular rejection in liver transplant recipients: a review. *Hepatol Res.* 2013;43:165–178.
9. Lo YM, Tein MS, Pang CC, et al. Presence of donor-specific DNA in plasma of kidney and liver-transplant recipients. *Lancet.* 1998;351:1329–1330.
10. De Vlaminck I, Valantine HA, Snyder TM, et al. Circulating cell-free DNA enables noninvasive diagnosis of heart transplant rejection. *Sci Transl Med.* 2014;6:241ra77.
11. De Vlaminck I, Martin L, Kertesz M, et al. Noninvasive monitoring of infection and rejection after lung transplantation. *Proc Natl Acad Sci U S A.* 2015;112:13336–13341.
12. 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.
13. Schütz E, Fischer A, Beck J, et al. Graft-derived cell-free DNA, a noninvasive early rejection and graft damage marker in liver transplantation: a prospective, observational, multicenter cohort study. *Plos Med.* 2017;14:e1002286.
14. García Moreira V, Prieto García B, Baltar Martín JM, et al. Cell-free DNA as a noninvasive acute rejection marker in renal transplantation. *Clin Chem.* 2009;55:1958–1966.
15. Sigdel TK, Vitalone MJ, Tran TQ, et al. A rapid noninvasive assay for the detection of renal transplant injury. *Transplantation.* 2013;96:97–101.
16. Macher HC, Suárez-Artacho G, Guerrero JM, et al. Monitoring of transplanted liver health by quantification of organ-specific genomic marker in circulating DNA from receptor. *PLOS One.* 2014;9:e113987.
17. Abecasis GR, Auton A, Brooks LD, et al; 1000 Genomes Project Consortium. An integrated map of genetic variation from 1,092 human genomes. *Nature.* 2012;491:56–65.
18. Weber JL, David D, Heil J, et al. Human diallelic insertion/deletion polymorphisms. *Am J Hum Genet.* 2002;71:854–862.
19. Beck J, Bierau S, Balzer S, et al. Digital droplet PCR for rapid quantification of donor DNA in the circulation of transplant recipients as a potential universal biomarker of graft injury. *Clin Chem.* 2013;59:1732–1741.
20. Grskovic M, Hiller DJ, Eubank LA, et al. Validation of a clinical-grade assay to measure donor-derived cell-free DNA in solid organ transplant recipients. *J Mol Diagn.* 2016;18:890–902.
21. Gordon PM, Khan A, Sajid U, et al. An algorithm measuring donor cell-free DNA in plasma of cellular and solid organ transplant recipients that does not require donor or recipient genotyping. *Front Cardiovasc Med.* 2016;3:33.
22. Snyder TM, Khush KK, Valantine HA, et al. Universal noninvasive detection of solid organ transplant rejection. *Proc Natl Acad Sci U S A.* 2011;108:6229–6234.
23. Lee H, Park YM, We YM, et al. Evaluation of digital PCR as a technique for monitoring acute rejection in kidney transplantation. *Genomics Inform.* 2017;15:2–10.
24. Hidestrand M, Tomita-Mitchell A, Hidestrand PM, et al. Highly sensitive noninvasive cardiac transplant rejection monitoring using targeted quantification of donor-specific cell-free deoxyribonucleic acid. *J Am Coll Cardiol.* 2014;63:1224–1226.
25. Goh SK, Muralidharan V, Christophi C, et al. Probe-free digital PCR quantitative methodology to measure donor-specific cell-free DNA after solid-organ transplantation. *Clin Chem.* 2017;63:742–750.
26. Adamek M, Opelz G, Klein K, et al. A fast and simple method for detecting and quantifying donor-derived cell-free DNA in sera of solid organ transplant recipients as a biomarker for graft function. *Clin Chem Lab Med.* 2016;54:1147–1155.
27. Bruno DL, Ganesamoorthy D, Thorne NP, et al. Use of copy number deletion polymorphisms to assess DNA chimerism. *Clin Chem.* 2014;60:1105–1114.
28. Gielis EM, Ledeganck KJ, De Winter BY, et al. Cell-free DNA: an upcoming biomarker in transplantation. *Am J Transplant.* 2015;15:2541–2551.
29. Hindson CM, Chevillet JR, Briggs HA, et al. Absolute quantification by droplet digital PCR versus analog real-time PCR. *Nat Methods.* 2013;10:1003–1005.
30. Zou J, Duffy B, Slade M, et al. Rapid detection of donor cell free DNA in lung transplant recipients with rejections using donor-recipient HLA mismatch. *Hum Immunol.* 2017;78:342–349.
31. Goh SK, Musafer A, Witkowski T, et al. Comparison of 3 methodologies for genotyping of small deletion and insertion polymorphisms. *Clin Chem.* 2016;62:1012–1019.
32. Agbor-Enoh S, Jackson AM, Tunc I, et al. Late manifestation of alloantibody-associated injury and clinical pulmonary antibody-mediated rejection: evidence from cell-free DNA analysis. *J Heart Lung Transplant.* 2018;37:925–932.
33. Bromberg JS, Brennan DC, Poggio E, et al. Biological variation of donor-derived cell-free DNA in renal transplant recipients: clinical implications. *J Appl Lab Med*. 2017;2:309–321.
34. Kanzow P, Kollmar O, Schütz E, et al. Graft-derived cell-free DNA as an early organ integrity biomarker after transplantation of a marginal HELLP syndrome donor liver. *Transplantation.* 2014;98:e43–e45.
35. Bartlett AS, Ramadas R, Furness S, et al. The natural history of acute histologic rejection without biochemical graft dysfunction in orthotopic liver transplantation: a systematic review. *Liver Transpl.* 2002;8:1147–1153.
36. Rodríguez-Perálvarez M, Germani G, Tsochatzis E, et al. Predicting severity and clinical course of acute rejection after liver transplantation using blood eosinophil count. *Transpl Int.* 2012;25:555–563.
37. De Simone P, Nevens F, De Carlis L, et al; H2304 Study Group. Everolimus with reduced tacrolimus improves renal function in de novo liver transplant recipients: a randomized controlled trial. *Am J Transplant.* 2012;12:3008–3020.
38. Saliba F, De Simone P, Nevens F, et al; H2304 Study Group. Renal function at two years in liver transplant patients receiving everolimus: results of a randomized, multicenter study. *Am J Transplant.* 2013;13:1734–1745.
39. Sood S, Haifer C, Yu L, et al. A novel immune function biomarker identifies patients at risk of clinical events early following liver transplantation. *Liver Transpl.* 2017;23:487–497.
40. Tug S, Helmig S, Deichmann ER, et al. Exercise-induced increases in cell free DNA in human plasma originate predominantly from cells of the haematopoietic lineage. *Exerc Immunol Rev.* 2015;21:164–173.
41. Wong D, Moturi S, Angkachatchai V, et al. Optimizing blood collection, transport and storage conditions for cell free DNA increases access to prenatal testing. *Clin Biochem.* 2013;46:1099–1104.
42. Kang Q, Henry NL, Paoletti C, et al. Comparative analysis of circulating tumor DNA stability in K3EDTA, streck, and cellsave blood collection tubes. *Clin Biochem.* 2016;49:1354–1360.
43. Beck J, Oellerich M, Schulz U, et al. Donor-derived cell-free DNA is a novel universal biomarker for allograft rejection in solid organ transplantation. *Transplant Proc.* 2015;47:2400–2403.
44. Goh SK, Muralidharan V, Christophi C, et al. Fresh frozen plasma transfusion can confound the analysis of circulating cell-free DNA. *Clin Chem.* 2018;64:749–751.