Monitoring of plasma circulating donor DNA reflects cardiac graft injury: Report of two cases

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Abstract. The current standard for graft rejection surveillance is endomyocardial biopsy (EMB), an invasive procedure with rare but potentially serious complications. Detection of circulating donor-derived cell-free DNA (ddcfDNA) is an option for noninvasive monitoring of graft injury and rejection. A 63-year-old man and a 65-year-old woman were monitored by EMB for allograft rejection. A total of 48 single-nucleotide polymorphisms with a minor allele frequency range of 0.4-0.5 were screened to distinguish donor and recipient DNA based on homozygosity, and digital droplet PCR was used to analyze ddcfDNA concentrations. Both subjects suffered rejection within the first 6 months after transplantation. The maximal ddcfDNA level of 270 copies (cp)/ml during EMB-confirmed acute cellular rejection (ACR; mild grade 1R/2, patient 1), and the maximal concentration of 1,846 cp/ml in the case of EMB-confirmed antibody-mediated rejection (AMR; grade 1+; patient 2), was detected. Individual monitoring of ddcfDNA dynamics from the 1st to the 6th month posttransplant reflected cardiac graft injury in patients suffering ACR or AMR, meaning that ddcfDNA may serve as a noninvasive biomarker.

Introduction

Improved surgical techniques and the development of new immunosuppressive drugs have significantly extended the survival of heart transplant (HTx) recipients. Acute rejection, which occurs in 25-32% of patients after HTx, still represents a mortality risk during the first 1-3 years following cardiac transplantation (1). The current standard for graft rejection surveillance is endomyocardial biopsy (EMB), an invasive procedure with rare but potentially serious complications (1). Therefore, the development of a new non-invasive method is required.

DNA is located mostly in the nuclei of cells, but at low concentrations, cell-free (cf)DNA is present in the plasma of all individuals (2). Donor-derived cfDNA (ddcfDNA) released into plasma from transplanted organ necrotic or apoptotic cells may serve as a non-invasive biomarker for the early detection of allograft rejection (3-5). This has been suggested for decades, but detection of a graft's DNA at the recipient's background has thus far proved challenging in terms of sufficient specificity and throughput. Different methods have been proposed [shotgun sequencing, target sequencing, digital droplet PCR (ddPCR)…] to quantify levels of ddcfDNA in recipient plasma using the detection of donor-specific genotypes (3,6,7). Changes in recipient cfDNA (e.g., due to leukopenia, leukocytosis and inflammatory illness) can affect the results of ddcfDNA fractional determination (%ddcfDNA), leading to falsely elevated or decreased results (8). This limitation may be overcome using absolute ddcfDNA quantification. The combination of fractional and absolute determination including total cfDNA is recommended for meaningful interpretation of the results. Fractional determination is possible with all mentioned methods but absolute quantification has only been validated for ddPCR (8-10). Circulating ddcfDNA has been investigated in studies of liver, kidney and heart transplants (2,3,5,11). It has been shown that immediately after engraftment, graft cfDNA reaches high values (>5% of total cfDNA). Within the first 2 weeks after transplantation, ddcfDNA typically exponentially declines to baseline levels (9). This may be used to discriminate graft injury. An abnormal non-exponential decline of ddcfDNA has been observed in certain patients due to urinary tract infections, hemodynamic problems or surgical complications (12). Episodes of rejection in heart and kidney transplants are accompanied by a significant increase of graft cfDNA (>5-fold) levels in patients without complications,

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occurring earlier than clinical or biochemical markers of rejection (4).

Patients and methods

Patient characteristics. The selection criteria in the present case report study were noncomplicated heart transplantation (HTx) and postoperative course with later EMB confirming acute cellular rejection (ACR) or antibody-mediated rejection (AMR) at the time of blood collection. The patients were subjected to standard clinical practice. The timeline for performing EMBs is presented in Table I. Patients who failed to appear at their scheduled blood draw appointment were not included in the study. Histology, immunohistochemistry and EMB were performed according standard protocols and guidelines (13-15).

Patient 1 (male; age, 63 years) underwent orthotopic HTx (OHT) in March 2016 at the Cardio Center of the Institute for Clinical and Experimental Medicine (Prague, Czech Republic) due to dilated cardiomyopathy. Standard immunosuppression was applied. The patient received induction with rabbit antithymocyte globulin (rATG; Fresenius). The maintenance therapy consisted of tacrolimus (Astellas Pharma Europe B.V., Leiderdorp), mycophenolate mofetil (Salutas Pharma) and corticosteroids (Zentiva a.s.). The patient was postoperatively treated for 10 days with Tamiflu (F. Hoffmann-La Roche Ag) due to a positive test for influenza AB in a donor. In the first EMB, there were histological findings without evidence of cellular or antibody-mediated rejection. Echocardiography showed good heart graft function and insignificant pericardial effusion, without progression. Cardiopulmonary compensation during hospitalization was without heart rhythm disturbance. Donor 1 (male; age, 36 years) had suffered from epilepsy since childhood and had suffered brain death due to post-hypoxic brain damage after an epileptic seizure.

Patient 2 (female; age, 65 years) underwent OHT in October 2017 at the Cardio Center of the Institute for Clinical and Experimental Medicine (Prague, Czech Republic) due to congenital cardiac malformation. The patient had been affected by an atrial septal defect and underwent surgical closure in 1959 and reoperation (surgical closure with bovine pericardial patch and tricuspid valve plastic surgery) in 2011. In 2014, the patient had been hospitalized multiple times due to right-sided heart failure. In 2016, treatment with sildenafil (Mylan) was started because of the detection of significant pulmonary arterial hypertension. In March 2017, the patient was added to the waiting list for HTx. The patient suffered from comorbidities such as permanent atrial fibrillation, diabetes mellitus and dyslipidemia. Up to the 7th postoperative day, the patient was supported with inotropic milrinone for right-sided decompensation due to problematic borderline diuresis with high furosemide (FSM; Zentiva a.s.) support/preoperatively administered FSM at a dose of 1 g per day for right-sided failure, without the need for an elimination method. The patient received the same induction with rATG (Fresenius) and maintenance therapy as patient 1. Corticoid-induced deterioration of diabetes was compensated for by insulin therapy. The first three EMBs showed histological findings without evidence of cellular or antibody-mediated rejection. Echocardiography showed a good heart graft with maximum moderate tissue relaxation with inversion recovery and mild right ventricular dysfunction and treated arterial hypertension. Donor 2 (female; age, 50 years) had suffered from hypertension, hypertensive encephalopathy and hypothyroidism, and brain death had occurred as a result of intracerebral hemorrhage.

DNA analysis. Aortic tissues for genomic DNA extraction were collected during OHT from donors and recipients from March 2016 to October 2017 at the Institute for Clinical and Experimental Medicine (Prague, Czech Republic). The screening of 48 single-nucleotide polymorphisms (SNPs; minor allele frequency range 0.4-0.5; Table SI) for identification of the homozygous status was performed using microfluidic chips (Fluidigm; cat. no. 48.48 IFC) on a BioMark™ system (Fluidigm). The protocol was performed according to manufacturer's recommendations stated in the Fluidigm SNP Genotyping User Guide (PN68000098 REV.18, Appendix C-SNP Type™ Assays for SNP Genotyping on the Dynamic Array™ IFCs).

Blood samples (10 ml) were collected (before their corresponding biopsy) in EDTA-containing tubes on the 10th day and at the end of the 1st, 6th and 12th month after OHT (from April 2016 to October 2018 at the Cardio Center of the Institute for Clinical and Experimental Medicine, Prague, Czech Republic), at the times when EMB was performed. Plasma was separated (centrifugation at 1,500 x g, 15 min, room temperature) within 30 min of blood collection and stored at -80˚C in RNase-/DNase-free tubes. cfDNA was extracted from 1 ml of plasma using a Plasma/Serum Cell-Free Circulating DNA Purification MIDI kit (cat. no. 55600; Norgen Biotek). DNA was eluted in a final volume of 45 μl. No artificial spike was used.

The experiment was performed according the protocol of Beck *et al* (16) with slight modifications. Preamplification of the cfDNA was conducted using the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs) with a final elution volume of 21 μl. Quantification of cfDNA after this step was performed using a Qubit 4 fluorimeter (Thermo Fisher Scientific, Inc.).

The total cfDNA concentration was determined using a droplet PCR assay. PCR oligonucleotides and fluorophore-conjugated hydrolysis probes were purchased from GeneriBiotech. ddPCR was performed using a QX200 Droplet DPCR System (Bio-Rad Laboratories, Inc.) in a volume of 20 μl containing 10 μl 2x ddPCR Supermix for probes (no dUTP) (Bio-Rad Laboratories, Inc.), 7 μl of preamplified DNA, 1.8 μl of primer mix (forward primer: 5'-TAGGCCATAATACTC TTGA-3'; reverse primer: 5'-ACTGGCATTCTAACTAGA-3'), 0.5 μl of FAM-labeled probe (5'-TTGACATTTGGCCATTTT ATAGGTCCA-3', and 0.5 μl of HEX-labeled probe (5'-TGA CATTTGGGCATTTTATAGGTCCA-3'), according to the manufacturer's instructions. The thermocycling conditions were 95˚C for 10 min and 40 cycles of 94˚C for 30 sec and 57˚C for 1 min, followed by one final step at 98˚C for 10 min. Sample analysis of each experiment was performed using QuantaSoft v1.7 SW (Bio-Rad Laboratories, Inc.). Rare event detection settings were used to calculate the positive droplet concentration. The fluorescence threshold was set manually based on validation data obtained from homozygous variants of both versions, the temperature gradient and the negativity of

Table I. Timeline for performing EMBs, routinely performed within the first year at our institute.

EMB	Time after HTx	Frequency
1st	Between 7th and 10th days	First EMB is performed on the 7th day at the earliest; then, they follow in the frequency of once every 10 days
2nd	On the 21st day	
3rd	On the 30th day	
4th and 5th	Within 2 months	Once every 14 days
6th	At the end of the 3rd month	Once a month
7th	At the end of the 6th month	3 months apart
8th	1 year	6 months apart

EMB, endomyocardial biopsy; HTx, heart transplantation.

no-template controls. All reactions were performed in duplicate. To account for the random distribution of target DNA into partitions, Poisson's statistical model was applied and the absolute quantity was calculated. Fractional abundance (%ddcfDNA) was calculated as the ratio of donor absolute quantity [copies (cp) per 1 ml] to absolute quantity of cfDNA (donor absolute quantity plus recipient absolute quantity).

Conversion of ddPCR results. The ddPCR concentration results were converted to a plasma-equivalent concentration (cp/ml) using the following equation:

$$C_{plasma} = \frac{C_{ddPCR}\ x\ V_{elution}\ x\ (V_{cfDNA} + V_{MM})}{V_{plasma}\ x\ V_{cfDNA}}$$

where C_{plasma} is the concentration of the target within the plasma in cp/ml; C_{ddPCR} is the concentration of the target within the PCR in cp/μl; $V_{elution}$ is the volume of eluent used during the cfDNA extraction step in μl; V_{cfDNA} is the volume of extracted cfDNA used in the PCR in μl; V_{MM} is the volume of all other PCR components in μl; and V_{plasma} is the volume of plasma used for cfDNA extraction in ml.

Demonstration of ddcfDNA measurement on two digital PCR instruments. For comparing the quantification of ddcfDNA, two digital PCR systems were used, namely the QX200 digital droplet PCR system (Bio-Rad Laboratories) and the Qiacuity One dPCR system (5plex; Qiagen GmbH). The measurements were conducted on samples of patient 2. The first method used for Bio-Rad is provided in the Methods section. For the second measurement, Qiacuity was used, which involved a volume of 40 μl with 10 μl of 4X Probe PCR Master Mix (Qiagen GmbH), 8.8 μl of preamplified DNA, 4 μl of 10X primer-probe mix (0.8 μM forward primer: 5'-TAGGCCATAATACTC TTGA-3'; 0.8 μM reverse primer: 5'-ACTGGCATTCTAACT AGA-3'; 0.4 μM FAM-labeled probe (5'-TTGACATTTGGC CATTTTATAGGTCCA-3') and 4 μl of 10X primer-probe mix (0.8 μM forward primer: 5'-TAGGCCATAATACTCTTGA-3'; 0.8 μM reverse primer: 5'-ACTGGCATTCTAACTAGA-3'; 0.4 μM HEX-labeled probe (5'-TGACATTTGGGCATTTTA TAGGTCCA-3') and 13.2 μl of RNAse free water, following the manufacturer's instructions. The thermocycling conditions were 95˚C for 2 min and 40 cycles of 95˚C for 15 sec and 60˚C for 30 min. Quiacuity Nanoplates 26k (Qiagen GmbH) with up to 26,000 partitions per well were used to conduct the measurements. The ddcfDNA absolute quantity and %ddcfDNA were calculated, and the Bland-Altman analysis was performed using GraphPad Prism 5 software, version 5.03 (GraphPad; Dotmatics) (17).

Results

SNP screening. SNP screening identified 10 (patient 1) and 7 (patient 2) different homozygous variants suitable for the detection of donor DNA in paired samples (Table SI). All selected SNP primers (for sequences see Table SI) were validated before measurement to eliminate false-positive droplets. Only variant rs521861 (within the *MYO5B* gene) passed validation for using the ddPCR method. Target sequences C/G within rs521861 were analyzed in plasma cfDNA of the patients.

Case reports. In patient 1, OHT was performed without any complications and the postoperative course was favorable. Echocardiographic parameters were normal except for mild dysfunction of the dilated right ventricle during the first days after Tx. Increased wall thickness was detected and was treated with intravenous (IV) pulse dose steroids. Other events were not observed during follow-up. EMB examination confirmed mild grades of ACR (1R/2) in the 1st month and (1R/1A) in the 6th month after OHT. The maximal ddcfDNA level of 270 cp/ml was detected in the 1st month after Tx. A decline in ddcfDNA to 80 cp/ml was then detected in the 6th month after OHT and 53 cp/ml (time without rejection; Fig. 1A). At the time of conclusion of the present study (October 2023), patient 1 is free of any cardiac problems and does not have any coronary graft disease.

In patient 2, the OHT was performed without any complications. Right-sided heart decompensation was treated with inotropic drugs (milrinone) until the 6th postoperative day. Echocardiography showed dilatation and dysfunction of the right ventricle and secondary tricuspid regurgitation, while all other parameters were normal. At one month after HTx, the right ventricle had a standard size and only mild dysfunction, but the wall thickness was detected to increase. EMBs were without any histological signs of acute cellular or humoral rejection at this point. In the 2nd month after transplantation, an oral pulse dose of steroids was indicated due to mild acute cellular rejection (grade 1R/2).

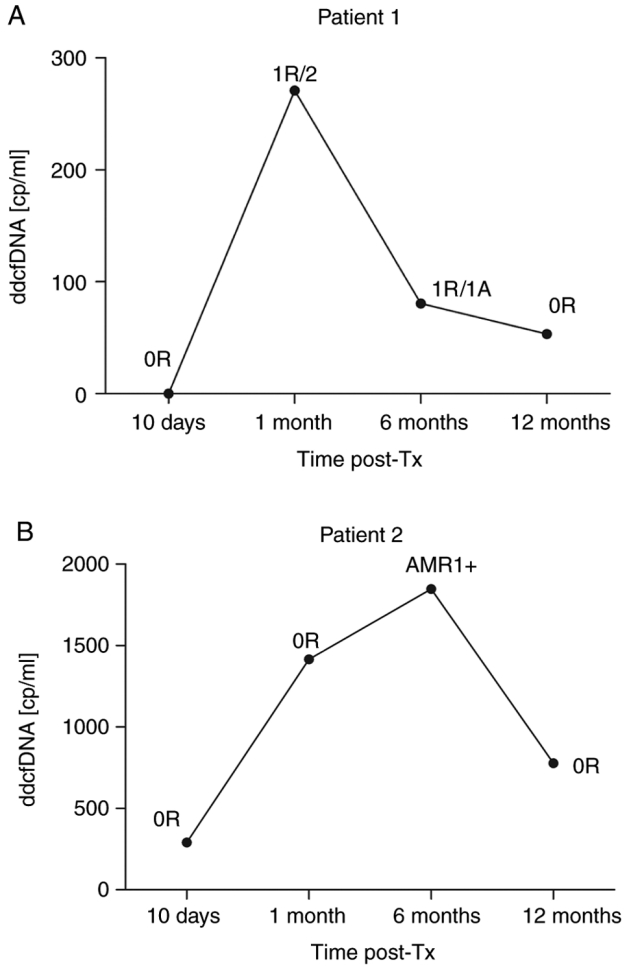

Figure 1. Quantity of ddcfDNA (copies/ml). (A) Patient 1 with confirmed ACR and (B) patient 2 with confirmed AMR. 0R, ACR and AMR grade 0; 10D, 10th day; 1M, at the end of the 1st month; 6M, at the end of the 6th month; 12M, at the end of the 12th month after orthotopic heart Tx; AMR, antibody-mediated rejection; ACR, acute cellular rejection; ddcfDNA, donor-derived cell-free DNA; cp, copies; Tx, transplantation.

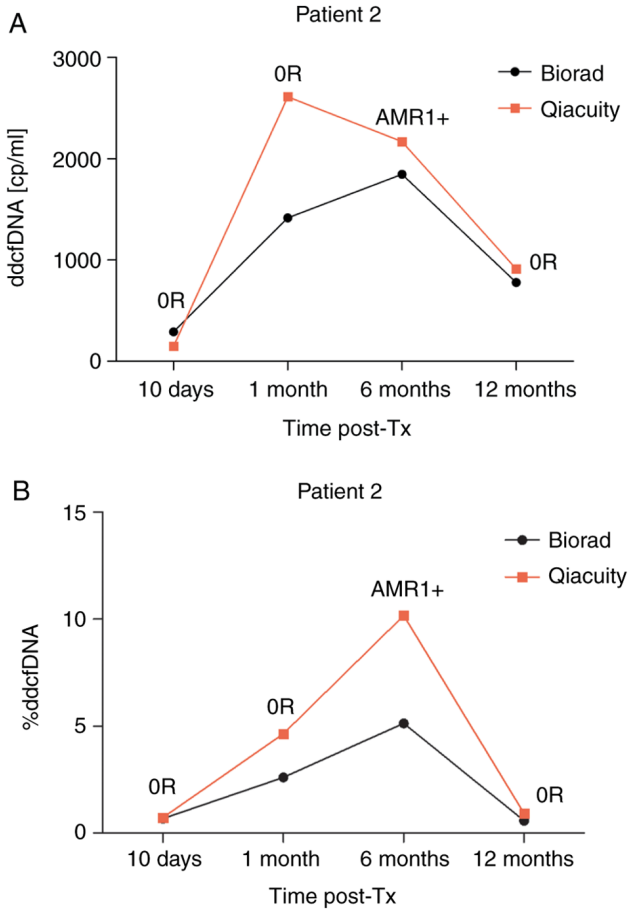

Figure 2. Comparison of ddcfDNA measurements using two different digital PCR systems. (A) ddcfDNA (cp/ml) and (B) ddcfDNA fraction (%). Differences between ddcfDNA (cp/ml) and ddcfDNA % reflect different sensitivities between instruments. 0R, ACR and AMR grade 0; 10D, 10th day; 1M, at the end of the 1st month; 6M, at the end of the 6th month; 12M, at the end of the 12th month after orthotopic heart Tx; ddcfDNA, donor-derived cell-free DNA; cp, copies; Tx, transplantation; AMR, antibody-mediated rejection.

Echocardiographic parameters were normal. EMB examination confirmed moderate-grade ACR (2R/3A) in the 3rd month after OHT, which was treated with 1,000 mg/day of IV methylprednisolone for 3 days. EMB examination at the end of the 6th month after OHT confirmed AMR grade 1+. Due to the histological findings of AMR, complement-dependent cytotoxicity (CDC) crossmatch and human leukocyte antigen antibody (HLA; Luminex® bead assay; Luminex Corp.) testing were added; this was performed according to standard procedures. The specificity of HLA antibodies was defined by LABScreen Mixed and Single Antigen class I and class II beads (One Lambda Inc.). CDC crossmatch was negative. Only non-significant positivity of anti-HLA class II antibodies (anti-DR 13, anti-DR 14 and anti-DR 17; One Lambda Inc.) was detected. In view of the above, the tacrolimus dose was increased and a higher target tacrolimus level was set. Echocardiography showed only a mild thickening of the interventricular septum (14 mm) without deterioration of ventricular function. The patient was treated with Metroprolol succinate (Astra Zeneca Spa), Perindoprilum Argininum/Amlodipinum (Servier Industries Ltd.) and FSM. This was the last time when a rejection episode was detected. Corresponding to the EMB results, a slight increase of ddcfDNA 1,416 cp/ml was identified early in the sample collected 1 month after OHT, without serious rejection. The highest value of ddcfDNA at 1,846 cp/ml was detected 6 months after transplantation when AMR grade 1+ was also detected. Subsequently, there was a decline of ddcfDNA to 777 cp/ml in the rejection-free time (Fig. 1B). At the time of conclusion of the present study (October 2023), patient 2 is in a stable clinical condition, but is classified as New York Heart Association functional class III.

After transplantation, each patient is monitored and treated until death in our institute. Regular medical follow-ups are scheduled every 6 months to assess the status and function of the graft.

Comparative analysis. The ddcfDNA measurements obtained using two different digital PCR instruments were compared. There was a similar trend in the %ddcfDNA and in the plasma quantity of ddcfDNA (Table II and Fig. 2). The slight differences in results may have been influenced by the different PCR reagents, efficiency and specificity of the instruments.

Table II. Comparison of measurements using two different digital PCR instruments.

A, ddcfDNA, %

Time post-OHT	Bio-Rad	Qiacuity	Average	Difference	Bias ± SD	95% Limit of agreement
10D	0.6772	0.731	0.704	-0.054	-1.866±2.288	-6.350 to 2.618
1M	2.6074	4.646	3.627	-2.038		
6M	5.1292	10.164	7.647	-5.035		
12M	0.5829	0.918	0.751	-0.335		

B, ddcfDNA, cp/ml

Time post-OHT	Bio-Rad	Qiacuity	Average	Difference	Bias ± SD	95% Limit of agreement
10D	291.6	147.54	219.57	144.06	-377.1±578.6	-1511 to 756.9
1M	1416.34	2612.25	2014.30	-1195.91		
6M	1846.8	2169.64	2008.22	-322.84		
12M	777.8	911.25	844.43	-133.65		

ddcfDNA fraction (%) and quantity of ddcfDNA (cp/ml) were determined by Bland-Altman analysis. Average is of the two measures (Bio-Rad and Qiacuity). Difference is between the two measures (Bio-Rad and Qiacuity). ddcfDNA, donor-derived cell-free DNA; cp, copies; 10D, 10th day; 1M, at the end of the 1st month; 6M, at the end of the 6th month; 12M, at the end of the 12th month after orthotopic heart Tx; SD, standard deviation.

Discussion

Gentle detection of acute rejection is one of the central tasks in transplantology. The standard invasive method is EMB, which is carried out using a vessel to catheterize the heart, which takes 3-4 biopsies from the right ventricle, and then graded histologically. All patients undergo at least 8-10 biopsies in the first year after transplantation, most within the first 3 months. The presence of cell-free human DNA in different body fluids gives new opportunities in the diagnosis of (not only) graft rejection. The release of ddcfDNA into recipients' blood due to myocardial cell damage makes these molecules potential biomarkers of graft health. The ddcfDNA kinetics seem to follow an L-shaped curve with high concentration immediately after transplantation, decreasing over a week to a stable level (6). CfDNA level monitoring using the newly established and more sensitive methods (such as ddPCR) may contribute to the early detection of allograft rejection.

In the present case study, the value of the ddPCR method combined with selected SNP typing for monitoring rejection was verified. The dynamics of ddcfDNA between the 1st and 6th months post-Tx reflected cardiac graft injury in both patients. A ddcfDNA fractional abundance of 0.84% was found during confirmed ACR (Fig. 3A) and 5.1% during confirmed AMR (Fig. 3B), which may well be in agreement with previous findings (18).

In patient 2, EMB examination confirmed mild- and moderate-grade ACR (1R/2 and 2R/3A) and mild right ventricular dysfunction within the time between the 1st and 6th months after Tx. Higher levels of ddcfDNA were able to reflect graft injury, as at the same time-points, increased levels of B-type natriuretic peptide (BNP); 1M=894 ng/l; 6M=402 ng/l and 12M=275 ng/l were detected. BNP is a neurohormone secreted from cardiac ventricles in response to ventricular strain (19). The BNP titer may be influenced by severe rejection episodes and diastolic dysfunction, and possibly intracardiac pressure derangement (20). Plasma BNP >90 pg/ml may serve as a marker for ventricular dysfunction. Elevated BNP was also associated with Grade ≥2 rejection (19,20).

Previous studies have described the posttransplant decay kinetics of ddcfDNA. Elevated median levels of ddcfDNA (approximately 4% in heart, 10% in renal, 26% in lung and 70% in liver Tx) within the first few days after surgery most likely reflect ischemia/reperfusion damage in the graft related to the transplant process. Within the first 10 days after Tx, in stable patients with no graft injury, the mean %ddcfDNA may decrease to a level below 0.06-10%, depending on the type of signs of transplanted organ (4,9,18,21,22). Agbor-Enoh *et al* (18) detected an increase in %ddcfDNA at 0.5 and 3.2 months before the histopathological diagnosis of ACR or AMR. Furthermore, in this study (18), the %ddcfDNA showed distinctive characteristics that varied between AMR and ACR.

In the present study, only two cases with different acute rejection were analyzed. The primary focus was on the trend of ddcfDNA in comparison to EMB results within the post-Tx time-points. However, the absence of statistical analyses may be a limitation of the current study. To identify the optimal threshold, a larger cohort needs to be measured. The limitation of this method may be the small amount of ddcfDNA in

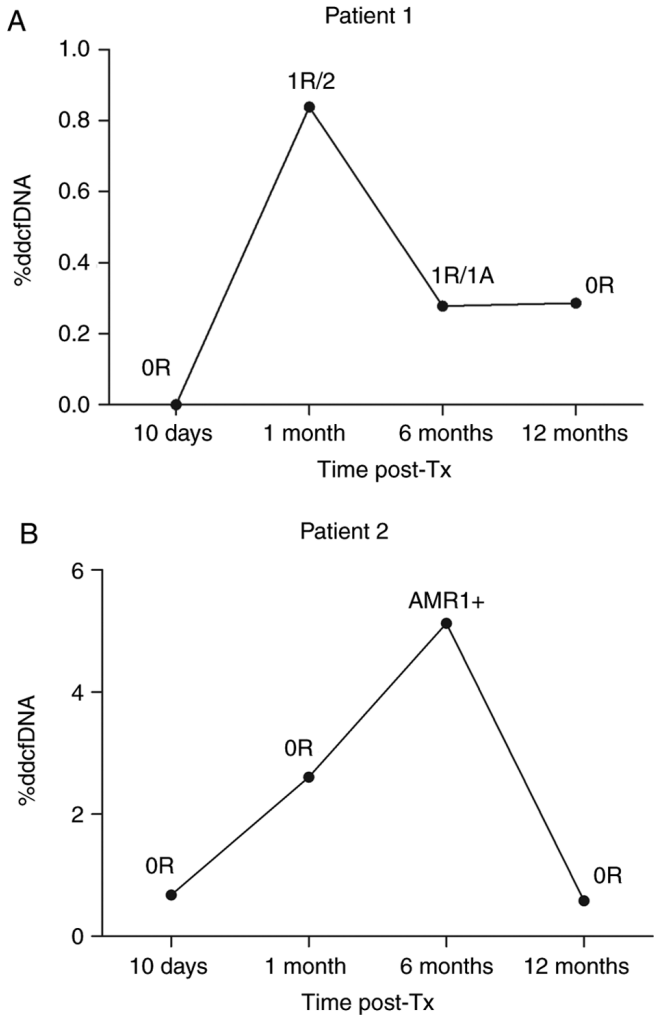

Figure 3. ddcfDNA fractional determination (%). (A) Patient 1 with confirmed ACR and (B) patient 2 with confirmed AMR. The fractional abundance (%ddcfDNA) was calculated as the ratio of donor absolute quantity (cp/ml) to absolute quantity of cfDNA (donor absolute quantity plus recipient absolute quantity). 0R, ACR and AMR grade 0; 10D, 10th day; 1M, at the end of the 1st month; 6M, at the end of the 6th month; 12M, at the end of the 12th month after orthotopic heart Tx; ddcfDNA, donor-derived cell-free DNA; cp, copies; Tx, transplantation; AMR, antibody-mediated rejection; ACR, acute cellular rejection.

plasma. In addition, increased release of recipient DNA into the bloodstream due to infections, exercise, medications or non-graft-associated vascular compromise may influence the lowering of %ddcfDNA (9). Using a preamplification step and absolute quantification (as copies per ml plasma), which are not affected by changes in recipient cfDNA, may avoid such biases.

The comparison of measurements obtained from two different digital PCRs is limited in the present study due to the lack of results from patient 1. To confirm the present findings, it is necessary to measure more genetic variants in a larger number of patients.

In the present case study, ddcfDNA was successfully measured as a marker for acute rejection in two patients. However, the present results need to undergo verification in a larger cohort to validate the reliability and generalizability of ddcfDNA as a biomarker.

In conclusion, in the present study, increased levels of ddcfDNA were detected during ongoing ACR and AMR. Individual monitoring of ddcfDNA dynamics from the 1st to the 6th- month post-Tx reflected cardiac graft injury in patients suffering ACR or AMR, meaning ddcfDNA may serve as a noninvasive biomarker.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

The original draft was written by DD and JAH. JV and DD designed and directed the study, summarized the clinical characteristics, contributed to the interpretation of the results and edited the article. ER and PH designed and performed the experiments, analyzed the data and wrote the article. DD and ER saw and verified all raw data. SN coordinated and created clinical databases. Data review was performed by JAH. All authors reviewed the original draft and approved the final version of the manuscript.

Ethics approval and consent to participate

The study was approved on 15 June 2015 (approval no. G-15-06-15) and on 12 June 2019 (approval no. G-19-37), both granted by the Ethics Committee of the Institute for Clinical and Experimental Medicine and Thomayer Hospital with Multicenter Competence in Prague, Czech Republic. Two ethics approvals are included because patients were included in two different studies. The second study, which focuses on circulating microRNA as biomarkers of graft dysfunction, has not yet been published.

Patient consent for publication

The study was performed according to the Declaration of Helsinki (2000) of the World Medical Association (24). Both patients provided written informed consent regarding analyses and examinations, as well as publication of case descriptions and results.

Competing interests

The authors declare that they have no competing interests.

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