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ORIGINAL CLINICAL SCIENCE

Donor-derived cell-free DNA as a new biomarker for cardiac allograft rejection: A prospective study (FreeDNA-CAR)

Marta Jiménez-Blanco, MD,[a,b,c] Maria Generosa Crespo-Leiro, MD, PhD,[c,d] Maria Dolores García-Cosío Carmena, MD, PhD,[c,e] Manuel Gómez Bueno, MD,[a] Raquel López-Vilella, MD,[f] Carlos Ortiz-Bautista, MD,[g] Marta Farrero-Torres, MD PhD,[h] Isabel Zegrí-Reiriz, MD, PhD,[i] Beatriz Díaz-Molina, MD PhD,[j] Elena García-Romero, MD,[c,k,l] Diego Rangel-Sousa, MD,[m] Nahikari Salterain, MD,[n] Iris Garrido Bravo, MD PhD,[o] and Javier Segovia-Cubero, MD, PhD[a,c]

From the [a]Hospital Universitario Puerta de Hierro Majadahonda, Madrid, Spain; [b]Hospital Universitario Ramón y Cajal, Madrid, Spain; [c]Centro de Investigación Cardiovascular en Red CIBER-CV, Madrid, Spain; [d]Complexo Hospitalario Universitario A Coruña (CHUAC), Instituto de Investigación Biomédica A Coruña (INIBIC), Universidad de A Coruña (UDC), A Coruña, Spain; [e]Servicio de Cardiología, Hospital Universitario 12 de Octubre, Instituto de Investigación Sanitaria Hospital 12 de Octubre (imas12), Madrid, Spain; [f]Hospital Universitario La Fe, Valencia, Spain; [g]Hospital Universitario Gregorio Marañón, Madrid, Spain; [h]Hospital Clinic de Barcelona, Barcelona, Spain; [i]Unidad de Insuficiencia cardiaca y Trasplante, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; [j]Hospital Universitario Central de Asturias, Asturias, Spain; [k]Cardiology Department, Hospital Universitari de Bellvitge, Bioheart Group, Cardiovascular, Respiratory and Systemic Diseases and Cellular Aging Program, Institut d'Investigació Biomèdica de Bellvitge (IDIBELL), L'Hospitalet de Llobregat, Spain; [l]Department of Clinical Sciences, School of Medicine, Universitat de Barcelona (UB), L'Hospitalet de Llobregat, Spain; [m]Hospital Universitario Virgen del Rocío, Sevilla, Spain; [n]Clínica Universitaria de Navarra, Navarra, Spain; and the [o]Hospital Virgen de la Arrixaca, Murcia, Spain.

KEYWORDS:
donor-derived cell-free DNA;
acute cardiac rejection;
heart transplantation;
biomarkers;
NTproBNP

BACKGROUND: There is a long-standing need for a noninvasive biomarker that allows monitoring of cardiac allograft rejection, avoiding the need for periodic endomyocardial biopsies (EMB).

METHODS: Multicenter, observational, prospective study, performed between 2019 and 2023 (NCT 04973943). All patients underwent 7 per-protocol surveillance EMB during the first postheart transplantation year. Donor-derived cell-free DNA (dd-cfDNA) levels were determined before each EMB, using Next Generation Sequencing Technology (Allonext assay, Eurofins Genome). The primary end-point was the association between dd-cfDNA levels and the presence of acute cellular rejection (ACR) in EMB.

RESULTS: The study included 206 patients from 12 centers, with 1,090 pairs of EMB/dd-cfDNA determinations available for analysis. EMB with ACR (n = 49) were associated with dd-cfDNA levels significantly higher than those without, median 0.189% (interquartilic range 0.05-0.70) vs 0.095% (0.04-0.23), p = 0.013. A dd-cfDNA threshold of 0.10% showed a negative predictive value for ACR of 97%. A statistically significant association between N-terminal prohormone of brain (NTProBNP) and dd-cfDNA

Reprint requests: Marta Jiménez-Blanco, MD, Hospital Universitario Puerta de Hierro, C/Manuel de Falla s/n, 28222 Majadahonda, Madrid, Spain.
E-mail address: martajbb@gmail.com.

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was also found, with an increase of 0.007% dd-cfDNA (95% confidence interval 0.003-0.011) for every 500 units of NTproBNP, p 0.001. The combination of both biomarkers for diagnosis of ACR showed an area under the receiver operating characteristic (ROC) curve of 0.681, and this combined approach was significantly better than dd-cfDNA alone (area under the ROC curve 0.603), $p = 0.016$. Using a cut-off point of 0.10% for dd-cfDNA and 1,000 UI/ml for NTproBNP, negative predictive value increased to 98.1%.

CONCLUSIONS: dd-cfDNA may be a useful biomarker to rule out significant ACR in a low-risk population. However, a dd-cfDNA value above normal threshold does not correlate robustly with the presence of disease. The combination with NTproBNP, a readily available biomarker, increased the discrimination power of dd-cfDNA alone.

CLINICAL TRIAL NOTATION: Donor-derived Cell-Free DNA as a New Biomarker in Cardiac Acute Rejection, NCT 04973943.

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Background

Heart transplantation (HT) is the treatment of choice for selected patients with advanced heart failure, and, to this date, is the one that has the most positive impact on the natural history of the disease.[1] However, despite advances in modern immunosuppressive treatment, acute rejection remains a major cause of graft failure, particularly during the first post-transplant year.[2]

Endomyocardial biopsy (EMB) is the current gold standard for acute rejection surveillance, but it is an invasive and expensive procedure with many setbacks, including high interobserver variability,[3] potential complications, and significant patient discomfort.[4] Therefore, a noninvasive surveillance method that could allow early diagnosis, monitoring of rejection, and eventually reduce the need of EMB would be of great clinical interest.

Cellular contents, including donor-derived DNA, are released when acute rejection leads to damage of graft cells.[5] In this context, donor-derived cell-free DNA (dd-cfDNA) could potentially serve as a very specific biomarker for allograft injury.[6-9] Several studies have already shown promising results in this sense.[10-12]

The main objective of FreeDNA-CAR study (Donor-derived Cell-Free DNA as a New Biomarker in Cardiac Acute Rejection) was to determine if there is a significant association between dd-cfDNA levels and the presence of acute cellular rejection (ACR) as determined by EMB during the first post-transplant year, using a novel Next Generation Sequencing (NGS) Technology test (Allonext assay, commercially available in Europe).

Methods

Design

FreeDNA-CAR was a prospective, multicenter, observational study. All consecutive patients who underwent HT in 1 of the 12 participating centers between April 2019 and April 2021 were offered to participate in the study.

The study was registered later at ClinicalTrials.gov (NCT 04973943) in April 2021, after the patients had been fully enrolled but before any analyses were conducted. The delay in registration was a result of the challenges experienced during the COVID-19 pandemic.

Exclusion criteria included age under 18 years, patients with moderate or severe primary graft dysfunction (according to International Society of Heart and Lung Transplantation 2014 criteria[13]), patients under mechanical ventilation or in need of renal substitution therapy at 30 days post-transplant, cardiomyopathy that could potentially recur in the transplanted organ (amyloidosis, Chagas disease, or cardiac sarcoidosis, among others) or multiorgan transplant.

According to study protocol, all patients underwent routine EMB on day 15, and at months 1, 2, 3, 4, 6, and 12 post-transplant (Figure 1). Patients with an EMB with ACR ≥2R underwent a control EMB 12 ± 4 days later, but these EMB were excluded from our analysis. Demographic and clinical data were collected for each patient.

A blood sample for determining dd-cfDNA levels was obtained on the same day before each EMB,[14] along with blood tests that included N-terminal prohormone of brain natriuretic peptide (NTproBNP), renal function, and cytomegalovirus (CMV) quantification. An echocardiogram was also performed in each visit. Moreover, at months 1, 6, and 12, patients were tested for anti-human leukocyte antigen (HLA) antibodies, both donor-specific and non–donor-specific (Luminex assay). The performance of a coronary angiogram at month 12 was encouraged, but not mandatory. Figure 1 shows an outline of the protocol.

The investigation conforms with the principles outlined in the Declaration of Helsinki. The study was approved by the local Institutional Review Board and all patients signed informed consent.

End-points

The primary end-point was the presence of significant ACR on EMB, defined as ACR ≥2R according to International Society of Heart and Lung Transplantation 2005 classification,[15] and its association with dd-cfDNA levels.

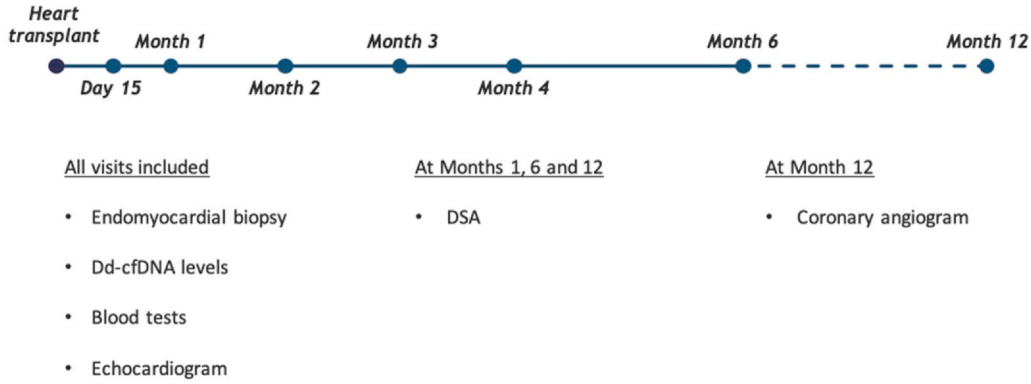

Figure 1 Study protocol. dd-cfDNA, donor-derived cell-free DNA. DSA, donor-specific antibodies

Secondary end-points included the association between dd-cfDNA and clinically relevant acute rejection (a combined end-point defined as the presence of ACR ≥2R and/or antibody-mediated rejection (pAMR) grade ≥1), as well as the correlation between dd-cfDNA and NTproBNP.

Sample processing and quantification of %dd-cfDNA

Blood was collected using Streck tubes, which are validated to limit cell lysis and thus genomic DNA contamination up to 10 days in the temperature range of +6º/+30º C. Test tubes were sent to a core lab facility (Eurofins Megalab, Madrid, Spain) through a shipping service immediately after being extracted. Once there, 2 geneticists inspected the samples to ensure they met the analysis criteria. Valid test tubes were then forwarded to Eurofins Genome's Core Laboratory in Rome (Italy) for sample processing in less than 48 hours. All laboratory staff was blinded to the patient's identity and EMB results.

The percentage of dd-cfDNA was determined with Allonext assay (Eurofins Genome). This test uses NGS technology to measure differential allele contributions in a panel of amplified single nucleotide polymorphisms (SNPs), avoiding the need to genotype the donor. An American version of this assay (known as TRAC, Viracor) has already been validated in a kidney transplant population.[16]

The assay involves a panel of over 500 SNPs, chosen for their high heterozygosity, low amplification errors, and minimal linkage, for amplification and sequencing. Cell-free DNA is extracted from 1 ml plasma and is then amplified using AmpliSeq protocol. The S5 NGS sequencer (Thermo-Fisher) is then used for sequencing the amplicons. A specialized NGS bioinformatics toolkit is employed to align the sequences to the SNP regions, identifying donor-derived sequences and determining dd-cfDNA percentages. Sequencing depth exceeds 1,000 unique reads per sample, averaging 4,000 reads. The core lab reports the donor-derived cfDNA fraction as a percentage of the total cfDNA. Average turnaround time is in the range of 72 hours.

Endomyocardial biopsies

EMBs were performed at local cath labs by experienced interventional cardiologists, who obtained 3 to 6 myocardial samples per procedure. Immunohistochemical studies (C3d, C4d, and CD68) were performed according to the pathologist's criteria or if AMR was suspected, based on histological, clinical, or immunological findings. All EMB were interpreted by an experienced local pathologist, who was blinded to dd-cfDNA results. Treating physicians were also blinded to dd-cfDNA results, and clinical decisions (e.g., treatment of rejection) were based solely on the pathological diagnosis.

Sample size calculation

The sample size calculation was carried out according to the formula previously published for this type of studies.[17] We assumed a 10% prevalence of acute rejection in the population. The main hypothesis consisted of a mean comparison between %dd-cfDNA in patients with and without acute rejection. The expected effect size based on previously published studies (mean difference between patients with and without ACR) was 1.8% and the standard deviation (SD) for the %dd-cfDNA in the population being 2%. We assumed a 0.05 type I error rate and a 0.2 type II rate. In a study with no clustering, 108 patients would be needed. However, each patient would be measured 7 times, so we assumed a 0.1 within-cluster correlation coefficient. Taking into account the cluster design, 184 patients would need to be recruited. Additionally, we assumed an 8% loss to follow-up, giving a final sample size of 199 patients.

Statistical analysis

Descriptive analysis for numerical variables was carried out through the mean and SD or median and percentiles 25 and 75 (p25; p75), depending on the variable distribution. For categorical variables, absolute and relative frequencies are shown. To test the association between the different approaches of acute rejection and levels of %dd-cfDNA, Kruskal-Wallis (for more than 2 groups) or Mann-Whitney-U tests (for 2-group comparison) were performed.

To assess the association between the levels of %dd-cfDNA and ACR considering that every patient has different biopsies along the time of follow-up, a generalized estimating equation (GEE) approach was followed.[18] We used a generalized linear model with a logit link and fitted the model using GEE. The correlation structure was assumed to be first-order autoregressive AR(1). The dependent variable was the presence or

not of ACR at each time point of follow-up (family binominal). The discriminative ability of %dd-cfDNA was tested utilizing the area under the receiver operating characteristic (ROC) curve (AUC) from the predicted values obtained in the generalized linear model with a logit link. We used the roctab command in Stata. The points on the nonparametric ROC curve are generated using each possible outcome of the diagnostic test as a classification cutpoint and computing the corresponding sensitivity and 1 – specificity. These points are then connected by straight lines, and the area under the resulting ROC curve is computed using the trapezoidal rule. The AUC ranges between 0.5 and 1, with higher values indicating better discrimination between patients who are high risk and low risk of ACR.

Finally, we developed a multivariable regression model by adding the NTproBNP to the %dd-cfDNA as independent variables to evaluate whether the 2 variables improved the discriminative ability of the %dd-cfDNA variable alone. For this purpose, we used an integrated discrimination improvement analysis to compare the discriminative ability between the 2 models by summarizing the extent to which the 2-variables model increased the risk in ACR and decreased risk in non-ACR. Significance level was set at 0.05. Statistical analysis was performed using Stata/IC software v18. (StataCorp. 2023. Stata Statistical Software: Release 18. StataCorp LLC, College Station, TX).

FreeDNA-CAR was an investigator-driven study, and the sponsor's role was solely to provide free dd-cfDNA determinations. Eurofins Megalab was not involved in the design or in the conduct of the study, nor did they have access to the database or data analysis. The original manuscript was written by M.J.B. and J.S.C. (first and last authors) and it was then reviewed by all participating authors.

Results

Between April 2019 and April 2021, a total of 316 patients underwent heart transplants in the 12 participating centers, of whom 206 were included. Mean recipient age was 53.7 years (SD 11.6 years), the majority were male (74%) and Caucasian (90%). The most frequent cause for transplant was ischemic cardiomyopathy (33%), followed by idiopathic/familial cardiomyopathy (29%) and hypertrophic cardiomyopathy (13%). Almost two-thirds of patients (64%) were transplanted on an elective list, and 39% had a ventricular assist device before HT. Table 1 shows the main donor and recipient demographic characteristics, as well as surgery-related factors. Fifteen patients died during follow-up (7.2%), 3 of them from COVID-19 pneumonia.

After excluding visits with missing data, mainly due to COVID-19 pandemic, a total of 1,090 pairs of EMB-dd-cfDNA were valid for analysis (Figure 2).

EMB results were as follows: ACR 0 was present in 606 EMB (56.4%), ACR 1 in 418 (38.9%), ACR ≥2R in 51 EMB (4.7%; of which 50 ACR 2R and 1 ACR 3R). Regarding AMR, 17 EMB revealed pAMR ≥1 (1.6%). Two samples had simultaneous ACR ≥2 and pAMR ≥1. These results and the corresponding dd-cfDNA levels are grouped in Table 2 for comparison.

Table 1 Donor, Recipient, and Surgery-Related Factors of the Free DNA-CAR population

Donor factors (n = 206)	
Mean age, years (SD)	45.2 ± 12.6
Male sex, %	59.2
Blood group, %	
O	49%
A	43%
AB	1%
B	7%
Cause of death, %	
Stroke	40%
Head trauma	22%
Other	38%
Recipient factors (n = 206)	
Mean age, years (SD)	53.7 ± 11.6
Male sex, %	74%
Mean body mass index, kg/m^2, median (IQR)	26.1 (23.9-28.6)
Race or ethnicity, %	
Caucasian	90%
Latin-American	6%
Others	4%
Blood group, %	
O	43%
A	44.5%
AB	3%
B	9.5%
Previous cardiomyopathy, %	
Ischemic cardiomyopathy	33%
Idiopathic/familial cardiomyopathy	29%
Hypertrophic cardiomyopathy	13%
Congenital heart disease	4%
Valvular	3%
Retransplant	1%
Other (restrictive, cardiotoxicity, etc.)	17%
Heart transplant waiting list, %	
Urgent	36%
Elective	64%
Presensitized patients, %	8.4%
Ventricular assist device pre-HT, %	39%
Surgical factors	
Procurement with standard cold storage, %	100%
Median bypass time, min, median (IQR)	120 (100-141.5)
Median total ischemia time, min, median (IQR)	190 (136-240)

Abbreviations: HT, heart transplantation; IQR, interquartilic range; SD, standard deviation.

Median values for each ACR and AMR group are shown in Figures 3A and 3B, respectively (dd-cfDNA log-transformed to reduce the skewness and facilitate data visualization).

Primary end-point: Acute cellular rejection

Patients with clinically relevant ACR had dd-cfDNA levels significantly higher than those without, median 0.189% (interquartilic range 0.05-0.7) vs 0.095% (0.04-0.23) respectively, $p = 0.013$ (Figure 4).

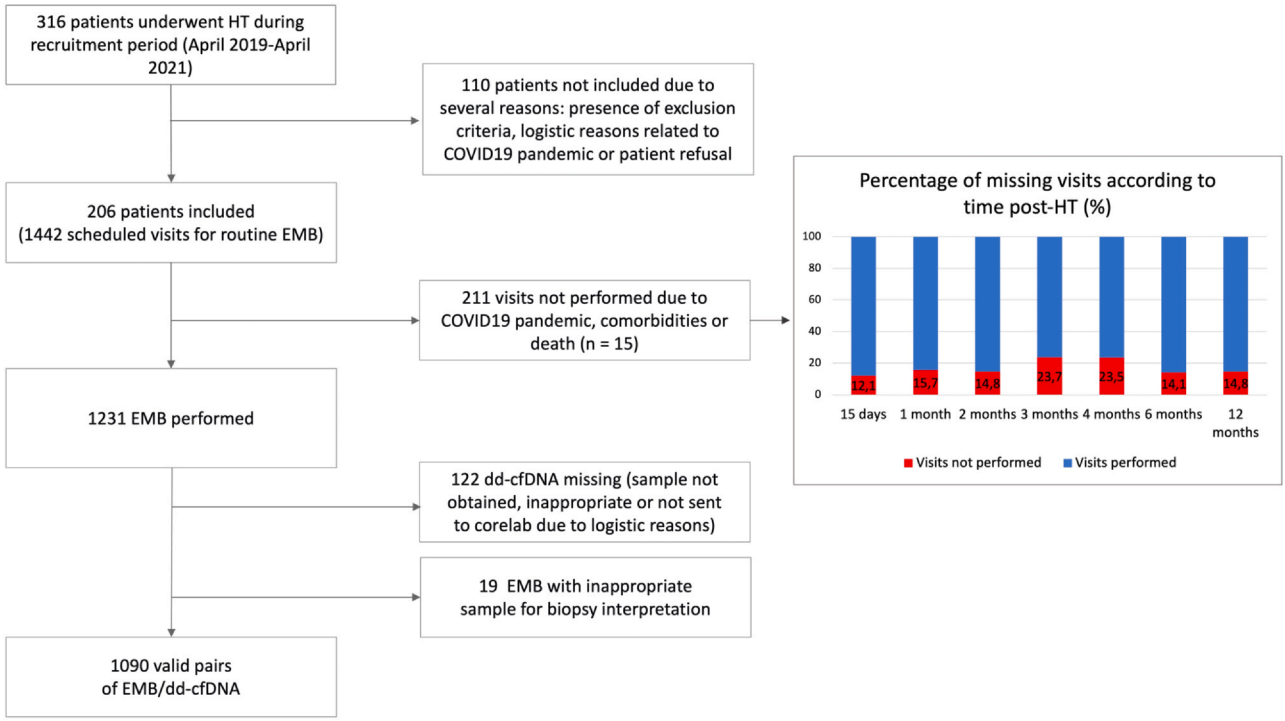

Figure 2 Study flowchart. dd-cfDNA, donor-derived cell-free DNA; EMB, endomyocardial biopsies; HT, heart transplantation.

Table 2 Endomyocardial Biopsies Results and Median dd-cfDNA for Each Category ($p = 0.0183$ for the Overall Comparison)

	No rejection (ACR 0-1 and AMR 0)	Acute cellular rejection (ACR ≥2R and pAMR 0)	Antibody-mediated rejection (pAMR ≥1 and ACR 0)	Mixed rejection (ACR ≥2 and pAMR ≥1)
n (%)	1,024 (93.9%)	49 (4.5%)	15 (1.4%)	2 (0.18%)
Median dd-cfDNA values (IQR)	0.0945 (0.04-0.23)	0.16 (0.048-0.6)	0.146 (0.06-0.56)	1.365 (0.43-2.3)

Abbreviations: ACR, acute cellular rejection; AMR, antibody-mediated rejection; dd-cfDNA, donor-derived cell-free DNA; IQR, interquartilic range.

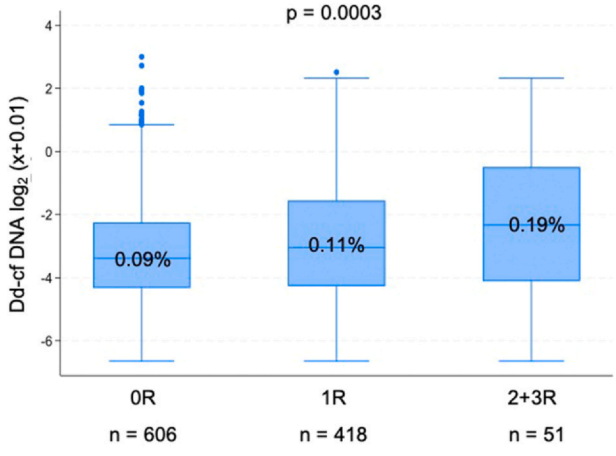

Figure 3A Median donor-derived cell-free DNA values in each ACR group ($p = 0.0003$). dd-cfDNA values are log-transformed to reduce skewness and facilitate data visualization. ACR, acute cellular rejection; dd-cfDNA, donor-derived cell-free DNA.

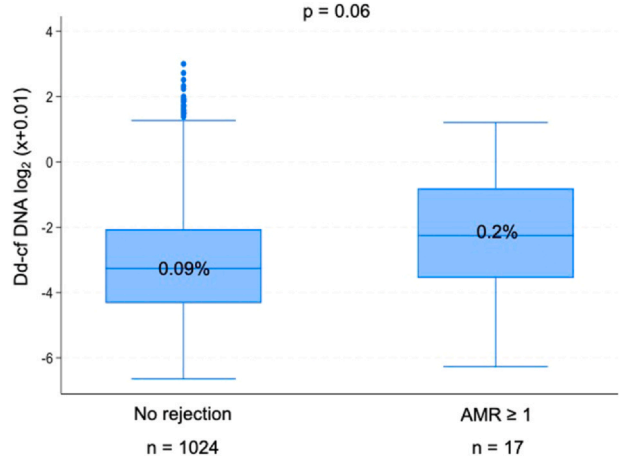

Figure 3B Median donor-derived cell-free DNA values in each AMR group ($p = 0.06$). dd-cfDNA values are log-transformed to reduce skewness and facilitate data visualization. AMR, antibody-mediated rejection; dd-cfDNA, donor-derived cell-free DNA.

Using GEE approach to account for repeated individual measures, a trend to a significant association between dd-cfDNA (%) and rejection was found (odds ratio 1.35, 95% confidence interval (CI) 0.99-1.84), with borderline statistical significance ($p = 0.054$).

The area under the ROC curve for the primary end-point was 0.603 (95% CI 0.513-0.692). The optimal cut-off point

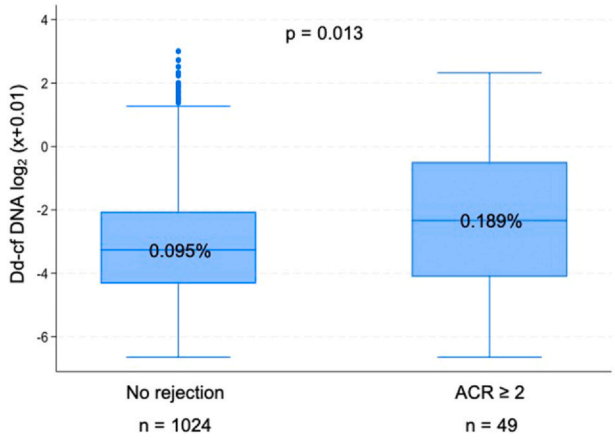

Figure 4A Median dd-cfDNA values in patients with and without clinically relevant ACR ($p = 0.013$). dd-cfDNA values are log-transformed to reduce skewness and facilitate data visualization. ACR, acute cellular rejection; dd-cfDNA, donor-derived cell-free DNA.

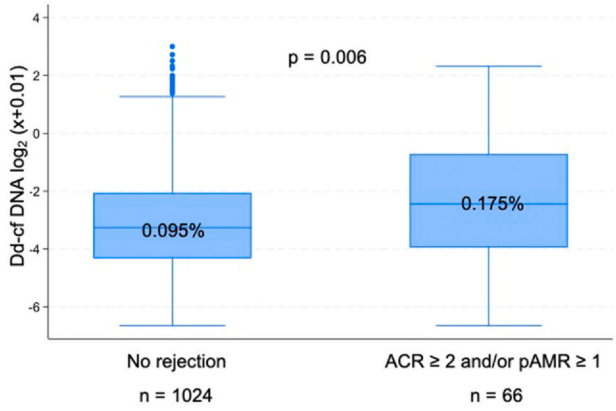

Figure 4B Median dd-cfDNA values in patients with and without clinically relevant rejection (ACR ≥2 and/or pAMR ≥1), $p = 0.006$. dd-cfDNA values are log-transformed to reduce skewness and facilitate data visualization. ACR, acute cellular rejection; dd-cfDNA, donor-derived cell-free DNA.

was a dd-cfDNA threshold of 0.10%, which revealed a negative predictive value (NPV) of 97%, a positive predictive value of 6.4%, a sensitivity of 67%, and a specificity of 51%.

Influence of time postheart transplant

The probability of rejection varied in each visit and was significantly higher at 15 days, 3 months, and 4 months post-HT (Figure 5). However, using GEE models, no interaction was found between dd-cfDNA and time post-HT.

Secondary end-points

Clinically relevant rejection

Patients with clinically relevant rejection (ACR ≥2 and/or pAMR ≥1; $n = 66$) had significantly higher dd-cfDNA values than those without, 0.175% (0.06-0.6) vs 0.095% (0.04-0.23) respectively, $p = 0.006$. Area under the ROC curve for the combined end-point was 0.609 (95% CI 0.53-0.688).

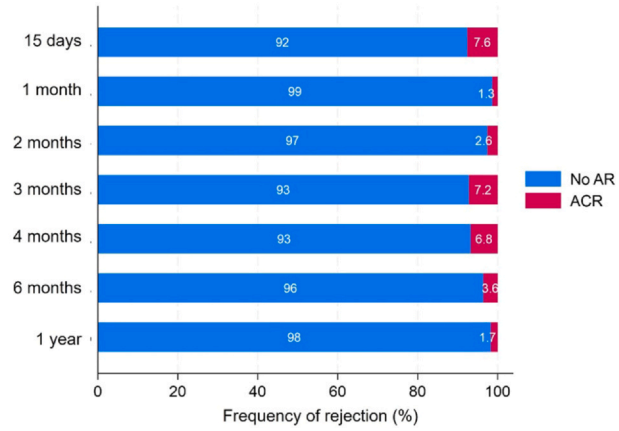

Figure 5 Percentage of biopsies with rejection, according to time postheart transplant. ACR, acute cellular rejection; AR, autoregressive.

Donor-derived cell-free DNA and NTproBNP

A total of 951 sets of EMB/both biomarkers were available for analysis. Median interquartilic range NTproBNP values for each ACR group were: 0R 1,041 pg/ml (450-2,450), 1R 1,260 pg/ml (535-3,009), and 2R-3R 2,032 pg/ml (919-6,645), $p < 0.001$ (Figure 6A). Regarding AMR, median NTproBNP in pAMR 0 was 1,110 pg/ml (481-2,696) and in pAMR ≥1, 2,230 (806-4,257), $p = 0.0581$ (Figure 6B).

Using a GEE approach to account for repeated measures in each individual, a statistically significant association between both biomarkers was found, with a coefficient of 0.007 (95% CI 0.003-0.011), meaning that for every increase of 500 units of NTproBNP, dd-cfDNA (%) increased in 0.007, $p < 0.001$ (Figure 7).

Moreover, in a multivariable analysis, both biomarkers were independently associated with the risk of ACR, with an odds ratio 1.46 (95% CI 1.05-2.02), $p = 0.023$ for dd-cfDNA, and OR 1.02 (95% CI 1.003-1.039) $p = 0.021$ for each additional 500 pg/ml of NTproBNP.

The area under the ROC curve using the predictive probability after the 2-variable regression model with both biomarkers improved to 0.681 (95% CI 0.59-0.77). Using a

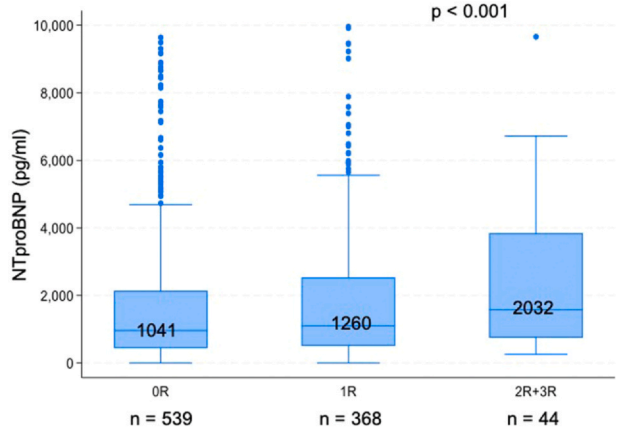

Figure 6A Median NTproBNP levels in each ACR group, $p < 0.001$ for comparison. ACR, acute cellular rejection; NTProBNP, N-terminal prohormone of brain natriuretic peptide

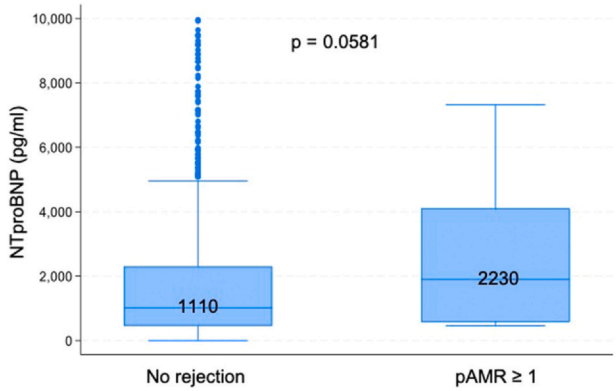

Figure 6B Median NTproBNP levels in each AMR group, $p = 0.0581$ for comparison. AMR, antibody-mediated rejection; NTProBNP, N-terminal prohormone of brain natriuretic peptide.

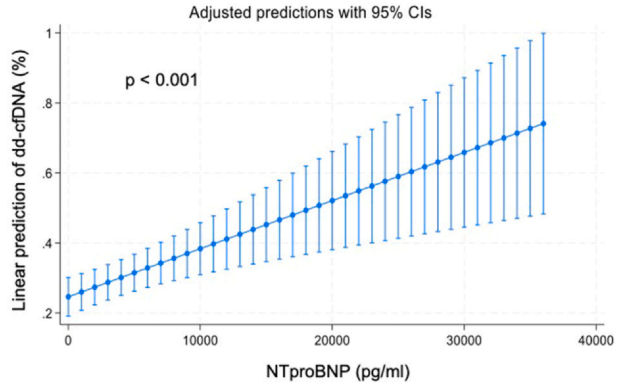

Figure 7 Linear prediction of %dd-cfDNA by NTproBNP. dd-cfDNA, donor-derived cell-free DNA; NTProBNP, N-terminal prohormone of brain natriuretic peptide.

cut-off point of 0.10% for dd-cfDNA and 1,000 UI/ml for NTproBNP, negative predictive value increased to 98.1% (Table 3). To test if the 2-variable model had a better discriminative ability than the dd-cfDNA (%) alone, we estimated the integrated discrimination improvement, resulting the combined approach being significantly better than dd-cfDNA alone, $p = 0.016$.

A total of 893 echocardiograms were performed. The incidence of graft dysfunction (defined as left ventricular ejection fraction < 50%) was extremely low during follow-up (1.7%), and the relationship with dd-cfDNA could not be established ($p = 0.11$). Similarly, significant CMV infection (defined as CMV copies > 1,000 UI/ml) was very infrequent (3.5% of all CMV determinations), and there were no statistically significant differences in dd-cfDNA levels between patients with CMV copies < 1,000 UI/ml or ≥1,000 UI/ml (0.094% vs 0.1375%, respectively, $p = 0.16$). In both cases, this data should not be interpreted as an absence of relationship, because the incidence of both events was very low and our study was not powered to detect significant differences in these variables.

Discussion

The present study reports the performance of a novel dd-cfDNA test (Allonext assay) in a cohort of HT patients. Our results show a significant difference in the biomarker levels between patients with and without ACR, and a high negative predictive value for a cut-off value of 0.10%. These findings align with those of previous studies using various dd-cfDNA tests. This supports the biomarker's role as a noninvasive tool for rejection surveillance in HT recipients, potentially reducing the number of EMB procedures currently performed.

The 3 main studies published to date in this field are summarized in Table 4 for comparison.[10-12] The DOAR trial included 841 dd-cfDNA determinations paired with EMB from 443 patients > 55 days post-HT. Median dd-cfDNA values in no rejection samples were 0.07% vs 0.17% in acute rejection (including both ACR and AMR, $p = 0.005$), with an AUC of 0.64. A cut-off value of 0.2% showed an NPV of 97%.[10] The similarities with our study in terms of rejection incidence, median values of the biomarker, AUC, and NPV are remarkable.

The GRAfT study, which included prospective and retrospective patients, explored dd-cfDNA performance using shotgun sequencing, a technique that requires donor and recipient genotyping. The test revealed an excellent discrimination between no rejection and acute rejection (0.03% vs 0.38%), with an NPV of 99% for a cut-off point of 0.25%.[11] These potentially superior results are counterbalanced by the fact that this method is more expensive, has a higher turnaround time, and is less applicable in practice.

The most recent study by Kim et al, the DEDUCE study (Prospera test, by Natera), included 811 samples from 223 patients (both retrospective and prospective): dd-cfDNA was significantly higher in AR (0.58%) compared to non-AR (0.04%). A cut-off point of 0.15% provided a 97.3% NPV. They did not include any samples from the first 4 weeks post-HT, and both surveillance and for-cause biopsies were included.[12]

The study by Richmond et al, which combined adult and pediatric patients, also shows good performance of the biomarker.[19] However, their definition of acute rejection includes ACR ≥1, and therefore the results are not comparable.

Table 3 Test Performance Characteristics of dd-cfDNA and dd-cfDNA/NTproBNP for ACR

	Sensitivity	Specificity	Positive predictive value	Negative predictive value
dd-cfDNA < 0.10%	67%	51%	6.4%	97%
dd-cfDNA < 0.10% and NTproBNP < 1,000 UI/ml	88%	28%	5.2%	98.1%

Abbreviations: ACR, acute cellular rejection; dd-cfDNA, donor-derived cell-free DNA; NTProBNP, N-terminal prohormone of brain natriuretic peptide.

Table 4 Main Results of dd-cfDNA Studies Comparing Biomarker's Levels in Patients With No Rejection vs Acute Rejection, Defined as ACR ≥2 and/or pAMR ≥1

	Assay	Number of pairs dd-cfDNA/EMB	Study design	Percentage of EMB with acute rejection (%) (ACR, AMR)	% no rejection vs acute rejection (%)	Cut-off point (%)	NPV (%)
DOAR[10]	Allosure (Targeted NGS)	841	Prospective	4.1% (2.0, 2.1)	0.07 vs 0.17	0.2	97
GRAfT[11]	Shotgun sequencing[a]	1141	Prospective	5.2% (2.9, 2.5)	0.03 vs 0.38	0.25	99
DEDUCE[12]	Prospera	811	Prospective and retrospective	6.0% (2.1, 3.9)	0.04 vs 0.58	0.15	97.3
FreeDNA-CAR	Allonext	1090	Prospective	6.0% (4.7, 1.6)	0.095 vs 0.175	0.10	97

Abbreviations: ACR, acute cellular rejection; AMR, antibody-mediated rejection; dd-cfDNA, donor-derived cell-free DNA; EMB, endomyocardial biopsies; NPV, negative predictive value.
[a]Requires donor and recipient genotyping.

There are some differences between our study design and previously published studies. First of all, our study only included prospective patients, all of whom underwent per-protocol EMB and dd-cfDNA sampling on the same day. Second, we included patients earlier after HT than in any other study, and all of our dd-cfDNA determinations have a paired biopsy. Last, it is one of the largest studies performed so far, with a total of 1,090 biopsy-paired samples available for analysis. Moreover, dd-cfDNA samples were all extracted on the same day of EMB, but before it, as various studies have already shown that the procedure can itself elevate the biomarker levels.[14,20]

Our cohort was an average-risk cohort. The prevalence of AR varies across the different studies, but most studies report an overall incidence of rejection between 2% and 6%.[10-12] However, AMR was much more infrequent in our cohort than in other studies (Table 3). We had a very low percentage of presensitized patients, and none of our patients developed DSA during the study. This low prevalence of AMR could potentially explain why dd-cfDNA was not significantly higher in AMR patients in our cohort (although there was a statistical trend to significance), most likely due to small sample size.

Each of the aforementioned studies used a different dd-cfDNA testing technique, from whole genome sequencing (that requires genotyping donor and recipient) in the GRAfT study to targeted NGS (that does not require to genotype the donor) in the DOAR (Allosure) or the DEDUCE trial (Prospera). The study by Richmond et al also used NGS, but with a different assay ($myTAI_{HEART}$) that is no longer commercially available.[19] Our assay (Allonext) uses NGS, but with a different number of SNPs than Allosure or Prospera, which could potentially explain the small differences seen in dd-cfDNA values in rejection and nonrejection patients and in the AUC ROC curve. However, both standard (Allosure) and expanded (Prospera) SNP dd-cfDNA tests have recently shown to be similar in their testing accuracy for significant AR.[21] Our AUC ROC curve is similar to the one published in DOAR, and the fact that we could potentially eliminate half of the currently performed EMB is already significant. However, the positive predictive value in our study is low, and discrimination between the various subtypes of rejection is poor.

NTproBNP subanalysis

Most NTproBNP studies in this field have shown significantly higher levels of the biomarker in patients with acute rejection.[22-27] Nonetheless, the discriminating power to detect clinically significant episodes of rejection in some brain natriuretic peptide (BNP) studies was low,[28,29] and based on this, a recent consensus document by the European Society for Organ Transplantation does not recommend the use of BNP/NTproBNP to rule out rejection.[30]

However, our study shows that NTproBNP is significantly correlated with the presence of both ACR and AMR, and that it is independently associated with dd-cfDNA levels. The combination of both dd-cfDNA and NTproBNP revealed a better discriminating power than dd-cfDNA alone. Even though the

improvement in the NPV was modest (from 97%-98%), NTproBNP is a commonly accessible and affordable biomarker that could be easily included in follow-up protocols.

This idea of a "combined biomarker approach" is not new, and it is evident that the interest in this field is steadily increasing.[31,32] Most studies have focused on the combination of gene expression profiling (GEP) and dd-cfDNA.[33] A single-center, retrospective analysis of adult heart transplant recipients compared post-transplant rejection surveillance with GEP alone vs GEP and dd-cfDNA, and found that the latter was associated with similar survival and rejection-free survival at 1 year while requiring significantly fewer biopsies.[34] More recently, the SHORE Registry revealed that the likelihood of detecting ACR following molecular testing was greatest among patients who tested positive for both dd-cfDNA and GEP.[35] This dual non-invasive testing strategy was also associated with fewer biopsies during follow-up, with low incidence of graft dysfunction over time. Finally, the Trifecta-Heart study investigated the correlations between genome-wide molecular changes in EMB and dd-cfDNA, and found that dd-cfDNA correlates with molecular rejection but also with injury and macrophage infiltration.[36]

Our study is the first to explore the combination of NTproBNP and dd-cfDNA, with very positive results. This approach could be very useful in clinical practice, as NTproBNP is a widely available and inexpensive biomarker.

Limitations

Our study has some limitations that must be taken into account. First of all, our follow-up is limited to 1 year, and even though most episodes of ACR happen during the first year post-HT, other events that happen later on (such as CAV or tumors) have not been registered.

Second, we did not ask the pathologists to describe histologic lesions differently than ACR or AMR (e.g., ischemic injury in early biopsies). Although more than 75% of biopsies were stained for AMR immunohistochemistry, this was not systematically performed in all samples. We cannot exclude that some episodes of AMR could have been underdiagnosed. In any case, this fact would result in an underestimation of the biomarker's performance.

One hundred and twenty-two dd-cfDNA samples were lost or not valid due to several reasons (sample not obtained, inappropriate, or not sent to the core lab), and could not be used for analysis. Given that these events happened randomly, we believe they did not introduce any bias in our results.

Finally, a total of 110 patients (34.8%) transplanted during the recruitment period of the study were not included. The main reasons for this were the presence of exclusion criteria, logistic reasons (mainly related to COVID-19), or patient's refusal to participate.

Conclusion

dd-cfDNA may be a useful biomarker to rule out significant ACR in a low-risk population. Moreover, the addition of NTproBNP, a readily available biomarker, increases the discrimination power of dd-cfDNA alone for excluding rejection. However, a dd-cfDNA value that exceeds a normal threshold does not correlate robustly with the presence of disease. Prospective randomized clinical trials are needed to determine if a dd-cfDNA surveillance strategy, alone or in combination with other biomarkers, is noninferior to EMB for acute rejection monitoring in HT patients.

Author contributions

Marta Jiménez-Blanco: study design, acquisition and interpretation of data, data analysis, writing of manuscript. Maria Generosa Crespo-Leiro, Maria Dolores García-Cosío Carmena, Manuel Gómez Bueno, Raquel López-Vilella, Carlos Ortiz-Bautista, Marta Farrero-Torres, Isabel Zegrí-Reiriz, Beatriz Díaz-Molina, Elena García-Romero, Diego Rangel-Sousa, Nahikari Salterain, and Iris Garrido Bravo: study design, acquisition and interpretation of data, reviewing the manuscript. Javier Segovia-Cubero: study design, acquisition and interpretation of data, and writing and reviewing the manuscript.

Disclosure statement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.healun.2024.11.009.

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