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Differentiation between lung allograft rejection and infection using donor-derived cell-free DNA and pathogen detection by metagenomic next-generation sequencing

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ABSTRACT

Background: In lung transplant recipients (LTRs), the primary causes of mortality are rejection and infection, which often present similar symptoms, making differentiation challenging. This study aimed to explore the diagnostic efficacy of plasma donor-derived cell-free DNA (dd-cfDNA) in conjunction with metagenomic next-generation sequencing (mNGS) for pathogen detection in differentiation between lung allograft rejection and infection in LTRs experiencing new-onset pulmonary complications.

Methods: We conducted a retrospective study on 188 LTRs who underwent lung or heart-lung transplantation at our institution from 2015 to 2021. The LTRs were categorized into three groups: stable, rejection, and infection. We measured plasma dd-cfDNA levels and utilized both mNGS and culture methods to identify pathogens in the bronchoalveolar lavage fluid (BALF).

Results: The rejection group exhibited the highest levels of plasma dd-cfDNA (median 1.34 %, interquartile range [IQR] 1.06–2.19 %) compared to the infection group (median 0.72 %, IQR 0.62–1.07 %) and the stable group (median 0.69 %, IQR 0.58–0.78 %) (both $p < 0.001$). Within the infection group, a significantly higher level of dd-cfDNA was observed in the cytomegalovirus infection subgroup ($p < 0.001$), but not in the fungal ($p > 0.05$) or bacterial infection subgroups ($p > 0.05$), when compared to the stable group. Elevated dd-cfDNA levels, in combination with negative mNGS results, strongly indicated lung allograft rejection, with a positive predictive value and negative predictive value of 88.7 % and 99.2 %, respectively.

Conclusions: Plasma dd-cfDNA in combination with BALF pathogen detection by mNGS shows satisfactory accuracy in differentiating lung allograft rejection from infectious complications.

1. Introduction

Lung transplantation is often the final treatment for progressive end-stage lung diseases, with over 4000 procedures performed

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annually worldwide [1]. However, the clinical outcomes of lung transplantation remain poor, with a median survival of less than 6 years. This is largely attributed to the high incidence of postoperative complications and the resulting poor outcomes [2].

Allograft rejection is a serious complication following lung transplantation, with an incidence of 28–65 % in the first year after transplantation [3,4]. Acute rejection is the most common and severe complication in the first two months after transplantation [5]. It can cause acute graft failure, which may lead to chronic lung allograft dysfunction, and increase mortality and morbidity in heart-lung transplant recipients [3]. Infection is also a significant complication in lung transplant recipients (LTRs) and represents the most common cause of death in the first year after transplantation. Appropriate and early management of these complications can significantly improve patient survival. However, at the early stage of an episode of rejection or infection, there are no differential features in symptoms, chest computed tomography (CT), or laboratory tests between LTRs with rejection and those with infection. Early diagnosis of rejection and infection after lung transplantation remains a challenge for clinicians.

Donor-derived cell-free deoxyribonucleic acid (dd-cfDNA) in plasma is an emerging biomarker for detecting injury in transplanted organs [6,7]. Necrosis and apoptosis of cells in the transplant can lead to the release of nucleosomes into the bloodstream. These nucleosomes are then degraded by various types of nucleases into cfDNA fragments [8]. However, dd-cfDNA accounts for only approximately 10 % of total plasma cfDNA, with the majority coming from the recipient's white blood cells [9]. Furthermore, plasma cfDNA seems to have a rapid turnover, as demonstrated by the transient increase in cfDNA levels after exercise, which lasts only 30 min [10]. This feature of cfDNA enables it to accurately reflect the real-time status of the transplant. However, the detailed mechanisms of cfDNA clearance, including and renal excretion, are still poorly understood.

In our previous study, we explored plasma dd-cfDNA levels and lung allograft rejection in Chinese LTRs, and our preliminary results suggest that elevated dd-cfDNA levels could be a promising tool for diagnosing rejection in lung transplantation [11]. Generally, lung allograft rejection should be differentiated from infection, with a negative pathogen result being required. Metagenomic next-generation sequencing (mNGS) is a new method that can detect pathogens with high sensitivity and short turnaround time, including bacteria, fungi, viruses, and other atypical pathogens [12]. It has recently been utilized for the identification of pathogens in various types of diseases, including respiratory, neurologic, urinary, pediatric, and orthopedic conditions [13].

The present study aimed to investigate the performance of plasma dd-cfDNA in combination with mNGS in differentiating rejection from infection in LTRs who experience new-onset pulmonary complications.

2. Patients and methods

2.1. Study design

We retrospectively screened the recipients who underwent lung or lung-heart transplantation between 2015 and 2021. All lungs were derived from deceased donors after cardiovascular or brain death. This study was carried out per the principles of the Declaration of Helsinki and theInternational Society for Heart and Lung Transplantation (ISHLT) ethics statement and was approved by the Ethics Review Committee of the First Affiliated Hospital of Guangzhou Medical University (k2021-98). No organs from executed prisoners were used in this study. Informed consent was obtained from each recipient.

The inclusion criteria were: 1) had single-lung, double-lung, or heart-lung transplantation; 2) recipients were in a stable state and had plasma dd-cfDNA assay; 3) recipients had an episode of exacerbation of pulmonary complication and plasma dd-cfDNA assay with or without bronchoalveolar lavage fluid (BALF) microorganism detected by using mNGS for diagnosing. The exclusion criteria were: 1) age under 18 years; 2) incomplete medical data; 3) transplantation of other organs such as heart, liver, and kidney; 4) hematopoietic stem cell transplantation; 52) death of unknown causes. Patients with previous transplantations were excluded to eliminate the potential effect of prior transplantations on the levels of cfDNA.

Patients were categorized according to their final diagnosis into the stable group (those in a stable state without any signs of infection or rejection), the rejection group (which includes cases of acute cellular rejection and antibody-mediated rejection), and the infection group. The conclusive diagnosis was established clinically by physicians, using an amalgamation of comprehensive clinical data and a review of the patient's response to treatment.

2.2. Data collection and pulmonary exacerbation defination

Clinical data of the patients were collected retrospectively. There is currently no international consensus on defining an exacerbation of clinical status post-lung transplantation. Thus, we employed definitions used for other chronic respiratory diseases, like chronic obstructive pulmonary diseases [14]. In this study, we defined a pulmonary exacerbation as the presence of any new clinical symptoms (including fever, cough, sputum production, dyspnea, chest discomfort, and shortness of breath), coupled with new lung lesions as confirmed by chest CT scans, irrespective of the presence or absence of clinical manifestations from other diseases.

Regarding differential diagnosis, a panel of at least three experts with significant clinical experience in managing complications in LTRs evaluated clinical symptoms, signs, imaging findings, lung function tests, laboratory tests, and findings from mNGS and traditional pathogen detection methods. A differential diagnosis between infection and rejection was established through a comprehensive review of test results and treatment outcomes.

2.3. Diagnostic criteria of lung allograft rejection and infection

Transbronchial biopsy was conducted when the patient's condition allowed and when consent for the invasive procedure was

obtained. Histopathological analysis of the transbronchial biopsy specimens was carried out by an expert panel of pulmonary pathologists in China, following theISHLTworking group recommendations [15]. Chronic rejection was determined based on the ISHLT consensus council guidelines for the classification of chronic lung allograft dysfunction [16].

Pulmonary infection was diagnosed following the guidelines adopted by the Infectious Disease Society of America [17]. The specific criteria for diagnosing suspected new-onset pulmonary infection included a new or deteriorating focal or diffuse infiltration on chest X-ray or CT, coupled with at least one of the following five factors: 1) fever (body temperature >37.5 °C); 2) symptoms such as cough, sputum production, chest tightness, hypoxia, or an exacerbation of existing respiratory symptoms; 3) leukocytosis; 4) clinical signs of lung consolidation or moist rales; 5) evidence of pathogen infection. Pathogen evidence was based on comprehensive microorganism detection methods, which included mNGS, culture, and smear. Furthermore, the diagnosis of Cytomegalovirus (CMV) infection was made based on established guidelines and our prior study [18,19].

2.4. Microorganism detection

Potential allograft infection was indicated by the detection of pathogens, including bacteria, fungi, and viruses, in BALF detected by mNGS or conventional methodsas described in our previous studies [20,21].

2.5. Plasma dd-cfDNA assay

For dd-cfDNA measurement, peripheral blood was collected on the day of the biopsy or within two days prior to the biopsy. The plasma samples from unique patients must first pass strict quality control. An adequate volume of plasma (2 mL) was assayed by using a clinical-grade NGS dd-cfDNA system at a certified laboratory (AlloDx Biotech, Co., Ltd). A total of 6200 human single nucleotide polymorphism loci were enriched through liquid hybridization [20]. Briefly, 8 mL of blood was drawn into a tube for the collection of cfDNA (Streck, Omaha, NE). After two rounds of centrifugation at 1600 g for 10 min, plasma was isolated from the blood. With 1.8 mL plasma, cfDNA was extracted using a Circulating Nucleic Acid kit (Qiagen, Cat.No55114). A sequencing library was constructed using 30 ng cfDNA (KAPA LTP Library Preparation Kit, KK8235). The captured libraries were sequenced on an Illumina (X-ten, 10 ± 5 million, PE 150 bp). Sequencing of the raw data was processed using BWA and Samtools. The Bayes approach was used to measure the dd-cfDNA levels. The sequence data was shared in the Sequence Read Archive (https://submit.ncbi.nlm.nih.gov/subs/bioproject/, accession no: PRJNA 791820, 790691 and PRJNA 801094).

2.6. Statistical analysis

A threshold was set for the categorization of dd-cfDNA scores indicating rejection or demonstrating stability or infection based on the data distribution. Continuous variables that met the criteria for normality and equal variance between groups were compared using the Student's t-test, while those that did not meet these criteria were compared using the rank-sum test. Categorical variables were tested for baseline comparability using the chi-square test or Fisher's exact test. Logistic regression analysis was used to create a model

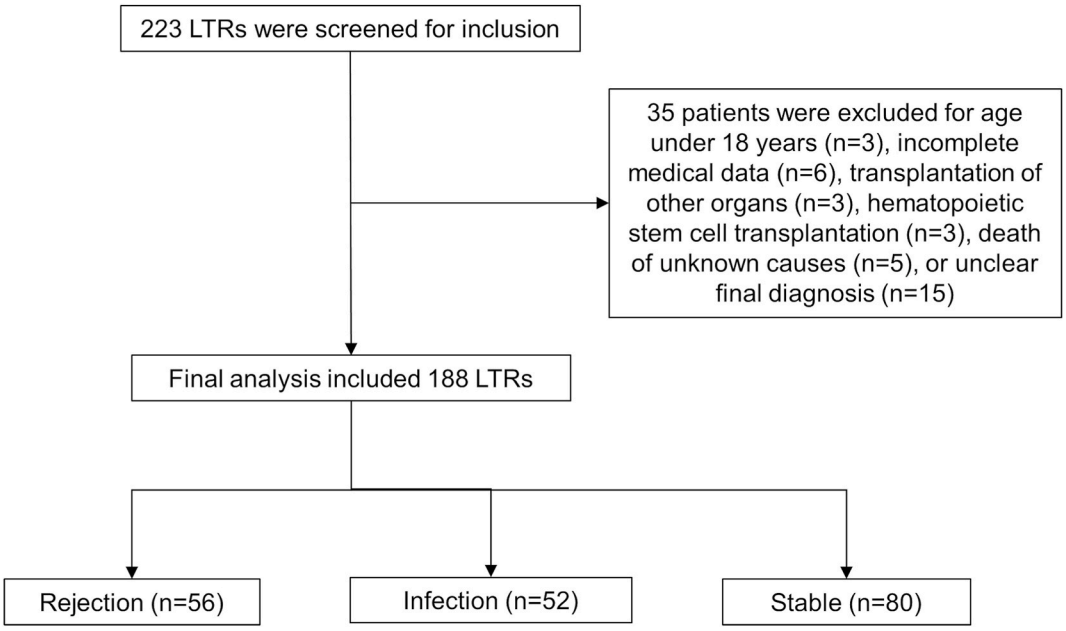

Fig. 1. A flow chart of patient inclusion and exclusion.

for diagnosing rejection employing both dd-cfDNA and mNGS. $p < 0.05$ indicates statistical significance. All statistical analyses were performed by using IBM SPSS Statistics 26.0 (IBM Corp., Armonk, NY), and graphs were made by using GraphPad Prism 8 (GraphPad Software, San Diego, CA) and R.

3. Results

3.1. Patient general characteristics

A total of 223 LTRs were screened for the study, and the final analysis included 188 subjects (Fig. 1). Patient demographic and clinical characteristics are shown in Table 1. Our patients included 97 unilateral-lung recipients, 84 bilateral-lung recipients, and 7 heart-lung recipients. All recipients received allograft rejection prophylaxis through a standard regimen of three immunosuppressants, consisting of a calcineurin inhibitor, a cell cycle inhibitor, and glucocorticoids.

3.2. Time trend in dd-cfDNA levels

A total of 188 plasma samples were assayed. An obvious decreasing trend was noticed in the plasma dd-cfDNA levels over time (Fig. 2). Specifically, the dd-cfDNA levels in the first month following transplantation were significantly elevated compared to those in the subsequent months (Fig. 3A). However, the dd-cfDNA levels did not differ significantly between the months following the first posttransplant month ($p = 0.075$, Fig. 3B).

3.3. Association of patient status with dd-cfDNA levels and pulmonary function

The rejection group had the highest plasma dd-cfDNA levels (median 1.34 %, interquartile range [IQR] 1.06–2.19 %) compared to the infection group (median 0.72 %, IQR 0.62–1.07 %), and the stable group (median 0.69 %, IQR 0.58–0.78 %) (both $p < 0.001$). However, the infection group and the stable group showed no significant difference in dd-cfDNA levels ($p = 0.084$) (Fig. 4A). The rejection group also had the greatest decline in FEV1%pred, from the baseline value (median −27.78 %, IQR -62.24 % to −15.55 %) compared to the infection group (median −10.38 %, IQR -27.41 % to −2.64 %, $p = 0.002$) and the stable group (median −6.83 %, IQR -36.54 % to −1.62 %, $p < 0.001$). The reduction in FEV1%pred was not significantly different between the infection group and the stable group (Fig. 4B).

3.4. Diagnosis of rejection by plasma dd-cfDNA combined with mNGS

Plasma dd-cfDNA and mNGS results were collected from 56 rejection and 132 non-rejection samples. Binary logistic regression analysis revealed that the plasma dd-cfDNA level was a significant independent predictor of rejection ($p < 0.001$). The logistic function was as follows:

Prediction score $= e^X/(1 + e^X)$

where X = −8.531 + 8.61 * dd-cfDNA level - 72.202 * mNGS (positive mNGS = 1, negative mNGS = 0).

Receiver operating curve (ROC) analysis was conducted based on the prediction score obtained from the combination of dd-cfDNA and mNGS results for diagnosing rejection. According to the ROC calculation results, the optimal cut-off value for diagnosing rejection

Table 1
General characteristics of the lung transplant recipients.

Demographics	Rejection (n = 56)	Infection (n = 52)	Stable (n = 80)	*p*-value
Age, year [median, (IQR)]	63.0 (52.0–66.3)	62.0 (54.0–67.0)	60.0 (52.0–66.0)	0.537
Male, n (%)	43 (76.8)	39 (75.0)	66 (83.5)	0.437
BMI, kg/m^2 [median, (IQR)]	20.31 (18.52–23.03)	20.48 (18.43–21.64)	20.08 (17.71–22.34)	0.725
Postoperative days, [median, (IQR)]	483.0 (207.25–920.75)	427.0 (115.50–812.25)	530.0 (174.0–861.0)	0.969
Panel reactive antibody +	2 (3.6 %)	6 (11.5 %)	3 (3.8 %)	0.149
Primary indications for lung transplantation, n (%)				0.411
Bronchiolitis obliterans	0	0	3 (3.8)	
Bronchiectasis	6 (10.7)	1 (1.9)	3 (3.8)	
COPD	17 (30.4)	20 (38.5)	27 (34.2)	
CTD-ILD	5 (8.9)	6 (11.5)	3 (3.8)	
IIP	21 (37.5)	20 (38.5)	31 (39.2)	
PLAM	1 (1.8)	2 (3.8)	1 (1.3)	
PVD	3 (5.4)	2 (3.8)	3 (3.8)	
WRLD	1 (1.8)	0	4 (5.1)	
Others	2 (3.6)	1 (1.9)	5 (6.3)	
CIM ≥ targeted levels, n (%)	21 (37.5)	21 (40.4)	25 (31.6)	0.566

IQR, interquartile range; BMI, body mass index; COPD: chronic obstructive pulmonary disease; CTD-ILD, connective tissue disease - interstitial lung disease; IIP, idiopathic interstitial pneumonia; PLAM, pulmonary lymphangiomyomatosis; PVD, pulmonary vascular disease; WRLD, work-related lung diseases; CIM, concentration of immunosuppressive medications.

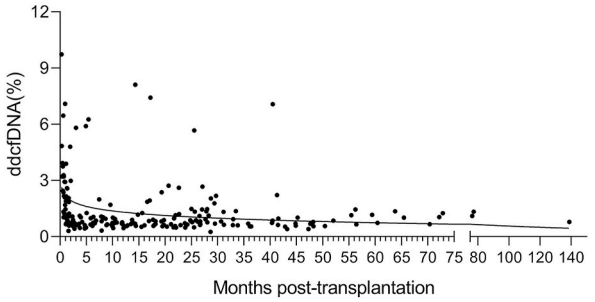

Fig. 2. Time trend in the plasma dd-cfDNA levels. A scatter plot of the dd-cfDNA concentrations against post-transplantation time (month) and the fitted curve show an obvious decreasing trend. Each dot represents a measurement of a plasma sample.

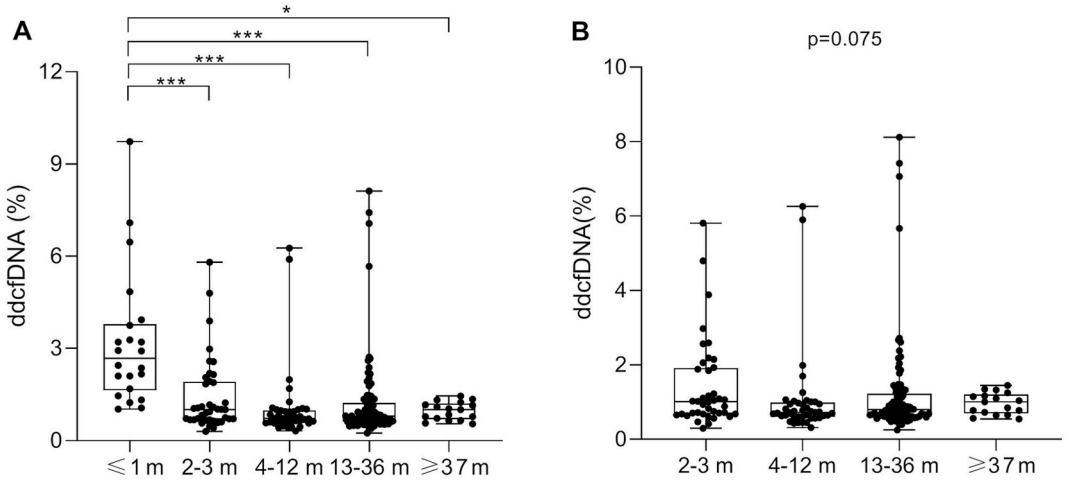

Fig. 3. Plasma dd-cfDNA levels in time intervals after transplantation. Kruskal-Wallis nonparametric comparison analysis was applied. Data are presented as box (25%–75 % interquartile range), whisker (minimum to maximum), and horizontal line (median). **(A)** The first month after transplantation had the highest plasma dd-cfDNA levels. **(B)** There were no significant differences in the plasma dd-cfDNA levels between the time intervals after the first month post-transplantation. m: month. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

was 0.2781 using the combined method. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the combined method for diagnosing rejection were 98.21 % (95 % confidence interval [CI] 90.4–100.0 %), 94.70 % (95 % CI 89.4–97.8 %), 88.7 % (95 % CI 79.2–94.2 %), and 99.2 % (95 % CI 94.7–99.9 %), respectively.

With a cut-off value of 0.89 %, the sensitivity, specificity, PPV, and NPV of dd-cfDNA alone for diagnosing rejection was 98.21 % (95 % CI 90.4–100.0 %), 82.58 % (95 % CI 75.0–88.6 %), 70.5 % (95 % CI 62.2–77.6 %), and 99.1 % (95 % CI 94.0–99.9 %), respectively. The area under the curve (AUC) of the combined detection method for diagnosing rejection was 0.986 (95 % CI 0.971–1.000), which was slightly but significantly higher than the AUC of dd-cfDNA alone (0.927, 95 % CI 0.891–0.964) (Delong test, $p = 0.0006$) (Fig. 5).

3.5. Association of dd-cfDNA levels with lung allograft infection

Out of the 98 BALF specimens from 98 LTRs, mNGS and traditional detection methods detected 91 (88.7 %) as positive and 8 (11.3 %) as negative. Fig. 6 shows the microorganisms detected by mNGS for pathogen detection across the stable, infection, and rejection groups. Of the 91 positive LTRs, 52 (57.1 %) were diagnosed with infectious diseases, and the pathogens were verified using clinically comprehensive criteria. The infection group had a slight elevation in dd-cfDNA levels compared to the stable group.

The plasma dd-cfDNA levels were significantly elevated in patients with CMV infection (n = 10, median 1.67 %, IQR 1.08–3.24 %, $p < 0.001$), especially in those with CMV pneumonia (Fig. 7). It seemed that a higher number of diffused lesions in CT images was associated with elevated plasma dd-cfDNA levels in patients with CMV pneumonia, which decreased subsequently after proper treatment. A 55-year-old woman had severe proven CMV pneumonia 10 months after lung transplantation. A dramatically high dd-cfDNA level of 7.42 % was recorded, which decreased to 0.48 % after treatment with ganciclovir, an anti-virus medication. Her symptoms and CT manifestations were also significantly improved. However, the plasma dd-cfDNA level was not significantly increased in patients infected with other viruses, such as Epstein Barr virus, Torque teno virus, adenovirus, and influenza virus (n = 4,

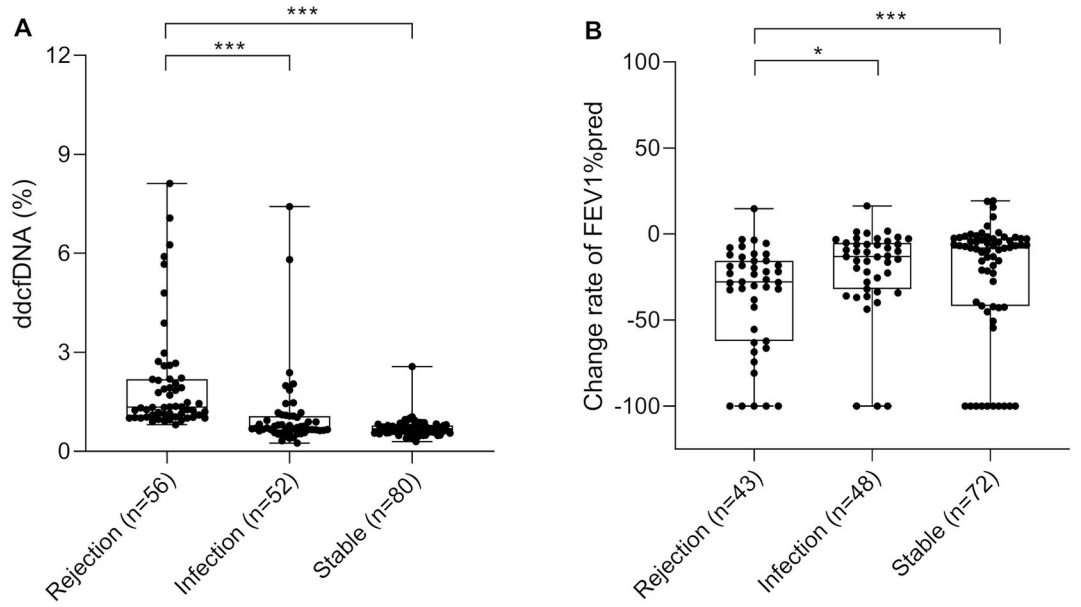

Fig. 4. Association of patient status with plasma dd-cfDNA levels and pulmonary function. Data are presented as box (25%–75 % interquartile range), whisker (minimum to maximum), and horizontal line (median). The rejection group had the highest plasma dd-cfDNA levels **(A)** and the greatest decline in pulmonary function **(B)**. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

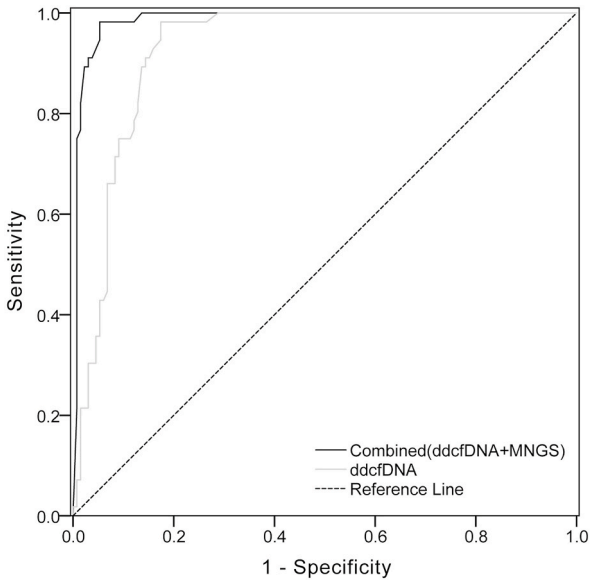

Fig. 5. Receiver operating characteristic curves of the selected biomarkers for diagnosing lung allograft rejection. The area under the curve was 0.927 for dd-cfDNA alone and 0.986 for dd-cfDNA in combination with mNGS ($p = 0.0006$).

median 0.82 %, IQR 0.80–1.0 %, $p > 0.999$).

Nine patients had proven invasive fungal infection (IFI), and 4 of them had elevated dd-cfDNA levels, consisting of 2 cases of *Aspergillus niger* and 2 cases of *Aspergillus flavus*. Plasma dd-cfDNA levels were mildly but significantly elevated in aspergillosis (n = 4, median 1.31 %, IQR 1.10–1.86 %, $p = 0.003$), but not in other IFIs, such as Pneumocystis jirovecii pneumonia, pulmonary candidiasis, and mucormycosis (n = 5, median 0.62 %, IQR 0.39–0.67 %).

The levels of dd-cfDNA did not differ significantly between LTRs with bacteria pneumonia (n = 29, median 0.66 %, IQR 0.56–0.74 %) and those in a stable state (n = 80, median 0.69, IQR 0.58–0.78 %, $p > 0.999$).

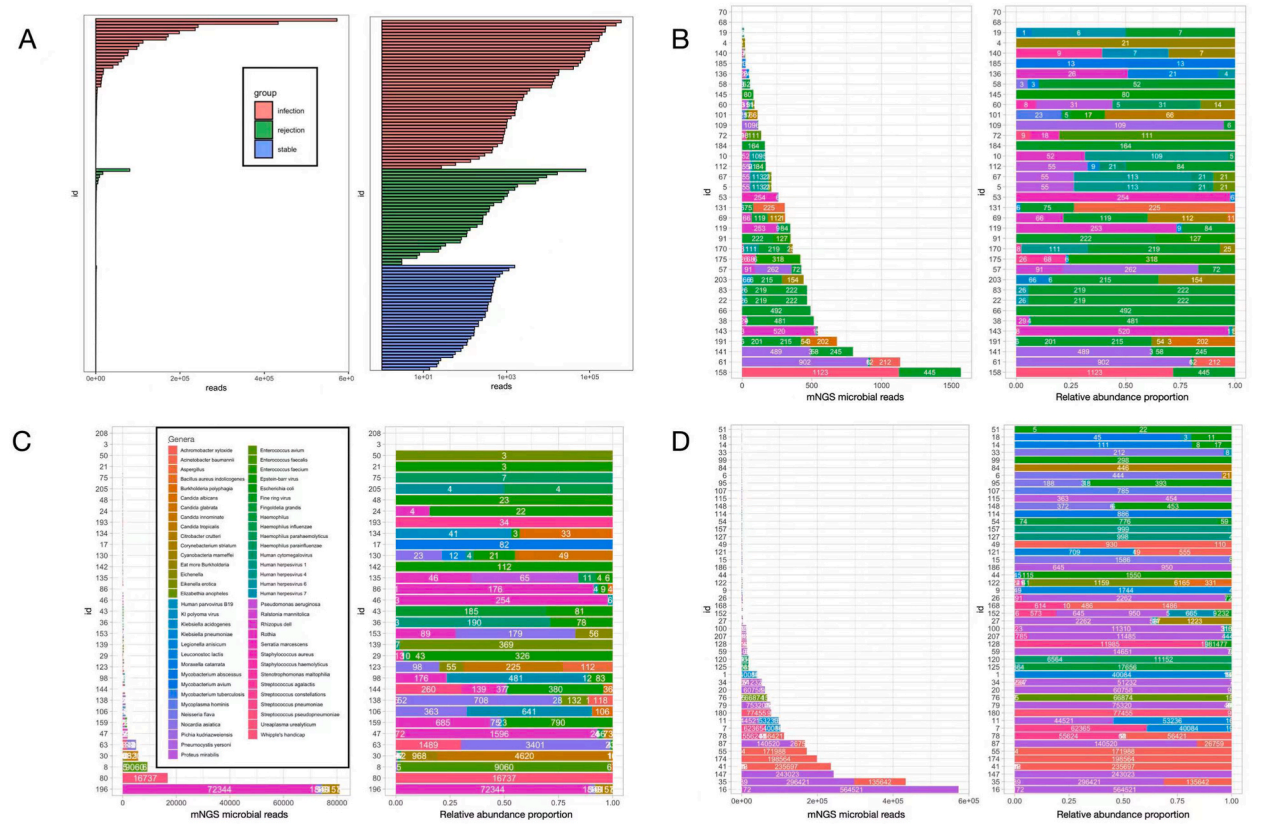

Fig. 6. Comparison of microbiome profiles between the infection, rejection, and stable groups. (A) Swimmer plot depicting microbial diversity in the infection, rejection, and stable groups. Untransformed (left) and log-transformed (right) data show reduced skewness after log transformation. (B) Stacked bar plots depicting absolute (left) and relative (right) abundances of microbial genera in the stable group. (C) Stacked bar plots showing microbial composition in the rejection group. (D) Stacked bar plots showing microbial composition in the infection group.

3.6. Treatment according to dd-cfDNA and mNGS

Twelve recipients (12%) had elevated plasma dd-cfDNA levels along with acute pulmonary complications, yet the pathogens presented by mNGS findings in BALF were negative. Based on their clinical presentations, these patients were diagnosed with non-infectious pulmonary diseases. Specifically, three patients were ultimately diagnosed with sirolimus-associated pneumonia. The other nine patients were suspected of having acute rejection, however, transbronchial biopsy confirmation could only be obtained in three patients. The remaining six patients did not undergo a biopsy due to poor clinical condition or refusal of consent. Without pathological evidence, the nine patients were clinically diagnosed with active rejection based on their elevated dd-cfDNA, negative mNGS, and presenting symptoms. Consequently, their treatment was changed from anti-infectious agents to escalated immunosuppression. This resulted in a rapid and significant improvement of symptoms and imaging findings, consistent with resolved rejection.

4. Discussion

Our study revealed that LTRs with pulmonary complications, including rejection and infection, had elevated plasma dd-cfDNA levels. In cases where mNGS and culture results in BALF were negative for pulmonary infection, elevated dd-cfDNA levels were a strong indication of lung allograft rejection. Additionally, a decrease in plasma dd-cfDNA levels had a strongly negative predictive value for allograft lung rejection.

Significant elevation in plasma dd-cfDNA levels was noticed in the first month after lung transplantation, regardless of the patient's stable or non-stable status (Fig. 2). It has been suggested that the increase in dd-cfDNA during the first two weeks after transplantation is related to ischemia-reperfusion injury [22]. These findings were also consistent with those of De Vlaminck et *al* [23], who demonstrated that dd-cfDNA levels were decreased along with time in LTRs after transplantation. The allograft lung is severely injured at the early stage after transplantation, which is characterized by diffused alveolar damage and robust inflammation [24]. Therefore, our results suggested that elevated plasma dd-cfDNA levels at the early stage after transplantation were an indicator of early allograft injury in primary graft dysfunction, which is consistent with previous findings [25].

Our study observed that a larger portion of recipients had plasma dd-cfDNA levels that were higher between 2 and 3 months than

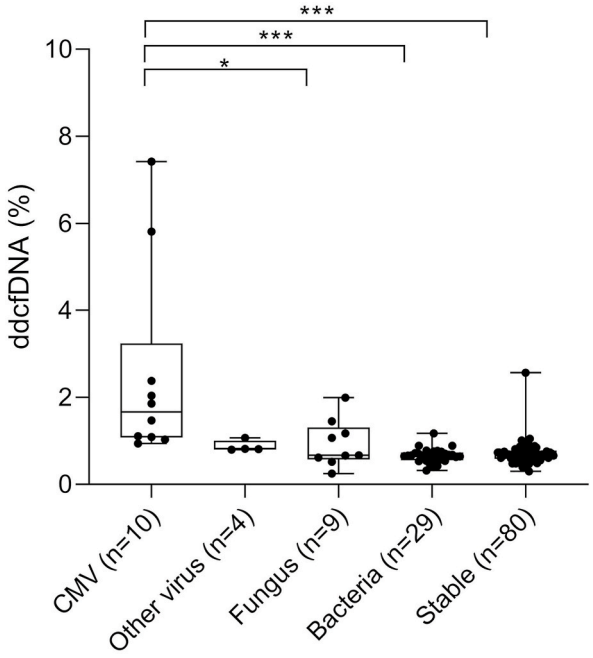

Fig. 7. Plasma dd-cfDNA level distribution in infectious complication. Data presented as box (25%–75 % interquartile range), whisker (min to max), and horizontal line (median) values. Kruskal-Wallis nonparametric comparison of CMV infection vs bacterial infections ($p < 0.001$), and stable ($p < 0.001$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

that at later stages posttransplant (Fig. 3A). This was likely due to the higher proportion of acute rejection recipients in this group. Acute rejection is most common and severe (54 % ≥ A1) within the first 3 months after lung transplantation [26]. Consistently, our study showed that 50 % (31/62) of the recipients had acute rejection within the first 3 months. Thus, it is expected a higher level of plasma dd-cfDNA to be observed between 2 and 3 months post-transplant. Our findings suggested that higher plasma dd-cfDNA levels are an indicator of acute allograft rejection, which is consistent with previous research indicating that cfDNA is a new clue of rejection in solid organ transplantation [5,23,27].

Additionally, our study showed that combining elevated plasma dd-cfDNA with negative mNGS results in BALF improved sensitivity and specificity in diagnosing rejection, compared to dd-cfDNA alone. ROC analysis showed the AUC for dd-cfDNA was 0.927 for detecting rejection, which significantly improved to 0.986 when incorporating negative mNGS findings. This enhancement in diagnostic performance stems from the complementary roles of the two tests. Plasma dd-cfDNA reflects allograft injury and mNGS can provide evidence of infectious etiology, together they give a more complete picture of the nature of posttransplant complications.

Elevated plasma dd-cfDNA levels in the rejection group are consistent with previous findings [7,11]. However, dd-cfDNA levels were also found to be slightly elevated in the infection group, especially in cases of CMV infection. Our study findings of significant lung allograft injury during CMV infection support previous reports of the interaction between CMV infection and allograft injury [28]. CMV reactivation and acute allograft rejection can intertwine and trigger one another. This highlights the critical need to rapidly differentiate between infection and rejection when an acute exacerbation occurs post-transplant. Making this distinction early is vital to guide appropriate treatment and optimize short and long-term outcomes. However, there is currently no definitive biomarker that can discriminate CMV infection from rejection at the outset of an acute presentation clinically. The symptoms, imaging findings, and routine laboratory tests lack specificity to differentiate these two complications. The application of dd-cfDNA levels or microorganism detection alone is not sufficient to yield an exact diagnosis. Our study showed that combining the dd-cfDNA assay with microorganism detection yielded satisfactory diagnostic performance.

Based on a comprehensive analysis of higher dd-cfDNA levels in plasma and negative mNGS findings in BALF, twelve recipients (12 %) were diagnosed with non-infectious pulmonary complications, including three cases of sirolimus-associated pneumonia and nine cases of acute rejection. Among the nine cases of acute rejection, six were without pathological evidence. Still, the treatment strategy, initially focusing on anti-infection, was changed to an escalation of the dose of immunosuppressants, based on the elevated plasma dd-cfDNA levels and negative mNGS findings in BALF. This led to a rapid improvement in clinical conditions and, significantly, a reduction in pulmonary infiltration, indicating reduced active rejection responses along with decreased plasma dd-cfDNA levels. These results suggest that the combined application of higher dd-cfDNA levels in plasma and negative mNGS findings in BALF strongly indicate active rejection, which can help clinicians determine the appropriate treatment strategy.

In addition to dd-cfDNA, procalcitonin is also a promising plasma biomarker for posttransplant organ monitoring, showing good discriminating power for bacterial infections after heart and lung transplantation [29,30]. Extracellular vesicles are also under study as potential biomarkers of allograft rejection and immunity [31,32]. More invasive approaches like BALF analysis, which contains

informative cytokines, remain a useful diagnostic method for pulmonary infections after lung transplantation [33].

5. Limitations

First, as a retrospective study, baseline plasma dd-cfDNA levels prior to transplantation were not available for recipients. Variability in baseline dd-cfDNA levels between recipients may have impacted the posttransplant measurements. Second, lung biopsy was not uniformly obtained across all recipients, potentially introducing bias into clinical diagnoses. Third, cfDNA fragment size was not measured, even though previous research indicates it impacts the accuracy of dd-cfDNA in diagnosing lung graft injury [34]. Fourth, the relatively small sample size from a single hospital and limited follow-up period restricts generalizability and longitudinal assessment. Finally, the lack of matching between groups raises the potential for confounding.

6. Conclusions

When used in combination with BALF mNGS and conventional detection methods, dd-cfDNA demonstrates satisfactory accuracy in differentiating lung allograft rejection from infection. Elevated dd-cfDNA levels and negative BALF mNGS results strongly suggest active rejection rather than infection.

Data availability statement

The data of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

CRediT authorship contribution statement

Chunrong Ju: Writing – original draft, Methodology, Investigation, Conceptualization. **Lulin Wang:** Methodology, Investigation. **Peihang Xu:** Methodology, Data curation. **Xiaohua Wang:** Validation, Methodology. **Dong Xiang:** Validation, Methodology. **Yu Xu:** Writing – review & editing, Validation. **Xin Xu:** Writing – review & editing, Visualization, Formal analysis, Data curation. **Rongchang Chen:** Writing – review & editing, Conceptualization. **Jianxing He:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Jianxing He reports financial support was provided by State Key Laboratory of Respiratory Disease. Jianxing He reports financial support was provided by Guangzhou Institute of Respiratory Health. Jianxing He reports financial support was provided by Zhong Nanshan Medical Foundation.

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