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Donor-derived Cell-free DNA Evaluation in Pediatric Heart Transplant Recipients: A Single-center 12-mo Experience

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Background. Endomyocardial biopsy (EMB) is considered the gold-standard method to diagnose rejection after heart transplantation. However, the many disadvantages and potential complications of this test restrict its routine application, particularly in pediatric patients. Donor-derived cell-free DNA (dd-cfDNA), released by the transplanted heart as result of cellular injury, is emerging as a biomarker of tissue damage involved in ischemia/reperfusion injury and posttransplant rejection. In the present study, we systematically evaluated dd-cfDNA levels in pediatric heart transplant patients coming for follow-up visits to our clinic for 12 mo, with the aim of determining whether dd-cfDNA monitoring could be efficiently applied and integrated into the posttransplant management of rejection in pediatric recipients. **Methods.** Twenty-nine patients were enrolled, and cfDNA was obtained from 158 blood samples collected during posttransplant follow-up. dd-cfDNA% was determined with a droplet-digital polymerase chain reaction assay. EMB scores, donor-specific antibody measurements, and distress marker quantification were correlated with dd-cfDNA, together with echocardiogram information. **Results.** The percentage of dd-cfDNA increased when EMBs scored positive for rejection ($P = 0.0002$) and donor-specific antibodies were present ($P = 0.0010$). N-terminal pro-B-type natriuretic peptide and high-sensitive troponin I elevation were significantly associated with dd-cfDNA release ($P = 0.02$ and $P < 0.0001$, respectively), as were reduced isovolumetric relaxation time ($P = 0.0031$), signs of heart failure ($P = 0.0018$), and treatment for rejection ($P = 0.0017$). By determining a positive threshold for rejection at 0.55%, the test had a negative predictive value maximized at 100%. **Conclusions.** Collectively, results indicate that dd-cfDNA monitoring has a high negative prognostic value, suggesting that in heart transplanted children with dd-cfDNA levels of <0.55% threshold, protocol EMBs may be postponed.

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Thanks to effective immunosuppressive therapies, the outcome and duration of heart transplantation in children have greatly improved.[1] Nevertheless, median survival remains between 11 and 22 y,[2] depending on the age of the patient at the time of transplantation. The main causes of loss of function of the transplanted organ include acute cellular rejection (ACR) or antibody-mediated rejection (AMR), chronic cardiac vasculopathy (CAV), and infections.[2] The current gold-standard test for the diagnosis of rejection is endomyocardial biopsy (EMB), which, however, is expensive,

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invasive, and causes stress and discomfort to the patient. In addition, it can be performed only a limited number of times because of the intervening fibrotic damage that occurs on repeated procedures.[3-5] Children typically have many more posttransplant years than adult recipients, leading to the potential need for EMB surveillance for >20 y.[3] Therefore, it is necessary to identify novel and less invasive rejection markers for monitoring pediatric heart transplantation.

Donor-derived cell-free DNA (dd-cfDNA), released as a result of cell death or graft injury by the transplanted organ into the recipient's blood, is a biomarker of tissue damage involved in ischemia/reperfusion injury and posttransplant rejection.[4-7] Recent studies on adult and pediatric patients receiving solid organ transplants have shown a direct correlation between the early increase in dd-cfDNA and the onset of organ damage.[3,8-10] By using a droplet-digital polymerase chain reaction (ddPCR) method based on single nucleotide polymorphisms of the *HLA-DRB1* gene, we showed that dd-cfDNA monitoring in adult heart recipients very effectively correlates with biopsy-proven episodes of rejection.[11] This method is rapid and cost-effective and could therefore be very easily implemented in routine posttransplant monitoring, even in relatively small centers.[10–12]

This study aimed at a population of pediatric heart transplant recipients who were being followed in our center for 12 mo. For these patients, we offered dd-cfDNA monitoring at every appointment, and after the study period, we correlated the results with their clinical, laboratory, and pathological findings to evaluate whether dd-cfDNA monitoring could be useful. Results from a 12-mo follow-up confirm that this noninvasive test is effective in detecting rejection and graft failure. Furthermore, results show a high negative prognostic value of the test, suggesting that it could be used to identify patients for whom EMB may be spaced out.

Our results open the possibility of introducing dd-cfDNA quantification in routine posttransplant management of children, therefore limiting the need for invasive tests to high-risk patient subsets.

MATERIALS AND METHODS

Patient Recruitment

The study was approved by the Institutional Ethical Committee (approval 6/2022; February 15, 2022). Fifty-one patients were transplanted at the Pediatric and Congenital Cardiac Surgery Department, Regina Margherita Children's Hospital, Torino, Italy, from 2006 to 2023. Of them, 9 patients (17.6%) died, 10 ended their follow-up when they turned 18 y (19.6%), and 3 (5.9%) were excluded for technical reasons. The remaining 29 patients were enrolled in the study after parents or legal representatives signed the informed consent form. Patient data were anonymized, and all sensitive information was stored on the RedCap online platform (https://www.medcap.unito.it/redcap/index.php; Table 1).

Immunosuppressive Treatment

At the time of transplant, patients received induction therapy (thymoglobuline 1–1, 5 mg/kg IV/die or 5–7 doses) and a brief corticosteroid intravenous therapy (prednisolone 20–25 mg/kg on day 0) tapered and discontinued in 7–10 d unless graft failure required prolongation. Immunosuppressive maintenance double therapy with tacrolimus and mycophenolate mofetil (MMF) reached therapeutic levels (tacrolimus 8–12 ng/mL, MMF 2–4 μg/mL) in the first 6 mo. Everolimus was used in case of renal failure, posttransplant lymphoproliferative disorder, or coronary allograft vasculopathy, in substitution to MMF, and with appropriate reduction of tacrolimus levels.

Upon finding ACR grade 1R, maintenance therapy was optimized. For AMR treatment, IVIG, rituximab, thymoglobuline, and plasmapheresis were used.[13,14]

Posttransplant Clinical Management

Signs and symptoms of heart failure were evaluated at every appointment. Echocardiograms were interpreted by a pediatric Cardiologist. Systolic dysfunction (ejection fraction reported as <50%), significantly increased mitral or tricuspid valve regurgitation, increase of tissue Doppler imaging data, individual patient reduction in isovolumetric relaxation time (IVRT; >20%),[15,16] and new pericardial effusions were considered abnormal and consistent with a suspicion of rejection.

ECG was evaluated by a pediatric cardiologist, including the sum of ECG total voltage (defined as the sum of I, II, III, V1, and V6 voltages); ECG abnormalities, new arrhythmias, and individual patient ECG total voltage reduction (>20%)[17] were considered abnormal and consistent with a suspicion of rejection.

Hematological Evaluation of Cardiac Distress

Blood tests included dosage of N-terminal pro-B-type natriuretic peptide (NT-proBNP) and high-sensitive troponin I (hs-troponin I) levels; an abnormal value for NT-proBNP was designated as ≥1000 ng/L and for hs-troponin I as ≥3 ng/L based on previous studies.[18–21]

Immunohistochemical Evaluation and Rejection Management

EMB was generally not performed in children weighing <8–10 kg. In the other patients, the first EMB was performed within 1–3 mo posttransplantation, the second in the first year, and then on a yearly basis unless a high suspicion of rejection was present. EMBs were evaluated by a single pathologist and graded according to the revised International Society for Heart and Lung Transplantation (ISHLT) biopsy grading system[22] for ACR and the ISHLT working formulation for AMR[23] for humoral one. C4d immunohistochemistry was performed on every specimen, whereas CD68 was tested only in the case of AMR.

Donor-specific Antibodies Screening

Patients underwent routine screening for anti-HLA class I and class II donor-specific antibodies (DSAs) at 1 and 3 mo posttransplant and every 4 mo thereafter unless there was clinical suspicion for rejection. Complement binding capacity (C1q-testing) was tested to identify harmful HLA antibodies. HLA antibodies and their specificity were determined using Luminex single antigen kit (One Lambda, West Hills, CA). Sera were considered positive for DSAs with a cutoff of >1000 mean fluorescent intensity. The definition of positive DSAs also includes unchanged DSAs or increasing DSAs over 2 separate measurements at any point after heart transplantation. Increased DSAs with clinical or echo abnormalities were considered highly suspicious for rejection.

TABLE 1.

Characteristics of the enrolled cohort

ID	Sex	Age, y	Diagnosis	Transplant date	Enrollment date	Time since transplant, y	No. of EMB	dd-cfDNA determinations
1	F	18.6	CHD	01/10/06	02/22/22	16.13	1	4
2	F	15.3	DCM	07/03/08	04/26/22	13.82	0	6
3	F	17.9	DCM	02/09/10	03/08/22	12.08	1	4
4	F	15.1	CHD	08/27/10	03/29/22	11.59	1	1
5	M	14.1	DCM	12/31/10	10/04/22	11.77	1	1
6	M	16.6	CHD	04/25/11	04/08/22	10.96	1	8
7	F	12.6	HCM	07/02/11	03/21/22	10.73	1	4
8	F	13.9	DCM	03/07/13	04/05/22	9.08	1	6
9	M	11.4	DCM	04/17/13	02/24/22	8.86	1	9
10	F	10.3	DCM	01/09/14	03/01/22	8.15	1	6
11	M	10.2	CHD	09/18/14	03/14/22	7.49	0	3
12	F	8.4	DCM	06/20/15	02/24/22	6.69	1	5
13	F	18.5	CHD	01/08/16	03/09/22	6.17	1	8
14	F	12.7	CHD	07/16/16	05/09/22	5.82	1	5
15	F	7.0	DCM	10/21/16	04/19/22	5.50	1	5
16	F	8.3	DCM	04/26/17	05/23/22	5.08	1	4
17	F	7.2	DCM	08/01/17	03/03/22	4.59	1	3
18	F	12.0	DCM	12/05/17	02/28/22	4.24	1	5
19	M	5.6	DCM	11/04/18	02/23/22	3.31	4	11
20	M	7.0	CHD	06/16/19	05/02/22	2.88	1	5
21	F	3.5	DCM	11/12/20	04/11/22	1.41	1	4
22	F	9.9	CHD	12/22/20	03/15/22	1.23	1	7
23	M	14.6	RCM	07/01/21	02/25/22	0.65	2	13
24	M	16.5	CHD	07/17/21	03/10/22	0.65	1	5
25	F	2.4	DCM	08/25/21	03/14/22	0.55	1	7
26	M	14.7	CHD	03/17/22	03/24/22	0.02	1	11
27	M	0.8	CHD	07/30/22	10/20/22	0.22	0	2
28	F	7.1	DCM	01/08/23	01/20/23	0.03	0	3
29	M	11.3	CHD	01/11/23	02/13/23	0.09	0	3

For each patient, sex, age, diagnosis, date of transplant and enrollment, and number of EMBs, and dd-cfDNA samples are listed.
CHD, congenital heart defect; DCM, dilated cardiomyopathy; dd-cfDNA, donor-derived cell-free DNA; EMB, endomyocardial biopsy; F, female; HCM, hypertrophic cardiomyopathy; M, male; RCM, restrictive cardiomyopathy.

Sample Collection and dd-cfDNA Analysis

Blood samples were collected using PAXgene tubes (768165, Qiagen, Hilden, Germany), plasma separated (centrifugation at 2000*g*, 15 min, 15 °C) and stored at –80 °C in the TESEO Biobank of the Department of Medical Sciences of the University of Turin, Italy (https://www.progettoeccellenzateseo.unito.it/it/node/46).

Total cell-free DNA (cfDNA) was extracted from 1 mL of plasma using the QIAmp MinElute ccfDNA Mini Kit (55204, Qiagen) and eluted in 30µl of ultra-clean water. cfDNA concentration was assessed by Qubit dsDNA HS Assay Kit (Q32854, Invitrogen, Waltham, MA). dd-cfDNA quantification was obtained using the HLA Expert Design Assay (Bio-Rad Laboratories Inc) as described, targeting the genetic polymorphisms present between donor and patient at the *HLA-DRB1* gene.[11,12,24] Donor and recipient HLA typing was performed at Immunogenetics and Transplant Biology Service, Città della Salute e della Scienza, Torino, Italy. Donor cfDNA contribution was expressed as a percentage of the total cfDNA, and results were correlated to clinical information.

Statistical Analysis

Continuous variables are reported as median and interquartile ranges or means ± SDs as appropriate, whereas categorical variables are indicated as numbers and percentages. Differences between median values were compared using the Mann-Whitney *U* test. *P* values of <0.05 were considered statistically significant. The correlation between 2 continuous variables was analyzed using the nonparametric Spearman test. Receiver operating characteristic (ROC) curves were calculated using the Wilson-Braun method to obtain a positive cutoff for rejection and heart injury. Statistical analyses were performed using GraphPad Prism version 8.0.2.

A random forest model was developed to predict organ rejection using laboratory and clinical parameters, such as NT-proBNP, hs-troponin I, ejection fraction, IVRT, DSA, failure, and dd-cfDNA. The model was trained using the randomForest R package (version 4.7-1.1) and evaluated using various metrics, including accuracy, precision, recall, and F1 score. The data were split into an 80/20 training test split using the caret R package (version 6.0-94) to maximize the training data. Additionally, linear regression models were fitted to analyze the relationship between NT-proBNP and dd-cfDNA, as well as hs-troponin I and dd-cfDNA levels, which were identified as the 2 most influential clinical features by the random forest algorithm. Graphs were generated by ggplot2 package (version 3.5.1). The R version 4.3.2 was used for these analyses (2023-10-31 ucrt) on an x86_64-w64-mingw32 platform. **Table S1** (**SDC,** http://links.lww.com/TXD/A683) provides further information on the performance of the model.

RESULTS

Description of the Recruited Cohort

Twenty-nine pediatric heart transplant recipients, 15 (55%) of whom were female individuals, were recruited at our Institution starting from February 2022 for the following 12 mo (Table 1). The mean age at recruitment was 11.0 ± 4.8 y, and the most represented conditions leading to heart failure were dilated cardiomyopathy (n = 15) and congenital heart disorders (n = 12). During the first posttransplant year, patients were checked monthly, whereas in later years, follow-ups decreased progressively up to 1 every 4 mo. No death and no retransplantation occurred during the study period; 2 patients were followed for CAV.

Correlation Between dd-cfDNA Values and Clinical Parameters

One hundred fifty-eight blood samples were collected during outpatient visits, with an average of 5.4 samples per patient. All the tests were blinded to the clinicians until the end of the study period. We obtained an optimal DNA yield from all samples (mean cfDNA concentration 0.24 ng/μL; Table 2), despite the reduced volume of blood that was drawn from younger patients. Dd-cfDNA was monitored by ddPCR capturing polymorphisms in the *HLA-DRB1* locus, as described.[10–12]

No differences in dd-cfDNA percentages were observed in relation to patient or donor weight ($P = 0.54$ and 0.39) and time after transplantation ($P = 0.34$), indicating that these variables do not impact dd-cfDNA release (Figure 1A–C).

During the study period, a total of 28 routine EMBs were performed on 24 patients; only in 1 case (the second relevant clinical case described below) EMBs were performed for suspected rejection. Eleven biopsies scored positive for rejection (39.3%), 9 of which were classified as ACR (all graded as 1R) and 2 as AMR (both pAMR2 score). Concomitant dd-cfDNA analysis was significantly different in samples showing no evidence of rejection (n = 17, median 0%) compared with those presenting signs of rejection, globally considered (n = 11, median 6.27%, $P = 0.0002$; Figure 2A). Among the negative group, 4 samples from 3 patients presented higher levels compared with the median value: the sample with the highest dd-cfDNA level was an outlier related to a patient (ID

TABLE 2.

Demographical and clinical features of recruited patients

Patient	N = 29
Male gender	13 (44.8%)
Age at transplant (mean ± SD)	4.2 ± 4.4
Age at recruitment (mean ± SD)	11.0 ± 4.8
Transplant survival (mean ± SD)	6.8 ± 5.0
Weight (mean ± SD)	33.6 ± 16.2
Diagnosis	
DCM	15 (51.7%)
RCM	1 (3.4%)
HCM	1 (3.4%)
CHD	12 (41.4%)
Positive cross-match	5 (17.2%)
Immunosuppression	
Tacrolimus	26 (89.7%)
Cyclosporine	3 (10.3%)
MMF	23 (79.3%)
Mycophenolic acid	2 (6.9%)
Everolimus	3 (10.3%)
Samples	158
Mean sample/patient	5.4
Mean cfDNA, ng/μL	0.24

Continuous variables are reported as mean ± SD, and categorical variables are indicated as number (percentage).
cfDNA, cell-free DNA; CHD, congenital heart defects; DCM, dilated cardiomyopathy; HCM, hypertrophic cardiomyopathy; MMF, mycophenolate mofetil; RCM, restrictive cardiomyopathy.

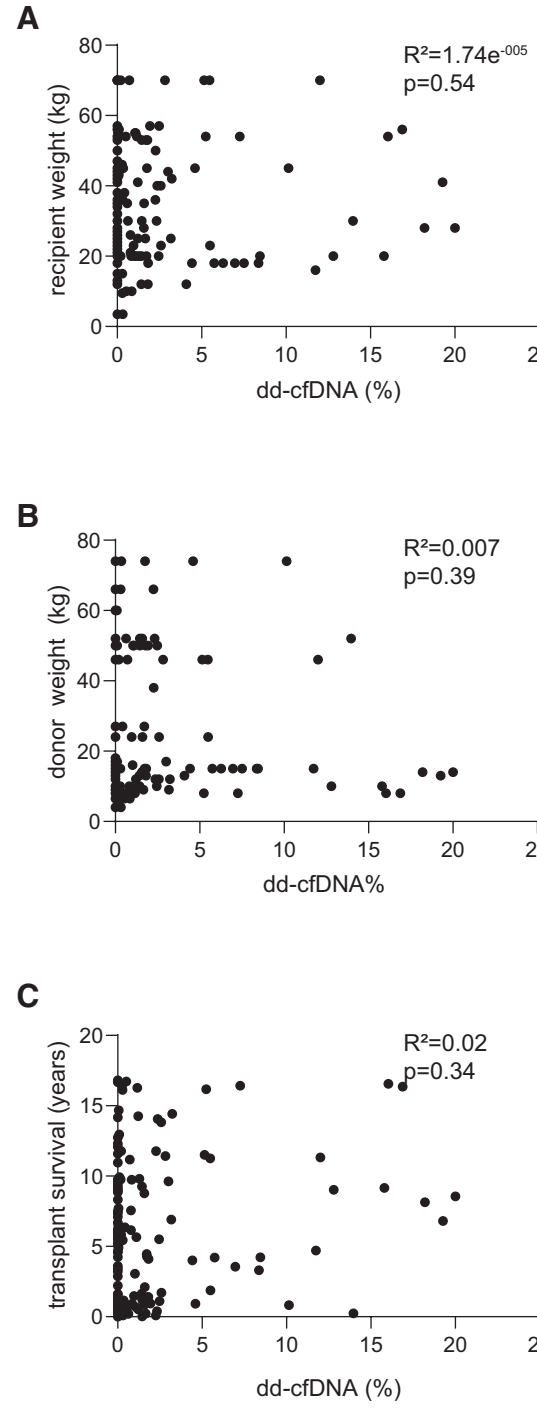

FIGURE 1. Distribution of dd-cfDNA levels based on patient (A), donor weight (B), and transplant survival (C). The correlation was performed using the nonparametric Spearman test. *P* values are 0.54, 0.39, and 0.34, respectively. dd-cfDNA, donor-derived cell-free DNA.

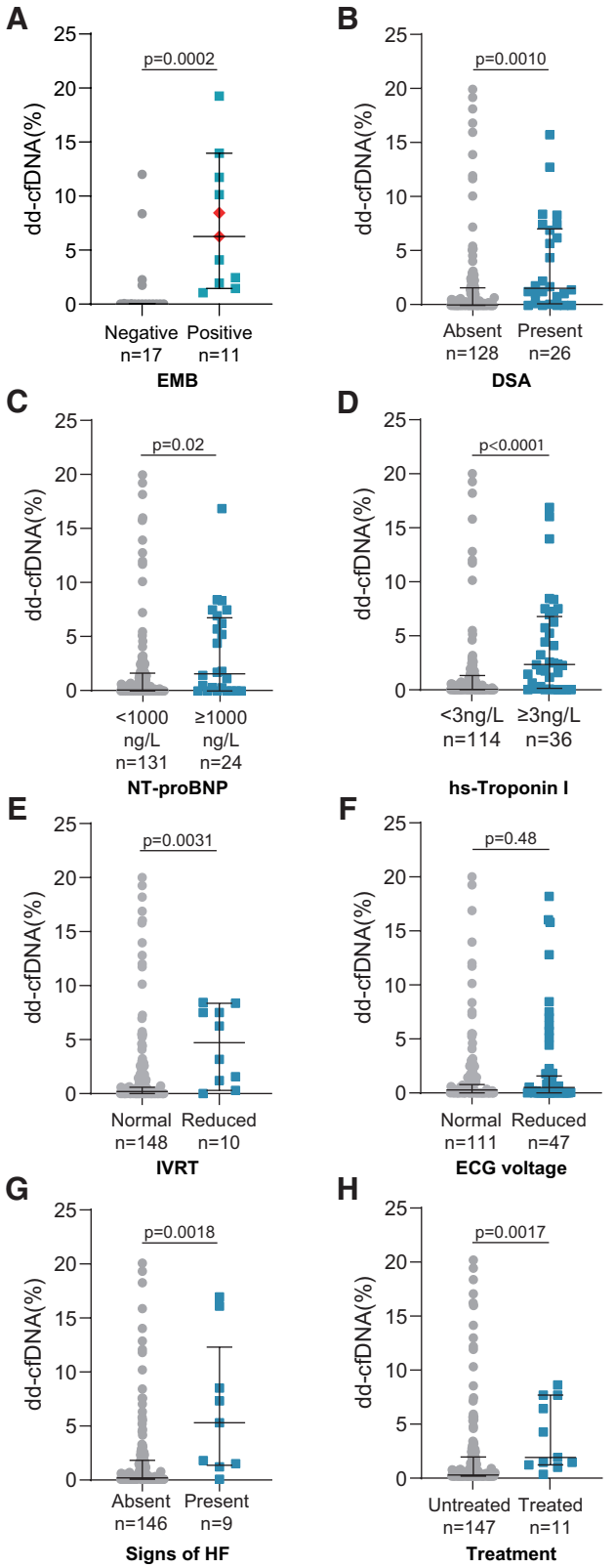

FIGURE 2. Correlation between dd-cfDNA values and clinical parameters of graft status. Dd-cfDNA percentages are plotted in each graph based on (A) EMB rejection grade, (B) presence of serum DSAs, (C) NT-proBNP levels, (D) hs-troponin I blood levels, (E) IVRT, (F) ECG voltage, (G) clinical signs of heart failure, and (H) for-cause treatment. The number of samples in each category is indicated below the graphs. EMBs that scored positive for AMR are highlighted in red in graph A. EMB, endomyocardial biopsy, dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibody; HF, heart failure; hs-troponin I, high-sensitive troponin I; IVRT, isovolumic relaxation time; NT-proBNP, N-terminal pro-brain natriuretic peptide.

6) showing elevated values in 4 different samples, without any other clinical or laboratory parameters pointing to heart damage. The plasma showing dd-cfDNA levels of 8.37% contains DSAs and has elevated NT-proBNP and hs-troponin I levels. The EMB performed at that time was negative for rejection, opening the question of whether the increase in dd-cfDNA was related to nonrejection caused cardiac injury, bad sampling, or the low sensitivity that affects EMB. From this same patient (ID 9), 2 more EMBs were collected; the first scored positive for AMR (pAMR2), then the patient was treated, and the following biopsy resulted in negative for rejection, with a decreasing dd-cfDNA value (1.73%). The last dd-cfDNA measure was related to a third patient (ID 5) who was dosed only once, and on that occasion, all parameters were negative. He entered the study in October 2022, and there were no further follow-ups until the end of the study period.

DSA analysis was performed in 154 samples from 29 different patients. Results demonstrated the presence of DSAs in 26 sera (16.9%), belonging to 6 different patients (Table 3). DSAs were specific for HLA class II in 4 patients, for HLA class I in 1 patient, and 1 patient developed DSAs specific for both HLA class I and HLA class II. Interestingly, only 1 patient developed signs of AMR in 2 different EMBs that scored pAMR2. In the other 5 patients, no signs of AMR were found but 3 presented signs of ACR at biopsy. No EMBs scored positive for complement deposition. The median value of dd-cfDNA was significantly increased in the DSA-positive group compared with the DSA-negative group (0.06% versus 1.58%, $P = 0.0010$; Figure 2B).

Despite controversial results present in the literature,[25,26] NT-proBNP and hs-troponin I are routinely analyzed at our Institution as further noninvasive markers of heart damage. We measured NT-proBNP plasma levels in 155 samples from 29 patients, setting a threshold for graft injury at 1000 ng/L.[18,19] We found higher levels of dd-cfDNA in samples with ≥1000 ng/L NT-proBNP (median levels: 1.59%), compared with the <1000 ng/L group (median levels: 0.10%, $P = 0.02$; Figure 2C). Hs-troponin I values were further considered an indirect marker of graft status, with a cutoff set at 3 ng/L.[20,21,27,28] The difference between median dd-cfDNA levels in low and high hs-troponin I groups was highly significant (median dd-cfDNA: 0.04% versus 2.35%, respectively; $P < 0.0001$; Figure 2D). Although dd-cfDNA nicely correlates with NT-proBNP and hs-troponin I, both are not considered specific rejection markers, even if they prove to be related to cardiac distress.[29–31]

We then correlated dd-cfDNA levels to ECG and echocardiogram parameters that are considered associated with heart failure. Dd-cfDNA levels increased significantly in relation to reduced IVRT ($P = 0.0031$; Figure 2E), which is generally associated with episodes of acute rejection,[15,16] whereas no differences were observed according to the ECG voltage ($P = 0.48$; Figure 2F).

Finally, 9 samples were collected from 4 patients presenting clinical signs and symptoms of heart failure, mainly poor weight gain, decreased oral intake, dyspnea at rest or with exercise, fatigue, hepatomegaly, abdominal pain, nausea, vomiting, and peripheral edema. In these samples, median dd-cfDNA was 5.24%, significantly higher compared with samples without any sign of failure (median dd-cfDNA: 0.14%, $P = 0.0018$; Figure 2G). We also considered 11 sera from 4 patients who received steroid treatment for ongoing rejection. Median dd-cfDNA% was 1.73%

TABLE 3.

DSAs in 26 sera from 6 patients

ID patient	Serum	Class	Antigen	MFI	AMR
4	1	I	A3	1811	No
29	2	II	DQ5	1521	No
		II	DQ8	2537	
		II	DR53	1759	
16	3	II	DR52	1322	No
9	4	II	DQ6	4326	No
		II	DQ7	5212	
	5	II	DQ6	3250	No
		II	DQ7	5186	
	6	II	DQ6	2840	No
		II	DQ7	4463	
	7	II	DQ6	2899	No
		II	DQ7	4673	
	8	II	DQ6	2458	Yes
		II	DQ7	3175	
	9	II	DQ6	1792	No
		II	DQ7	1414	
	10	II	DQ6	2202	No
		II	DQ7	2612	
	11	II	DQ6	2279	No
		II	DQ7	3505	
	12	II	DQ6	2076	No
		II	DQ7	3296	
	13	II	DQ6	2596	Yes
		II	DQ7	2917	
	14	II	DQ6	1139	No
23	15	I	A1	1325	No
		II	DQ8	2650	
	16	II	DQ8	3012	No
	17	II	DQ8	2988	No
	18	II	DQ8	2883	No
12	19	II	DR4	1660	No
	20	II	DR4	1682	No
	21	II	DR4	1745	No
	22	II	DR4	1363	No
		II	DR53	1316	
	23	II	DR4	2672	No
		II	DR53	1332	
	24	II	DR4	1971	No
	25	II	DR4	1565	No
	26	II	DR53	1803	No

Specificity for class I or II and relative MFI is reported for each serum resulted containing DSA. Biopsy-proven AMR and ACR are indicated.
ACR, acute cellular rejection; AMR, antibody-mediated rejection; DSA, donor-specific antibody; MFI, mean fluorescent intensity.

compared with 0.11% in the untreated group (147 samples, $P = 0.0017$; Figure 2H). Collectively, these results show that dd-cfDNA is highly associated with every currently used marker of rejection, independent of the time elapsed since transplantation.

Determination of a Rejection Score Based on dd-cfDNA

Next, we sought to calculate the performance of dd-cfDNA to distinguish between rejection and no rejection, considering each laboratory and clinical marker and performing ROC analyses. ROC curve of dd-cfDNA related to EMB grade resulted in an area under the curve (AUC) of 0.89 (95% confidence interval [CI], 0.77-1.0; $P = 0.0005$; Figure 3), similar to previously reported data on adult and pediatric patients.[32] With a cutoff of 0.55%, dd-cfDNA had 100% sensitivity and 76.5% specificity in distinguishing rejection from no rejection, with a negative predictive value (NPV) of 100% and a positive predictive value of 73%. We then performed the same analysis considering other laboratory and clinical parameters: DSA measurement resulted in an AUC of 0.69 (95% CI, 0.58-0.80; $P = 0.0019$), NT-proBNP resulted in an AUC of 0.64 (95% CI, 0.51-0.77; $P = 0.03$), hs-troponin I resulted in an AUC of 0.73 (95% CI, 0.63-0.83; $P < 0.0001$), IVRT resulted in an AUC of 0.76 (95% CI, 0.61-0.91; $P = 0.0060$), and clinically defined heart failure resulted in an AUC of 0.78 (95% CI, 0.63-0.94; $P = 0.0045$; Figure 3). Moreover, dd-cfDNA outperformed the combination of all the other cardiac biomarkers considered in the study in detecting rejection, as

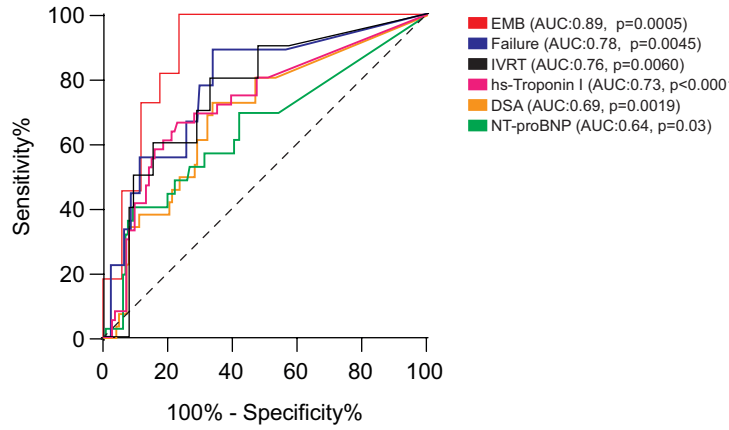

FIGURE 3. ROC curves of dd-cfDNA related to clinical and blood parameters of allograft injury. ROC analysis of rejection markers and clinical parameters of cardiac injury is calculated and compared. AUC and *P* values are reported in the legend in brackets. AUC, area under the curve; dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibody; EMB, endomyocardial biopsy; hs-troponin I, high-sensitive troponin I; IVRT, isovolumic relaxation time; NT-proBNP, N-terminal pro-brain natriuretic peptide; ROC, receiver operating characteristic.

TABLE 4.

Receiver operating characteristics analysis of the combination of different cardiac markers in identifying rejection in heart transplant

	AUC	*P*
EMB + hs-troponin I	0.63	0.23
EMB + NT-proBNP	0.67	0.14
EMB + IVRT	0.63	0.24
EMB + failure	0.52	0.89
EMB + DSA	0.59	0.41
EMB + dd-cfDNA	**0.89**	**0.0005**
hs-Troponin I + NT-proBNP	0.59	0.44
hs-Troponin I + IVRT	0.52	0.82
hs-Troponin I + failure	0.60	0.37
hs-Troponin I + DSA	0.67	0.14
hs-Troponin I + dd-cfDNA	**0.73**	**<0.0001**
DSA + NT-proBNP	0.56	0.57
DSA + IVRT	0.54	0.7
DSA + failure	0.61	0.33
DSA + dd-cfDNA	**0.69**	**0.0019**
NT-proBNP + failure	0.52	0.87
NT-proBNP + IVRT	0.54	0.73
NT-proBNP + dd-cfDNA	**0.64**	**0.03**
Failure + IVRT	0.52	0.83
Failure + dd-cfDNA	**0.78**	**0.0045**

The AUC and *P* values were assessed by integrating the combination of the cardiac markers listed.
Bold values indicate statistical significance.AUC, area under the curve; DSA, donor-specific antibody; EMB, endomyocardial biopsy; hs-troponin I, high-sensitive troponin I; IVRT, isovolumetric relaxation time; NT-proBNP, N-terminal pro B-type natriuretic peptide.

highlighted by performing ROC analysis combining different biomarkers (Table 4).

We then applied 0.55% as a positive threshold for rejection to estimate the power of the test on samples from our recruited cohort. A total of 88 samples fell under the positive threshold (median dd-cfDNA 0%; range, 0–0.54). Among them, 13 were collected before a biopsy was performed, and none scored positive for rejection; DSAs were present in 7 samples, NT-proBNP was above the threshold in 8 samples, hs-troponin I was elevated in 4 samples, 2 samples were related to a reduced IVRT, and only 1 sample was collected from a patient who was still under treatment for a past event of rejection. Globally, no samples were associated with signs of rejection and no modification in the immunosuppressive regimen was made.

In contrast, samples with dd-cfDNA% >0.55 were 70 (median dd-cfDNA 2.35%; range, 0.59–20). Of them, 15 samples were collected before biopsies and 11 resulted positive for rejection; DSAs were present in 19 samples, NT-proBNP was elevated in 14 samples, hs-troponin I was above the threshold in 22 samples, and reduced IVRT was present in 8 samples. Moreover, 10 samples from 4 patients were collected when additional steroid treatment for ongoing rejection was administered.

We then checked dd-cfDNA values according to the number of parameters positive for rejection (EMB, DSA, NT-proBNP, hs-troponin I, and heart failure). Of the 158 samples, 92 measurements did not present any marker of rejection (median dd-cfDNA 0.04%), 39 had 1 parameter indicating rejection (median dd-cfDNA 0.51%), 14 presented 2 parameters

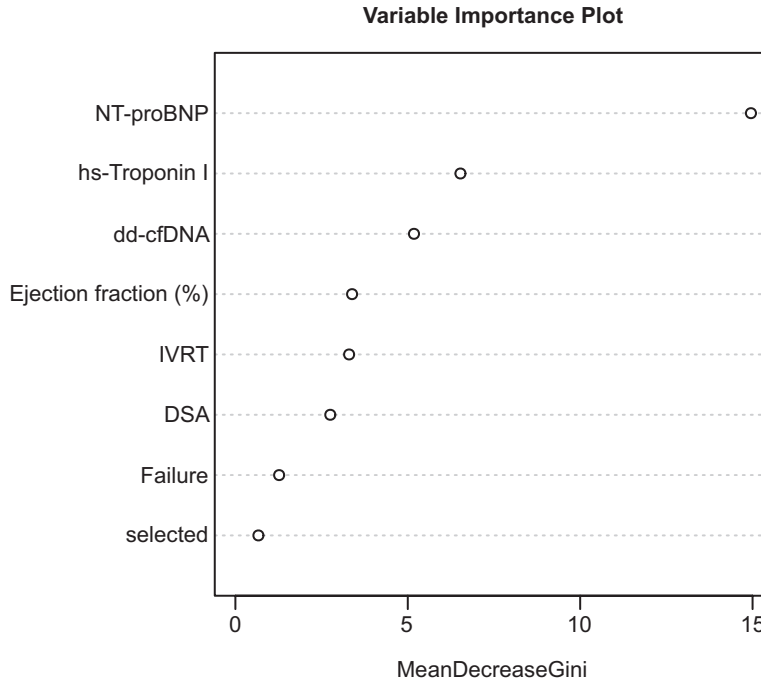

FIGURE 4. Variable importance plot of the random forest model showing the most significant variables (clinical features) contributing to rejection prediction. The most significant ones are ordered in descending order (from top to bottom) by a mean decrease in the Gini coefficient. The top variables contribute more to the model than the bottom ones. dd-cfDNA, donor-derived cell-free DNA; DSA, donor-specific antibody; hs-troponin I, high-sensitive troponin I; IVRT, isovolumic relaxation time; NT-proBNP, N-terminal pro-brain natriuretic peptide.

(median dd-cfDNA 1.24%), 11 samples presented 3 parameters (median dd-cfDNA 5.74%), and 2 samples presented 4 markers out of 5 indicating rejection (median dd-cfDNA 7.36%), reflecting a step-wise increment of dd-cfDNA related to the number of rejection and injury markers and, therefore, the severity of the allograft damage.

Then, we tried to integrate all clinical and hematological parameters to score the risk of rejection. We reassessed the power of our test by applying 0.55% as a positive threshold for rejection. Considering all noninvasive blood parameters of heart injury to distinguish between healthy allograft and rejection, the NPV resulted in 100% and the positive predictive value in 60%.

Finally, we trained a random forest algorithm to distinguish samples associated with rejection from healthy allographs to build a predictive model. NT-proBNP, hs-troponin I, and dd-cfDNA resulted as the most relevant parameters (Figure 4; **Table S1, SDC,** http://links.lww.com/TXD/A683), although none of them seemed to be linearly related (**Figure S1, SDC,** http://links.lww.com/TXD/A683).

Relevant Clinical Cases of Rejection

We selected 2 patients who developed CAV and were followed during the 12 mo of the project; their dd-cfDNA values were dosed multiple times. The first patient is a 17-y-old girl with complex congenital heart disorder, transplanted in 2006 (ID 13; Figure 5A).

After developing chronic allograft rejection in 2021 with heart failure, high NT-proBNP levels, and discrete coronary stenosis (classified as CAV2 from ISHLT),[33] she was treated with angioplasty and switched to everolimus. In 2022, because of therapy noncompliance, she experienced chest pain and heart failure, requiring a new angioplasty. She was enrolled in March 2022, and DSA measurements were always negative during the entire follow-up. Dd-cfDNA% was evaluated monthly and the result was above the threshold for rejection until patient's condition stabilized after treatment in July 2022.

The second patient is a 5-y-old boy, transplanted in 2018 for dilated cardiomyopathy (ID 19; Figure 5B). DSAs were present since 2020 and were specific for HLA class II DQ antigens; one of them showed C1q binding capacity (DQ6, mean fluorescent intensity = 9223). In 2020–2021, he was treated with pulse steroids, intravenous rituximab, IVIG, and thymoglobulin. In the study period, he experienced 2 rejection events in September 2022 and January 2023, respectively. The first one scored for mixed ACR/AMR rejection (biopsy grade 1R(1A), pAMR2), and the second event scored for a pAMR2 grade rejection, with NT-proBNP and hs-troponin I levels above the reference values. Cardiac catheterization revealed high filling pressure in September 2022 and January 2023; diffuse coronary lesions and heart failure (ISHLT CAV3)[33] were present in January 2023. Biopsy-proven rejections were treated with rituximab, plasmapheresis, IVIG, and thymoglobuline. Dd-cfDNA values were >0.55% in association with rejection events and partially decreased after the 2 additional steroid treatments administered between July and October 2022 and between January and February 2023.

In contrast, Figure 5C shows dd-cfDNA monitoring in 3 patients (ID 1, 14, and 24) with no clinical signs of rejection and heart injury: their dd-cfDNA values were <0.55% cutoff in all measurements collected during follow-up time, reflecting the stable condition of the graft.

DISCUSSION

EMB is the gold-standard method to diagnose rejection after heart transplantation. However, considering its invasiveness and related risks as well as the limited number of EMBs

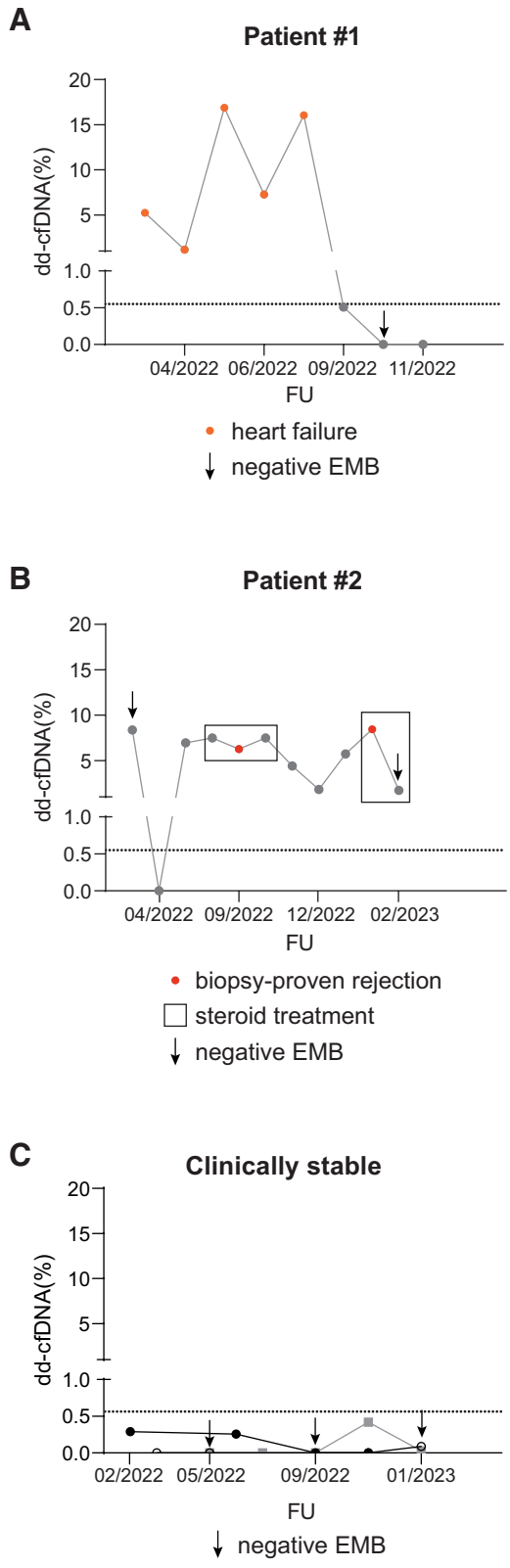

FIGURE 5. Dd-cfDNA trend in patients with clinical signs of rejection or with healthy allograft. The percentage of dd-cfDNA is reported for each measurement performed. Samples were collected monthly during routine posttransplant follow-up. Clinically and biopsy-proven heart failure and rejection are highlighted in orange and red, respectively. EMBs that scored negative are indicated by a black arrow. A, Patient 1 (ID 13) experienced heart failure with high NT-proBNP levels and coronary lesions as a consequence of chronic allograft rejection, then stabilized after treatment with (*Continued*)

that can be performed during posttransplant follow-up, there is a clinical need for noninvasive and alternative markers of rejection that are able to complement this method.

This study was undertaken to evaluate whether dd-cfDNA monitoring confirmed its effectiveness in posttransplant detection of rejection and graft failure in pediatric patients, opening up the possibility to apply the test as a first-line check for rejection, therefore limiting EMBs only to patients at risk (ie, patients presenting with clinical signs of failure, CAV, rejection, or those noncompliant with the immunosuppressive therapy).[34-37] Dd-cfDNA quantification has been introduced as a valuable marker of ACR and AMR in several transplanted organs, but most of the studies on heart transplant recipients have been performed on adult cohorts of patients or mixed cohorts of adults and children.[3,8,9]

We monitored dd-cfDNA using a rapid and easy-to-use test based on ddPCR, previously optimized and validated in our laboratory.[10-12] This method is based on the *HLA-DRB1* mismatch between the donor and the recipient and, unlike other approaches, does not require a screening step to identify relevant polymorphisms because we have access to the HLA typing routinely performed in transplantation centers. This feature avoids pretest screening, thereby saving valuable samples, time, and money. Following this protocol, we assessed dd-cfDNA levels in pediatric heart recipients undergoing follow-up at our Institution for 12 mo. Patients were included in the study regardless of the time of transplantation, enabling us to investigate dd-cfDNA levels in the chronic setting as well. Results were then correlated to other clinical and instrumental parameters usually considered in the follow-up.

The first result is that dd-cfDNA can be measured in every patient, including infants. The second result is that dd-cfDNA levels are strongly correlated to every pathological, clinical, or laboratory parameter of rejection and heart damage, confirming its potential as a biomarker in this patient population. Based on these findings, we determined a threshold with a strong NPV for rejection. Analysis was based on the correlation with EMB grade, and our test was best performed when the cutoff was set at 0.55% dd-cfDNA, with 100% sensitivity and 76.5% specificity. It should be noted that in our cohort, the percentage of patients showing signs of rejection is relatively high (39%). None of the 11 EMBs positive for rejection showed dd-cfDNA levels <0.55% cutoff, whereas in the EMB negative counterpart, only 4 of 18 samples were above the cutoff. If we had applied this threshold to our patient population during the study period, 7 patients had dd-cfDNA levels consistently below the cutoff value. Among them, 5 patients underwent surveillance EMB as part of their routine follow-up, and all were negative for rejection. Based on our data, these 5 patients could have postponed their EMB. However,

FIGURE 5. Continued. a decrease in dd-cfDNA%. B, Patient 2 (ID 19) had a mixed ACR/AMR rejection in September 2022 and an AMR rejection in January 2023, heavily treated. DSAs were present during all follow-up time. NT-proBNP and hs-troponin I levels were above the reference values. Cardiac catheterization revealed high filling pressure and, at the last follow-up, coronary allograft rejection, and heart failure. C, In contrast, dd-cfDNA values of patients (N = 3; ID 1, 14, 24) with no signs of rejection were all <0.55% cutoff. ACR, acute cellular rejection; AMR, antibody-mediated rejection; dd-cfDNA%, donor-derived cell-free DNA percentage; DSA, donor-specific antibody; EMB, endomyocardial biopsy; FU, follow-up; hs-troponin I, high-sensitive troponin I; NT-proBNP, N-terminal pro-brain natriuretic peptide.

further randomized studies with more patients are needed to understand whether in the future, dd-cfDNA quantification can inform clinicians in their decision to perform or postpone EMBs in stable patients. In this context, by using a machine learning algorithm, it was revealed that besides dd-cfDNA, both NT-proBNP and hs-troponin I greatly correlated with rejection, underlining the importance of these 2 distress markers as clinical features of cardiac alteration compared with other clinical data. Given the controversial nonspecific nature of these molecules,[29–31] this analysis suggests that dd-cfDNA results should be integrated into the wider clinical picture of each patient to obtain a clear idea of the graft status, keeping in mind the idea of saving patients from invasive investigation when not needed.

Notably, the threshold we propose is higher than the data reported in the literature.[4,32] The main explanation could be found in the different techniques applied in the studies, which mainly involve shotgun or next-generation sequencing,[4,32] and in their methodologies and characteristics.

Despite encouraging results, our study shows several limitations. First, the reduced number of patients and samples considered in the analysis reflects the experience of the 12-mo rejection monitoring program at our Institution. Second, the limited number of EMBs collected and considered for positive cutoff determination, which is directly related to the small size of the recruited cohort and also to the fact that the time from transplant was >1 y when acute rejection is reported to be less frequent.[38] Similarly, the third limitation is the number of ACR and AMR events that occurred in our cohort, preventing the possibility to further investigate the dd-cfDNA differences between the 2 types of rejection. Moreover, the data retrieved from EMBs are generally biased because of low sensitivity derived from bad sampling and are affected by interobserver variability between pathologists.[39-41]

Despite these limitations, we demonstrate a significant association between dd-cfDNA levels and clinical parameters of rejection, with the assessment of a positive threshold that maximizes the NPV of the test. The validity of these results should be confirmed in larger cohorts of patients, but we believe that the integration of dd-cfDNA evaluation in posttransplant monitoring can improve quality of life and survival. Furthermore, longitudinal measurements of molecular transplantation precision markers can improve the outcome prediction within a patient.[6,42] By applying dd-cfDNA quantification to routine patient management, clinicians may have a powerful tool to be integrated with markers of cardiac distress and clinical evaluation. The decision to perform an invasive biopsy will be based on multiple parameters, but the finding of a dd-cfDNA value below the threshold could argue in favor of postponing a surveillance EMB.

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