Exploring acute cellular rejection in lung transplantation: insights from donor-derived cell-free DNA analysis

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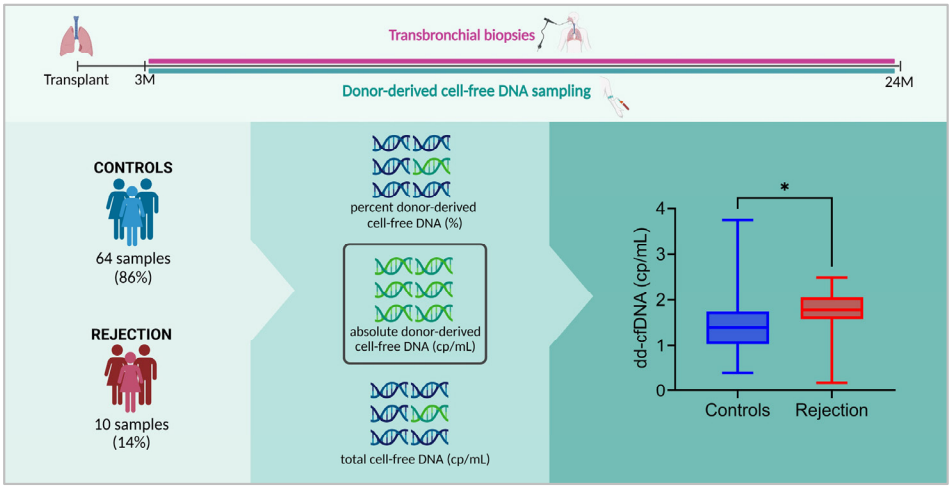
Background. Acute cellular rejection (ACR) is a frequent complication following lung transplantation, yet standardized guidelines for ACR screening remain lacking. This study aimed to compare the current gold standard for ACR evaluation – histological assessment of transbronchial biopsies – with a novel biomarker for allograft monitoring: donor-derived cell-free DNA (dd-cfDNA). Specifically, we investigated whether total cell-free DNA (cfDNA) and both the absolute and percentage values of dd-cfDNA (dd-cfDNA and dd-cfDNA%) could provide valuable insights into detecting ACR and assessing allograft health.

Methods. Patients after bilateral lung transplantation between May 2021 and March 2024 were included. Clinically significant ACR cases (ACR+) were defined as samples with histological ACR grade ≥A2 or ACR grade A1 in patients who have received antirejection therapy due to symptoms, CT findings, or lung function decline. Samples with A0 or A1 rejection in clinically stable, untreated patients were classified as controls. Measurements of dd-cfDNA%, dd-cfDNA (cp/mL) and total cfDNA (cp/mL) were obtained at the time of biopsy and compared between cohorts.

Results. The median dd-cfDNA concentration was significantly higher in the ACR+ group (61.2 cp/mL, IQR: 38.7–114.1) compared to controls (25.8 cp/mL, IQR: 10.7–65.7; *P*=0.04). However, no significant differences were observed for dd-cfDNA% and cfDNA.

Conclusion. Dd-cfDNA shows promise as a valuable tool for ruling out ACR; however, further research is necessary in order to validate its clinical utility and optimize its implementation. Its negative predictive value supports dd-cfDNA as an effective screening tool for allograft health, nevertheless, further investigation is required.

Exploring Acute Cellular Rejection in Lung Transplantation: Insights from Donor-Derived Cell-Free DNA Analyses



Dd-cfDNA shows promise as a valuable tool for ruling out acute cellular rejection – its high negative predictive value supports dd-cfDNA as an effective screening tool for allograft health, nevertheless, further investigation is required.

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INTRODUCTION

Lung transplant (LuTx) recipients face the shortest median survival among solid organ transplant recipients[1]. Continuous exposure to the outer environment leads to development of a unique and powerful immune system, resulting in higher incidence of immune-related complications, of which acute cellular rejection (ACR) is the most prevalent[2,3].

ACR is one of the primary immune-mediated etiologies of morbidity and mortality following LuTx. According to data from the International Society for Heart and Lung Transplantation (ISHLT) registry, the incidence of at least one episode of treated rejection in LuTx recipients is 28% (ref.[4]), whereas in randomized controlled trials reported rejection rates range from 44 to 57% (ref.[5-6]), with a declining trend over time[7].

The gold standard for ACR diagnosis is histopathological evaluation of a tissue obtained via transbronchial biopsies (TBB), in which rejection-related changes are assessed based on perivascular and interstitial lymphocytic infiltrates, following the consensus guidelines established by the ISHLT in 2007 (ref.[8]). However, this method does not provide any information regarding the functional status of these lymphocytes, making it impossible to distinguish regulatory lymphocyte infiltrates (favorable) from rejection lymphocytes (unfavorable).

Currently there are no standardized international recommendations for ideal frequency of screening for ACR. At our center, a proactive approach is implemented, with ACR surveillance via TBB at standardised time-points. We also perform for-cause TBB based on clinical indications, such as declining lung function, new findings on a CT scan, or newly arising subjective patient complaints, possibly indicative of the development of acute allograft rejection. Nevertheless, TBBs are not ideal for screening or diagnosis, as they have numerous limitations, such as their invasive nature, sampling errors, economic burden, and patient discomfort[9]. This further highlights an urgent need to develop sensitive, preferably non-invasive biomarkers for ACR surveillance and diagnosis.

Significant progress has been made in the field of biomarker research in recent years. As described in our previous work, tissue transcriptomics may provide superior information in comparison to standard histological evaluation of ACR (ref.[9]). Nevertheless, it still requires tissue obtained through invasive procedures. Among non-invasive biomarkers for monitoring lung allograft health, the most promising seems to be fractional donor-derived cell-free DNA (dd-cfDNA%), which represents the fraction of donor-derived cfDNA (dd-cfDNA) within the total cell-free DNA (cfDNA) (ref.[10]) (Fig. 1).

Several studies have demonstrated elevated levels of dd-cfDNA% in histologically confirmed ACR compared

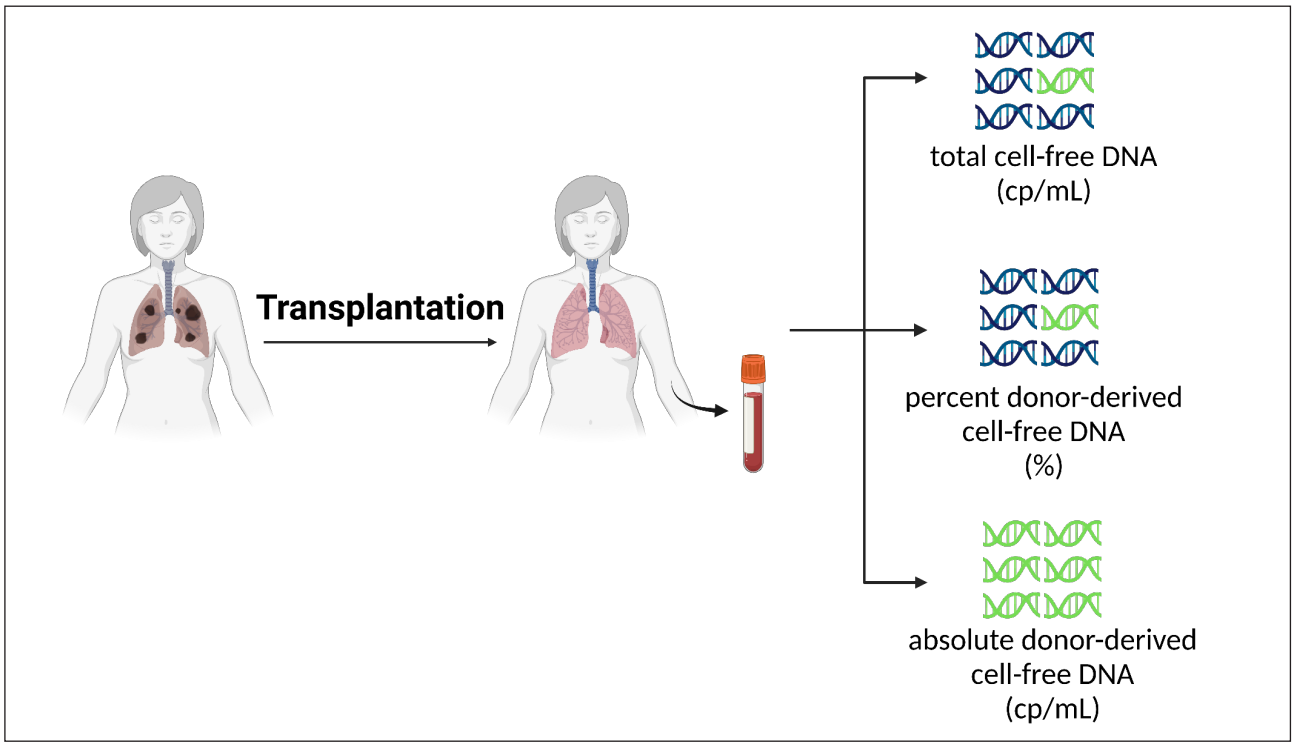

Fig. 1. Depiction of differences between total cfDNA, dd-cfDNA%, and dd-cfDNA.

to stable LuTx recipients[11-13]. Rosenheck et al. proposed a dd-cfDNA% threshold of ≥1% in combination with standard clinical ACR assessment. This approach exhibited a sensitivity of 89%, specificity of 83%, a positive predictive value of 52%, and a negative predictive value of 97%, highlighting the utility of dd-cfDNA% in ruling out ACR. However, it also showed limited specificity for diagnosing ACR (ref.[14]). The optimal dd-cfDNA% threshold for ACR diagnosis varies across different diagnostic assays, and current data remain insufficient to establish a definitive cutoff value to prompt further clinical investigation[15]. Dd-cfDNA% levels were also elevated in LuTx recipients affected by pulmonary infection complications. However, no significant difference in values allowing discrimination between infection and rejection was observed[10,14,16]. This further highlights one of the main limitations of dd-cfDNA% – its low specificity. An increase in dd-cfDNA% levels alone is insufficient for diagnosing lung allograft pathology and must be interpreted in conjunction with standard clinical assessments[10].

Moreover, expressing dd-cfDNA% solely as a percentage has notable limitations. Since nearly 55% of cfDNA is derived from white blood cells, systemic inflammation could increase overall cfDNA levels, potentially reducing dd-cfDNA% (ref.[17]). In our single-center prospective study, we aimed to evaluate the clinical utility of dd-cfDNA%, dd-cfDNA, and total cfDNA as biomarkers for distinguishing patients affected by ACR from ACR-free controls.

MATERIALS AND METHODS

Demography

All patients after bilateral LuTx who were followed up after transplantation at the University Hospital Motol between May 2021 and March 2024 were considered for inclusion in the study. These included age > 18 years, negative oncological history and informed consent. Post-transplant follow-up in another centre was considered as an exclusion criterion. The study was approved by the Local Ethics Committee (EK-500/21).

For patients included in the study, histological samples obtained by TBBs between September 2021 and July 2024 were analyzed. Evaluation of dd-cfDNA and anti-HLA antibody testing (Luminex®) were performed along with TBBs. TBBs included in the study were performed both as part of the surveillance follow-up (3, 6 and 12 months post-transplant) as well as for-cause indications depending on the clinical condition of the patient, which included deterioration in clinical status, worsening pulmonary function testing results and/or the presence of a radiological correlate. In order to avoid bias from clinically significant respiratory infection (clinical symptoms and/or requirement for anti-infective therapy given the presence of clear etiological agents) or donor-specific antibodies (DSA; cut-off for mean fluorescence intensity of 1000 for class I and 2000 for class II, as determined by the local immunogenetic laboratory), these samples were excluded from the analyses.

In the case of ACR, samples were considered to be clinically significant events (ACR+) with:

1) ACR ≥ grade A2
2) ACR grade A1 in patients who received antirejection therapy due to symptoms, CT correlate and/or decline in lung function

Samples with A0 or A1 rejection in clinically stable patients who were not treated were not considered a clinical event (controls).

These samples were combined with clinical data including laboratory findings (C-reactive protein, white blood cell count, anti-HLA antibodies, tacrolimus trough levels), microbiological findings (bronchoalveolar lavage samples obtained during TBBs) and medication records (mycophenolate mofetil doses), along with dd-cfDNA%, dd-cfDNA, and total cfDNA.

Histological evaluation of samples

TBBs were assayed in a standard 4% formaldehyde solution and then postfixed and embedded in paraffin. Paraffin blocks were cut into 4 μm sections and stained with hematoxylin and eosin, Masson's trichrome, orcein, Prussian blue and Schiff's stain. Further, immunohistochemical labelling for the following markers was used: CD45RO, CD8, CD4, CD20 and C4d. Samples were evaluated based on the 2007 ISHLT consensus (grade A0–A4) (ref.[8]).

Evaluation of cell-free DNA

Blood samples were collected between 3rd and 24th month after LuTx as part of routine post-transplant monitoring, with monthly withdrawals during the first year. Additionally, blood was drawn during TBBs, which were scheduled for surveillance or performed at the discretion of the clinician in response to factors such as declining lung function or abnormal CT findings. Consequently, the timing and frequency of blood collection varied among patients.

For each patient, two Streck tubes® (each containing 10 mL of blood) were drawn from peripheral veins and processed according to the manufacturer's guidelines to maintain stability at room temperature. The samples were then transported at room temperature to Natera (Austin, TX, USA), where plasma was separated and stored at -80°C for future batch processing. This processing included the analysis of donor-derived cell-free DNA (dd-cfDNA) fraction and an estimate of the total cfDNA concentration.

The procedure involved isolating cfDNA from plasma, followed by amplification using a highly multiplexed PCR assay targeting 13,926 single nucleotide polymorphisms (SNPs). The resulting amplicons were sequenced on the Illumina NextSeq500 platform, generating an average of 15 million reads per sample. Next-generation sequencing data, combined with SNP genotyping, were analyzed using proprietary algorithms to quantify dd-cfDNA and total cfDNA. The dd-cfDNA percentage represents the proportion of donor-derived cfDNA relative to the total cfDNA present in plasma, while the total cfDNA concentration is reported in genomic copies per milliliter (cp/mL).

Statical methods

Analyses and graphs were generated using GraphPad Prism 10.3.1®. Fisher's exact test was used for comparisons of categorical variables, and the Mann-Whitney and Kruskal-Wallis tests were used for comparisons of continuous variables.

RESULTS

Demography

Of 40 patients fulfilling inclusion criteria, a total of 91 samples were available. 74 of the TBBs (81%) were included in the study – 12 samples (13%) were excluded due to clinically significant infection and 5 (6%) based on the presence of DSA (Fig. 2). Histological examination of the samples showed grade A0 ACR in 22 (30%), grade A1 in 46 (62%), and grade A2 in 6 (8%) samples. No specimen was graded as ACR grade A3 or A4.

The group with ACR A0 or A1 without therapy (controls) included 64 samples (86%), while the treatment group (ACR+) consisted of 10 samples (14%) – 6 with A2 rejection (60%) and 4 with A1 rejection (40%) (Fig. 2). The therapeutic regimen included methylprednisolone pulses (500 mg daily for three days), followed by oral corticosteroid escalation (prednisone 50 mg, tapered by 5 mg every three days to a maintenance dose) in seven patients (70%). The remaining three patients (30%) received only oral corticosteroid escalation.

All patients diagnosed with ACR grade A2 received methylprednisolone pulses followed by oral escalation of corticoid treatment. Of the 4 patients treated for A1 rejection, 1 received methylprednisolone pulses (25%), while 3 received therapy with escalation of oral prednisone (75%). See Table 1 for more detailed demographics.

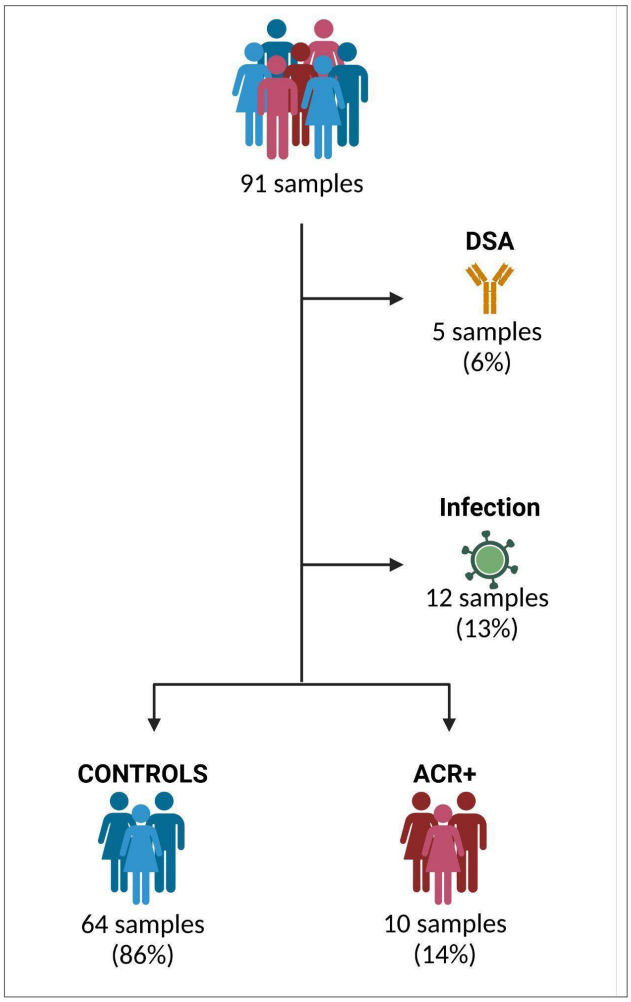

Fig. 2. Consort diagram.

Table 1. Demography of the included cohorts.

Demographics	Controls (n=64)	ACR+ (n=10)	*P*
Age at LuTx (year; median, IQR)	56.9 (42.9–63.7)	62.8 (42–64.4)	0.38
Time from Tx to TBB (months; median, IQR)	7.4 (4.7–12.3)	3.3 (3.1–7.5)	**0.006***
Female (n, %)	28 (44)	3 (30)	0.15
BMI (kg/m^2; median, IQR)	26.2 (23.3–29.9)	28.7 (24.8–31.2)	0.26
Grade 3 PGD at 72h, n (%)	5 (8)	2 (20)	**0.02***
CMV match between donor and recipient			0.66
Match, n (%)	41 (64)	6 (60)	
Mismatch, n (%)	23 (36)	4 (40)	
Diagnosis			0.89
Chronic obstructive pulmonary disease, n (%)	24 (38)	4 (40)	
Interstitial lung disease, n (%)	27 (42)	4 (40)	
Cystic fibrosis, n (%)	3 (5)	0	
Pulmonary hypertension, n (%)	10 (15)	2 (20)	
Indication for biopsy			0.14
Surveillance (n, %)	64 (100)	9 (90)	
For cause (n, %)	0	1 (10)	
Tacrolimus – trough level (ug/L; median, IQR)	12.8 (10.4–16.3)	14.6 (7.8–16.8)	0.74
Mycophenolate mofetil – daily dose (g; median, IQR)	2 (1–2)	2 (2–2)	0.15
C-reactive protein (mg/L; median, IQR)	0 (0–1.7)	0.4 (0–4.6)	0.7
White blood cells (10*9; median, IQR)	6 (4.2–8)	7.1 (6–9.7)	0.18
Creatinine (µmol/L; median, IQR)	113 (89–125)	90 (72–132)	0.3

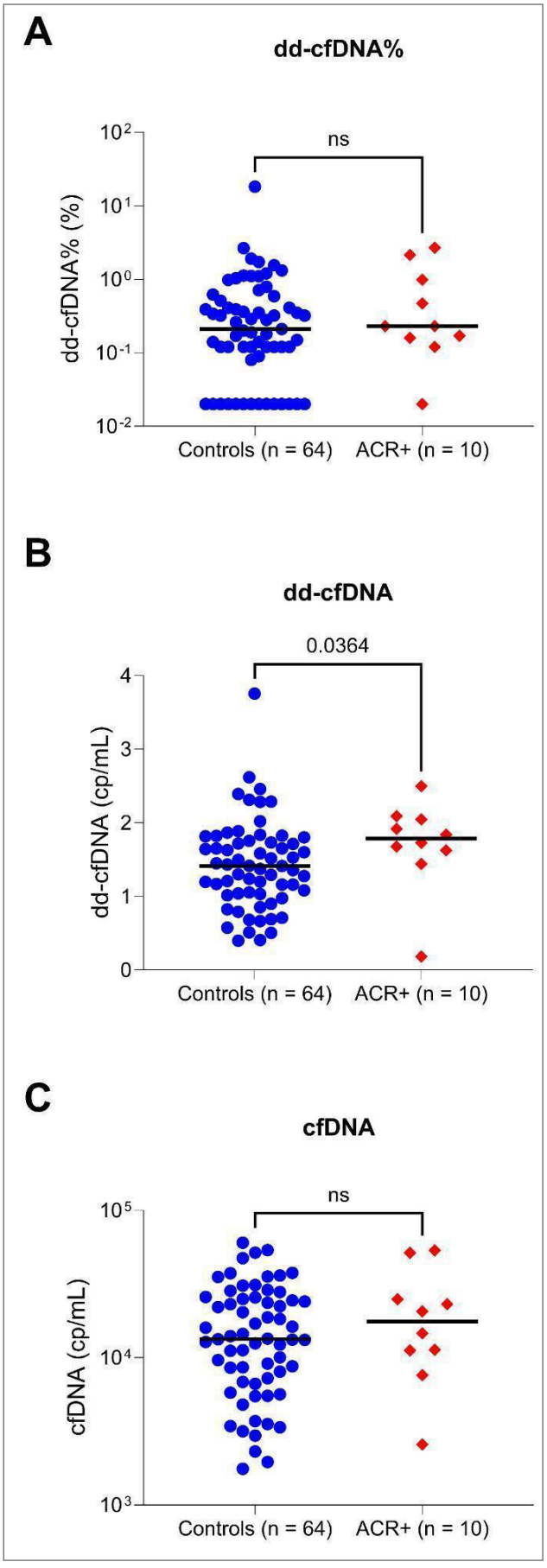

Fig. 3. Mann-Whitney analyses of dd-cfDNA% (A), dd-cfDNA (B) and cf-DNA (C) between controls and ACR+.

Evaluation of cell-free DNA

The median dd-cfDNA% for the ACR+ group was 0.23% (IQR 0.15–1.28), compared to 0.21% for controls (0.09–0.59; *P*=0.41). In terms of absolute values, the median dd-cfDNA for the ACR+ group was 61.2 cp/mL (IQR 38.7–114.1) compared to 25.8 cp/mL for controls (10.7–65.7; *P*=0.04). The median cfDNA levels were 17,621 cp/mL (10,319–31,598) and 13,404 cp/mL (6,954–25,511; *P*=0.54) for ACR+ and controls, respectively. Comparisons of the levels between ACR+ patients and controls are shown in Fig. 3.

DISCUSSION

The ability to distinguish between self and foreign is essential for all organisms in order to adequately respond to potentially harmful noxious agents. The major histocompatibility complex (MHC), which presents antigenic peptides to leukocytes, plays an important role in initiating an immune response[18]. When the recipient's immune system is exposed to a foreign donor MHC, one of the first responders are effector T-cells that recognize and destroy cells containing foreign MHC, leading to damage and loss of function of the allograft[19].

In our cohort, the incidence of treated ACR was 14%. However, if we also include untreated samples with minimal rejection (grade A1) from stable patients, the overall incidence of ACR rises to a striking 70% of samples, which is a very high figure in the context of the available data for the LuTx population. There are two possible explanations for these findings. Firstly, cryobiopsies are the standard of care at our centre, however, at most other centres, forceps biopsies are considered the gold standard. One possible reason for this difference could be that cryobiopsies provide larger tissue samples, increasing the likelihood of detecting rejection lesions. This hypothesis is supported by a publication of Steinack et al., which demonstrated that cryobiopsies enhanced the diagnostic yield for ACR, leading to reclassification and treatment strategy adjustments in 28.6% of cases[20]. Secondly, our centre uses multiple immunohistochemical markers that are not globally applied[8]. These additional markers may potentially overestimate the presence of lymphocytes, but further research is required to confirm this hypothesis.

Of the investigated biomarkers, only dd-cfDNA demonstrated its utility in discriminating between ACR+ patients and healthy controls. In terms of dd-cfDNA%, even in the ACR+ cohort, only 2 samples exceeded the threshold of 1%, which is the cutoff recommended by the European Society for Organ Transplantation consensus for the diagnosis of ACR (ref.[15]). However, these findings are inconsistent with the published literature, supporting our hypothesis of overestimation of ACR in our cohort due to the use of cryobiopsy and advanced immunohistochemical staining, which requires further analysis for confirmation[10-15].

Consistently with the available literature, the highest risk of ACR has been demonstrated in the first six months

after LuTx (ref.[4]) – the time since transplantation was significantly shorter in samples with treated ACR episodes.

In contrast, higher incidence of grade 3 primary graft dysfunction (PGD) at 72h was observed in controls – this finding might be explained by the centre's protocol of prolonged extracorporeal membrane oxygenation support in patients transplanted for pulmonary hypertension, equalling to PGD grade 3 based on ISHLT criteria[21].

Our study faces several limitations, given the modest sample size and the possible overestimation of ACR incidence. Additionally, it is crucial to note that dd-cfDNA is a calculated value rather than a direct measurement, which may introduce further sources of error. Therefore, additional validation of the methodology for quantifying cfDNA is essential.

In summary, dd-cfDNA demonstrates significant potential as a non-invasive tool for ruling out ACR. Current findings suggest that absolute dd-cfDNA values offer superior diagnostic accuracy compared to fractional measurements, likely due to their greater stability and reduced susceptibility to variability. The concept of tissue transcriptomics, introduced in the context of this study, warrants further consideration in the discussion, as it could provide valuable insights in relation to dd-cfDNA.

However, despite these promising results, further research is warranted to refine the methodology, enhance its clinical utility, and establish standardized thresholds for broader clinical application. Moreover, it is important to acknowledge the variability in analytical methods, including both alternative NGS approaches and in-house PCR techniques, which have been discussed in previous studies[10,13,22]. Future studies should focus on validating these findings across diverse patient populations and investigating potential confounding factors that may influence dd-cfDNA levels.

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Author contributions: AZ, MA, JH: conceptualisation, manuscript writing; GF, MG, DR, KV, TK, AD, ED, RL, LF, JH: critically revised the manuscript.

Conflict of interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

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