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When BAL meets CT scan: enhancing noninvasive diagnosis of acute cellular rejection after lung transplantation

Elena Pordon[1], Marco Guerrieri[1], Felice Perillo[1], Elisa Salvadori[1], Matteo Fanetti[1], Laura Bergantini[1], Claudia Ghiribelli[2], Luca Luzzi[2], Chiara Catelli[2], Elena Bargagli[1], Antonella Fossi[1], Cristiana Bellan[3], Chiara Piscitello[4], Maria Antonietta Mazzei[4] and David Bennett[1*]

Abstract

Background Acute cellular rejection (ACR) is a common complication after lung transplantation (LTX) and it is considered a risk factor for chronic lung allograft dysfunction (CLAD). Lung transbronchial biopsy is still the gold standard for a correct diagnosis of ACR. The aim of the present study was to evaluate the predictive role of bronchoalveolar lavage (BAL) cellular composition in combination with CT scan features for the diagnosis of ACR.

Method We retrospectively evaluated all LTX recipients who underwent transbronchial biopsies combined with BAL procedures and CT scan at a single Institution between January 2019 and October 2024 ($n=169$). ACR histological diagnosis was made according to current guidelines, BAL analysis included percentage of cellular composition, lymphocytes' typing and microbiology. A qualitative analysis of specific CT was conducted by an expert thoracic radiologist.

Results Among the 169 biopsies analyzed, 34% showed acute cellular rejection (ACR), predominantly grade A1 (68%). Patients with ACR exhibited significantly higher lymphocyte percentages in BAL ($p=0.025$), and the cutoff of 25% showed 22% sensibility and 92% specificity for the diagnosis of ACR. Combing BAL findings with CT features, patients with lymphocyte ≥ 25% in BAL and concomitant pleural effusion showed 95.7% specificity of ACR. Infections were associated with elevated neutrophil levels in BAL ($p=0.026$); eosinophil levels were significantly higher in patients with significant ACR (grade ≥ 2) and concomitant infection than those with infection only ($p=0.0014$).

Conclusion BAL cellular composition proved to be a strong predictive tool for the diagnosis of ACR. The lymphocyte threshold of 25% was able to distinguish patients with ACR, while the combination of increased BAL lymphocytes with ACR associated CT scan abnormalities especially pleural effusion significantly enhanced diagnostic accuracy. Elevated eosinophil levels were associated to more severe rejection and concomitant infection, highlighting their crucial role in the alloreactive immune response. These findings suggest the role of BAL and CT scan in combination as a valuable diagnostic tool in ACR diagnosis, although histological confirmation remains the gold standard.

Keywords Lung transplant, Acute cellular rejection, Transbronchial biopsy, Bronchoalveolar lavage, CT scan

*Correspondence:
David Bennett
David.btt@gmail.com
[1]Respiratory Diseases Unit, University Hospital of Siena, Viale Bracci, 14, Siena 53100, Italy
[2]Thoracic Surgery and Lung Transplantation Unit, University Hospital of Siena, Siena, Italy
[3]Anatomy Pathology Unit, University Hospital of Siena, Siena, Italy
[4]Imaging Diagnostic, University Hospital of Siena, Siena, Italy

Introduction

Lung transplantation (LTX) is a valid therapeutic approach for selected patients with end-stage pulmonary diseases ensuring increased survival and improved quality of life. Acute cellular rejection (ACR) is a common complication after LTX, approximately 30–50% of patients experience at least one episode of acute rejection within the first 12 months. It primarily represents a cell-mediated inflammatory response due to newly transplanted organ by the recipient's immune system [1, 2].

The clinical presentation of ACR is highly variable, with nonspecific signs and symptoms that may appear independently of an evident decline in lung function, however up to 40% of patients can be asymptomatic. Relying on the presence of symptoms implies that the diagnosis is often made late in the disease progression, at a point when the condition has already become severe [3, 4].

Recognizing acute rejection is crucial, as multiple studies demonstrated its correlation with development of bronchiolitis obliterans syndrome (BOS), the main form of chronic lung allograft dysfunction (CLAD) [5, 6].

Diagnosis is made through histological specific features obtained by endoscopic transbronchial lung biopsies (TBBx) [7]. The procedure is not free of risks and can be source of severe complication, particularly in patients with reduced respiratory functional reserve and those with single-lung transplants [8, 9]. Bronchoalveolar lavage (BAL) is often performed together with TBBx mainly for microbiological purposes, differential counts often reveal lymphocytosis during acute cellular rejection, though diagnostic sensitivity remains limited [10].

Radiology imaging is a key source in the puzzle of post-transplant complications. Specific High-resolution computed tomography (HRCT) features knowledge can improve clinical management by enabling timely diagnosis and guiding bronchoscopic procedures, particularly in cases where disease distribution is irregular and requires sampling from different sites than the lower lobes [11].

Several studies have assessed the diagnostic performance of HRCT for detecting acute rejection after lung transplantation, and certain radiographic abnormalities—such as ground-glass opacities, septal thickening, and pleural effusion—have been associated with ACR. However, its diagnostic accuracy remains limited because these findings are heterogeneous and lack specificity, frequently overlapping with those seen in infectious complications [12–14].

Recently, some emerging biomarker for non-invasive monitoring of allograft damage such as donor-derived cell-free DNA (dd-cfDNA) have been proposed. However, it still lacks specificity, as its elevation has been associated to ACR and other acute and chronic complications as well [15, 16].

The purpose of this study was to investigate, for the first time, the integration of BAL and HRCT in order to improve diagnostic accuracy of ACR in a cohort of lung transplant patients.

Materials and methods

Design of the study

To evaluate the contribution of (BAL) to the diagnosis of ACR, all TBBx included in the analysis were first classified according to the presence or absence of ACR on histopathology and subsequently subdivided based on the presence of concomitant infection. First, we assessed the diagnostic performance of BAL cellular analysis for identifying ACR. Second, chest CT features were incorporated into the analysis to explore their potential added value in improving diagnostic accuracy.

All transbronchial biopsies (TBBx) were classified according to the following classification:

1. Patients were first categorized based on the presence or absence of acute cellular rejection (ACR) on histopathological examination and, when ACR was present, further subdivided according to rejection severity using the ISHLT classification system [7].
2. Based on the presence of concomitant infection, patients were further stratified into three subgroups:
   - Diagnosis of ACR without infection
   - Diagnosis of ACR with concurrent infection
   - Microbiological and/or histopathological evidence of infection in the absence of ACR

All patients provided informed consent to participate in this study, which was approved by the Local Ethics Committee (protocol Respir1).

Population

We conducted a retrospective analysis on all patients who underwent lung transplantation at the Lung Transplant Center of the University Hospital of Siena, Italy and had at least one TBBx between January 2019 and October 2024. Only patients with available results for BAL cytology evaluation, lymphocyte typing, and lavage culture obtained during the same respiratory endoscopic procedure together with TBBx and a CT scan available were included in the study.

Data from 59 adult patients (34 males, 25 females, 63.74 ± 10.76 years old) were collected. Among these, 15 patients underwent single lung transplantation (9 left, 6 right lung), while 44 had bilateral LTX. All patients received standard immunosuppressive therapy consisting of basiliximab for induction and prednisone, tacrolimus, and mycophenolate mofetil for maintenance. In frail patients at high risk of infection, mycophenolate

mofetil was withheld. The use of everolimus is reserved (after 3–6 months post-transplant) for patients with renal dysfunction—with concomitant minimization of tacrolimus—and for those with concomitant malignancies.

Treatment of acute cellular rejection episodes consists of high-dose steroid pulses, and antithymocyte globulin (ATG) in selected cases.

Our surveillance protocol consists of performing transbronchial biopsies in all patients at postoperative days 30–40, and at 3, 6, and 12 months, as well as whenever clinically indicated (lung function decline and/or respiratory symptoms and/or new radiological changes). In these latter cases, and particularly in frail patients, deviations from the standard surveillance schedule may occur.

We collected pre-transplant data for all patients, including the diagnosis of the respiratory disease leading to transplantation, body mass index at the time of transplantation, smoking history, type of the transplant, and the presence of post-transplant primary graft dysfunction (PGD).

Endoscopic procedure

Biopsy samples were collected using a flexible bronchoscope and flat-valve forceps. The procedures were performed under conscious sedation, subsegmental branch for sampling were selected based on prior CT scan findings. A post-procedure chest X-ray to monitor possible complications was always conducted within six to eight hours.

During the same procedure, bronchoalveolar lavage (BAL) was performed by instilling 2–3 aliquots of 50 ml saline solution in segments of the middle lobe or lingula and gentle aspired according to guidelines [14]. BAL analysis included total and differential cell counts (macrophages, neutrophils, lymphocytes, eosinophils, basophils), lymphocyte typing (CD3+, CD4+, CD8+, CD19+, CD56+), and microbiological examination. Microbiological assessments included cultures for common bacteria, fungi and Mycobacterias. Depending on the clinical suspicion, testing for galactomannan antigen and PCR for *P. jirovecii* and pneumotropic viruses was also performed.

169 endoscopic procedures with corresponding transbronchial lung biopsies (2.9 TBBx per patient) and BAL (cytology and lymphocyte typing and complete biological results) were considered in this study.

Radiological imaging

Data from HRCT chest scans performed before endoscopic procedures were collected for 165 out of the 169 cases considered in this study.

An expert thoracic radiologist performed a qualitative analysis (0 = absent, 1 = present) to identify radiological signs potentially indicative of ACR, including ground-glass opacities, consolidations, micronodules, septal thickening, air trapping, pleural effusion, and lymphadenopathy.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 9.3.1 for Macintosh. Parametric tests were used (T-test and ANOVA) and differences were considered significant at $p \leq 0.05$. The Fisher's or Chi-square test was employed to study prevalence differences in contingency tables. All data are expressed as mean ± standard deviation unless otherwise specified.

ROC analysis was performed to evaluate the diagnostic performance of the test by calculating the area under the curve (AUC). Likelihood ratio (LR) analysis, taking into account the clinical priority of minimizing false-positive results, was used to identify the cut-off value for BAL lymphocyte percentage.

Results

This study included 59 LTX patients (34 males, 25 females, 63.74 ± 10.76 years old) who underwent 169 endoscopic procedures with corresponding transbronchial lung biopsies (2.9 TBBx per patient) and BAL (cytology and lymphocyte typing and complete biological results). Fifteen patients (25.4%) underwent single-lung transplant, 44 patients had bilateral lung transplant. Sixteen patients (27.3%) were affected by IPF and 18 by other form of pulmonary fibrosis (30.5%), 14 (23.7%) by COPD, 5 (8.5%) by cystic fibrosis, 4 (6.8%) by other diseases and two (3.4%) redo transplant due to CLAD (both were bilateral LTX). Baseline characteristics are reported in Table 1.

138 out 169 (81.66%) TBBx were considered adequate for diagnostic purpose according to guidelines [5, 8]. Eleven patients underwent only one lung biopsy procedure, 15 underwent two procedures, 17 underwent three, and 14 patients (23.7%) underwent more than three procedures (Table 2).

Histopathology

In 47 cases (34%) ACR was present, classified as: A1 in 32 cases, A2 in 12 cases, and A3 in 3 cases. No patients showed an A4 rejection grade.

Five biopsies showed signs compatible with antibody-mediated rejection (AMR), but in 4 cases this was associated with cellular rejection as well. In 4 ACR cases, lymphocytic bronchitis/bronchiolitis (B) was also present: 3 of type B1R and 1 B2R. In 5 cases we found an overlap of ACR and organizing pneumonia.

Nine biopsies showed direct signs of infection at histological evaluation, all confirmed at microbiological results that showed: 3 bacterial and viral, 2 bacterial and fungal, 2 viral and fungal, 3 viral, and 2 bacterial only.

Table 1 Pre-transplant characteristics, type of transplant, PGD (primary graft dysfunction) and CMV (cytomegalovirus) status. BMI = body mass index, IPF = idiopathic pulmonary fibrosis, COPD = chronic obstructive pulmonary disease

	Population
Number	59
Age at transplant	63,74 ± 10,76
BMI pre-transplant	25,08 ± 4,14
Recipient smoking history	30 (50.8%)
Donor smoking history	41 (69.5%)
Pre-transplant diagnosis	
• IPF	16 (27.2%)
• COPD	14 (23.7%)
• Cystic fibrosis	5 (8.5%)
• Other pulmonary fibrosis	18 (30.5%)
• Other diagnosis	4 (6.8%)
• Re-do LTX	2 (3.4%)
N° transplant	59
• Single LTX	15 (25.4%)
• Bilateral LTX	44 (74.6%)
PGD at 72 h (total)	41/59 (69.4%)
• Grade 1	15 (36.6%)
• Grade 2	17 (41.4%)
• Grade 3	9 (22%)
CMV status	
• D+/R+	50 (84.8%)
• D+/R-	3 (5%
• D-/R+	5 (8.5%)
• D-/R-	1 (1.7%)

In five cases, biopsies revealed alternative diagnoses: organizing pneumonia ($n = 2$), desquamative pneumonia ($n = 1$), EBV-associated large-cell lymphoma ($n = 1$), and early obliterative bronchiolitis ($n = 1$); notably, the latter patient showed only a 8% reduction from post-operative best FEV_1.

Seventy-six (55%) of adequate biopsies resulted negative either for lung reject or any other features.

Microbiology

Microbiological analyses were positive in 72 cases (45 bacterial, 12 viral, and 15 fungal). In two cases, concomitant bacterial and viral positivity was observed. Among the 15 cases with fungal positivity, 11 also showed concomitant bacterial infection, and in three cases a mixed infection involving fungi, bacteria, and viruses was identified (see Table 2).

The following microorganisms were isolated:

- Bacteria: Pseudomonas aeruginosa (21 cases), Acinetobacter spp. (6 cases), Klebsiella pneumoniae (5 cases), Staphylococcus epidermidis (5 cases), Escherichia coli (4 cases), Stenotrophomonas maltophilia (3 cases), Staphylococcus aureus (3 cases), Enterococcus faecalis (2 cases), Serratia spp. (1 case), Proteus spp. (1 case), Mycoplasma spp. (1 case), Klebsiella variicola (1 case), and Corynebacterium spp. (1 case).
- Fungi: Aspergillus fumigatus (10 cases), Aspergillus terreus (1 case), Aspergillus nidulans (1 case), Aspergillus flavus (1 case), Pneumocystis jirovecii (1 case), Fusarium spp. (1 case), and Candida spp. (1 case).
- Viruses: Cytomegalovirus (CMV) (7 cases), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (2 cases), herpes simplex virus (HSV) (2 cases), and respiratory syncytial virus (RSV) (1 case).

Table 2 Details regarding the bronchoscopic procedures, including the number of interventions, post-procedural complications, and diagnostic yield

	Population	
Patients	59	
Procedures	169	
Diagnostic procedures %	138 (81.6%)	
One procedure (%)	11 (18.6%)	
Two procedures (%)	15 (25.4%)	
Three procedures (%)	17 (28.8%)	
More than three procedures (%)	14 (23.7%)	
TBBx indication		
• Surveillance	83 (60.2%)	
• Clinical needed - ALAD	55 (39.8%)	
Sample localization (Lower lobes/upper and middle lobes)	165 (97.6%) / 134 (79.3%)	
Post procedural bleeding	104 (61.54%) 80 mild (77%), 24 moderate-severe (23%)	
Pneumothorax post procedural	3 (1.8%)	
Adequate for pathology evaluation	138 (81.66%)	
ACR positive	47 (34%)	
A grade		
• ACR 1	32 (68%)	
• ACR ≥ 2	15 (32%)	
B grade		
• B1r	3 (6.4%)	
• B2r	2 (4.3%)	
ACR-directed therapy		
• Steroids	47 (100%)	
• ATG	3 (2.1%)	
Microbiology		
• Bacterial	45 (32.6%)	
• Viral	12 (8.7%)	
• Fungal	15 (10.1%)	
Antimicrobic therapy modification after Bronchoscopy	43/56 (76.8%)	
LTX-to-bronchoscopy time (days)		
• ACR	335 ± 401	($p = 0.47$)
• ACR plus infection	117.6 ± 81.66	
• Infection	210.7 ± 222.4	

Table 3 Comparison of cytology and lymphocyte typing between patients with and without ACR, and between those with mild (A1 grade) and moderate-to-severe (≥ A2 grades) acute rejection. *Statistically significant

	ACR	No ACR	*p*	ACR A1	ACR ≥A2	*p*
Number	47	91		32	15	
Macrophages %	60.72 ± 29.87	56.88 ± 30.73	0.48	66.22 ± 27.02	49 ± 33.16	0.06
Neutrophils %	25.34 ± 28.74	31.76 ± 30.99	0.24	25.81 ± 27.53	27.93 ± 32.65	0.82
Lymphocytes %	15.34 ± 17.49	8.75 ± 14.61	0.025*	11.90 ± 14.23	23.47 ± 21.64	0.033*
Eosinophils %	0.70 ± 2.11	0.37 ± 1.30	0.15	0.37 ± 1.95	1.5 ± 2.40	0.10
	45	75		29	15	
CD3+	91.70 ± 5.06	89.50 ± 8.02	0.09	90.46 ± 5.31	93.93 ± 3.92	0.031*
CD4+	29.69 ± 18.35	32.74 ± 16.81	0.33	29.36 ± 16.59	31.57 ± 21.89	0.70
CD8+	63.01 ± 16.61	54.71 ± 17.27	0.008*	62.08 ± 16.60	63.26 ± 16.60	0.82
CD19+	1.59 ± 3.54	1.64 ± 2.97	0.93	2.04 ± 4.3	0.81 ± 0.88	0.28

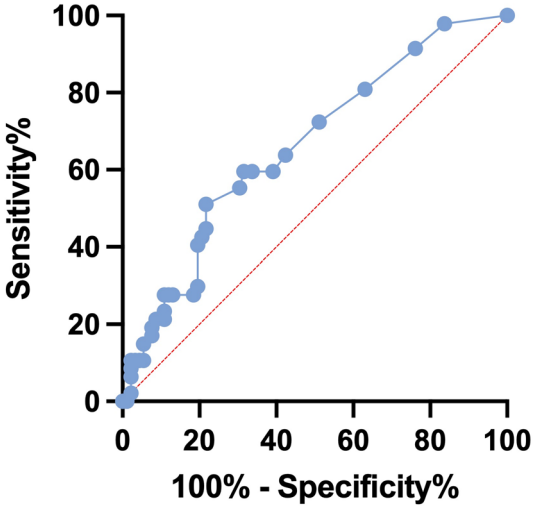

Fig. 1 Receiver Operating Characteristic (ROC) curve of percentage of lymphocytes in BAL from patients with and without ACR

Based on these data, the following groups were defined:

1. A total of 47 TBBx (34%) showed histopathological evidence of ACR (32 graded as A1 and 15 graded as A2 or higher). In the remaining 91 cases, ACR was not identified.
2. Thirty-three patients were diagnosed with ACR without evidence of infection, 14 with ACR and concomitant infection, and 42 with infection alone. Forty-nine TBBx were excluded from this subanalysis because the diagnosis was neither ACR nor infection.

In our cohort, 68% of ACR diagnoses were made during the first year post-LTX. However, no statistically significant differences were observed among the time points from LTX for diagnoses of ACR, ACR plus infection, and infection only.

Comparison of patients with and without ACR

Cell composition and lymphocyte typing in BAL

Patients with histological evidence of ACR exhibited significantly higher lymphocyte percentages than those without ACR ($p = 0.025$) (Table 3).

Among patients with ACR, those with rejection grade ≥ 2 had higher lymphocyte levels than those with grade 1 rejection ($p = 0.033$), with CD3 + lymphocytes levels also elevated ($p = 0.031$). Analysis of lymphocyte subpopulations revealed significantly elevated CD8 + levels in patients with ACR ($p = 0.008$) (Table 3).

ROC curve analysis identified a lymphocyte cutoff of 25% to differentiate ACR patients from no ACR patients (AUC 0.66 ± 0.04, $p = 0.012$), with sensitivity and specificity values of 22% and 92%, respectively, and a likelihood ratio of 2.5 (Fig. 1).

Among basal characteristics, no statistically significant differences emerged between those who experienced at least one rejection episode and those who did not. We did not observe any significant differences in BAL cellular composition between patients who received grafts from smoking versus non-smoking donors.

Regarding intra-individual variability across serial measurements, we observed that in cases diagnosed with ACR in which follow-up histopathology after treatment was negative and no infection was present, BAL lymphocyte percentages significantly decreased from 12.6 ± 8.6% to 2.9 ± 2.4% ($p = 0.019$). Conversely, no statistically significant differences were observed with respect to the

immunosuppressive regimens in place at the time of acute rejection diagnosis.

Radiological analysis

This study included 165 HRCT thoracic scans performed immediately before endoscopic examinations with BAL and transbronchial lung biopsy. In 129 cases the CT scans regarded bilateral transplanted patients (78.2%), whether 36 were about single ones.

The following findings were overall observed: ground-glass opacity in 108 cases (65.5%), parenchymal consolidations in 85 cases (51.5%), micronodules in 49 cases (29.7%), septal thickening in 50 cases (30%), air trapping in 77 cases (46.6%), pleural effusion in 84 cases (51%), lymphadenopathy in 6 cases (3.3%) (Fig. 2).

Patients with ACR showed a statistically significant higher incidence of ground-glass opacities respect to other different histological diagnosis ($p = 0.032$, sensitivity 76.6%, specificity 43.5%) respect to patients with other diagnosis. The presence of septal thickening and pleural effusion was more frequent in ACR, not reaching significance (Table 4).

Among patients who underwent bilateral lung transplantation and had histological evidence of acute cellular rejection, HRCT demonstrated bilateral involvement in 85.7% of cases. Lesions were predominantly distributed in the lower lung fields (57.1%) or diffusely throughout the lungs (42.85%), with upper lobe predominance observed in only 3.6% of cases. In patients with single-lung transplantation, lesions were mainly located in the lower lung fields (73.68%) or diffusely distributed (21%), while no cases showed upper lobe predominance.

Combination of BAL and radiological findings

Presence/absence of the three most expressed alterations reported in the ACR group at CT scan (ground-glass opacities, septal thickening and pleural effusion) was combined with BAL findings, in particularly lymphocytosis that represented the main observed features in BAL from patients with ACR.

We observed a significant further increase of specificity for the diagnosis of ACR in patients exhibiting BAL lymphocytes greater than 25% together with pleural effusion at CT ($p = 0.043$, sensitivity 24%, specificity 95.7%, likelihood ratio 5.595). No other CT features could increase diagnostic yield.

In a multivariable logistic regression model adjusted for the presence of diagnoses other than acute rejection, the odds ratio increased from 2.53 ($p = 0.034$) for the presence of lymphocytic alveolitis alone (BAL lymphocytes > 25%) to 4.54 ($p = 0.05$) when lymphocytic alveolitis was associated with pleural effusion on CT. No other radiological feature improved the diagnostic performance of the model.

Comparison of patients with ACR, ACR plus infection, infection only

In examining the impact of infection on the BAL cytological profile, significantly higher neutrophil levels were observed in patients with infection compared to those with ACR in absence of infection ($p = 0.026$). Neutrophil levels consistently remained elevated in patients with ACR and concurrent infection despite at limit of significance (Table 5; Fig. 3A).

In contrast, lymphocyte levels were higher at limit of significance in patients with ACR and ACR with infection compared to those with infection only ($p = 0.061$) (Fig. 3B).

CD3 + lymphocytes were significantly elevated in ACR and ACR with infection compared to infection without ACR ($p = 0.009$), while CD8 + lymphocytes were notably higher in ACR *plus* infection compared to patients with infection without ACR ($p = 0.006$) (see Table 5).

There were no differences in eosinophil counts between patients with acute cellular rejection (ACR) and those without ACR, nor among the ACR, ACR plus infection, and infection-only groups (Tables 3 and 5). However, when focusing on patients with grade ≥ 2 ACR, eosinophil levels were significantly higher in patients with acute rejection compared with those without ACR ($p = 0.049$) (Fig. 3C) and were markedly higher in patients with ACR and concomitant infection compared with those with infection alone ($p = 0.0014$) (Fig. 3D).

From the analysis of chest CT images, no statistically significant differences were observed in the prevalence of each radiological finding among the study groups (Table 6).

Discussion

ACR remain a significant complication in LTX recipients with higher incidence compared to other solid organ transplants. It's early recognition and treatment is of great importance to preserve graft function as it is considered a key risk factor for CLAD development, which remains the primary barrier to long-term survival [1–3]. It is characterized by perivascular lymphocytic infiltration, sometimes involving the bronchioles. Despite TBBx remains the gold standard for the diagnosis, the actual interpretation of pathology remains challenging [7]. In a recent study on 1,566 transbronchial biopsies from 20 different centers, limited reproducibility and high variability in the diagnosis and histological classification of acute rejection emerged. This variability was attributed to irregular lesion distribution, limited sample size, restricted access to small airways, and technique-related artifacts [8]. Otherwise, transbronchial biopsy (TBB) is not a risk-free procedure, especially in fragile patients as transplanted ones [17].

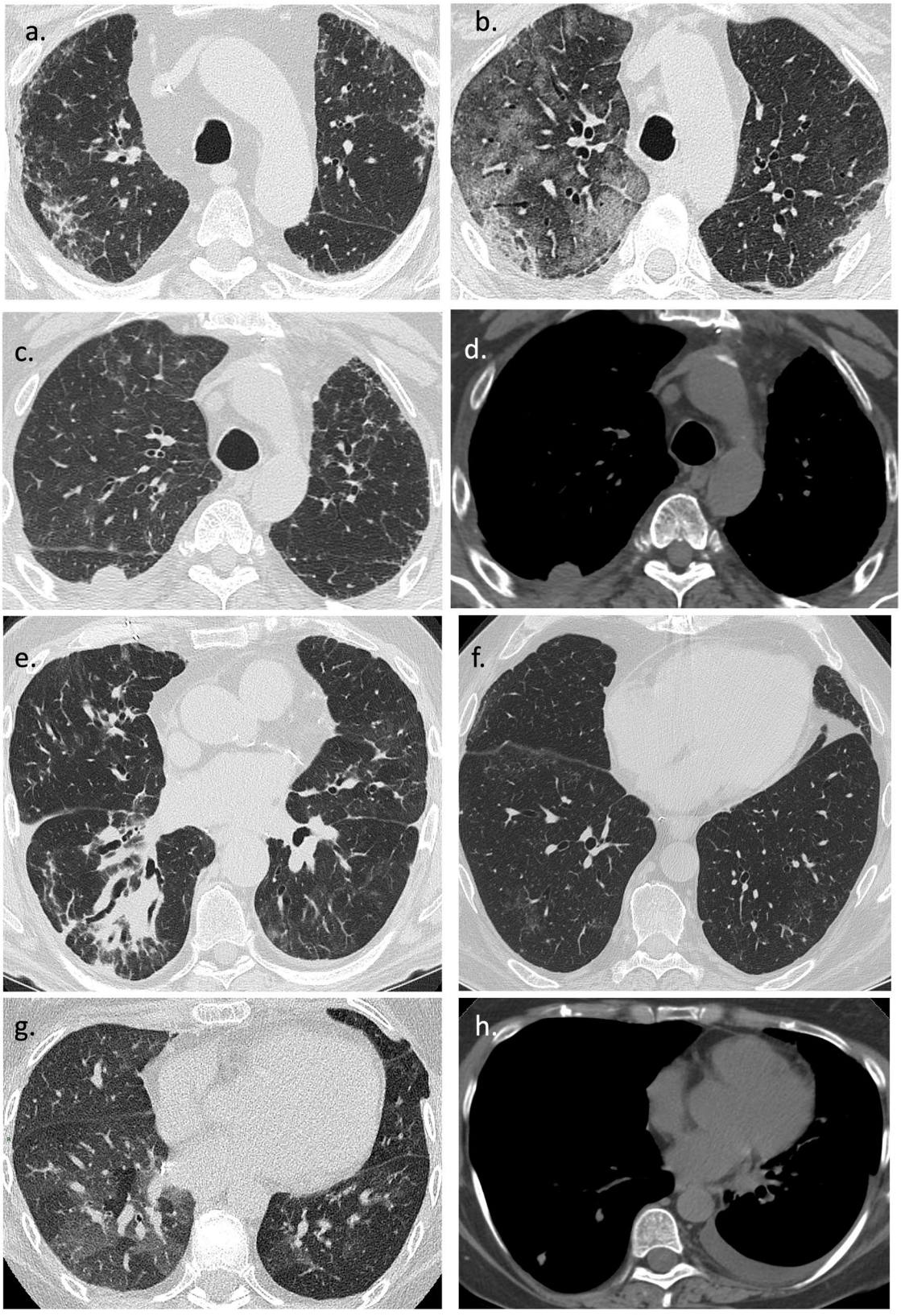

Fig. 2 The main radiological features of acute rejection on high-resolution CT (HRCT) are as follows: (**A**) Bilateral irregular subpleural septal thickening. **B** Subpleural septal thickening associated with areas of ground-glass opacity on the right lung. **C**, **D**. Smooth septal thickening in lung parenchyma reconstructions and enlarged lymph nodes in the mediastinum. **E** Peribronchial consolidations. **F** Bilateral pulmonary micronodules. **G** Areas of air trapping on expiratory scans. **H** Left pleural effusion

Table 4 CT abnormalities observed in ACR, as well as in other conditions, are reported as the percentage of cases in which each finding was present

	ACR	Other diagnosis	*p*
Ground glass	76.6%	59%	0.032*
Air trapping	51%	43.4%	0.19
Parenchymal consolidations	57.4%	47.5%	0.44
Micronodules	23.4%	23%	0.87
Pleural effusion	44.7%	51.6%	0.59
Septal thickening	36%	27%	0.19
Lymphadenopathy	4.3%	5.7%	0.87

Table 5 Comparison of cytology and lymphocyte typing among patients with ACR, ACR *plus* concomitant microbiological infection and infection only. * Statistically significant

	ACR	ACR plus infection	Infection	*p*
Number	33	14	42	
Macrophages %	65.03 ±29.19	50.57 ±34.35	49.47 ±32.80	0.088
Neutrophils %	20.15 ±26.15	37.57 ±31.77	39.17 ±33.76	0.026*
Lymphocytes %	14.24 ±17.48	17.93 ±18.24	7.17 ±15.63	0.061
Eosinophils %	0.56 ±2.18	1.25 ±2.18	0.17 ±0.57	0.15
	30	14	27	
CD3+	91.13 ±5.68	92.74 ±3.53	85.51 ±11.41	0.009*
CD4+	31.44 ±17.62	27.27 ±20.17	32.22 ±16.73	0.68
CD8+	59.75 ±17.28	68.33 ±13.07	50.86 ±17.01	0.006*
CD19+	1.84 ±4.18	1.16 ±1.71	1.82 ±3.56	0.82

Laboratory tests, pulmonary function assessments, imaging studies, BAL findings and some emerging biomarkers such as dd-cfDNA can adjuvate the diagnosis of ACR, however they still they lack standardization [15, 16].

HRCT of the chest is a key tool in post-transplant follow-up [11]. Several studies have evaluated the accuracy of HRCT in detecting acute rejection after lung transplantation. Loubeyre et al. [12] reported a 65% sensitivity of ground-glass opacities, which was higher at one month post-transplant and correlated with the extent of the lesions. Park et al. [13] described ground-glass opacities and septal thickening as findings associated with acute rejection, predominantly bilateral and basal, with an overall low sensitivity (50%) but increasing according to histopathologic grade (50% for A2 rejection and 100% for A3 rejection). However, Di Piazza et al. [14] demonstrated that HRCT may appear normal in histologically proven cases of acute rejection and, conversely, may reveal abnormalities in patients without rejection, underscoring the heterogeneity and limited specificity of HRCT findings, which often overlap with infectious complications.

Considering these observations, the present study was drawn to explore the contribution of BAL findings in combination with HRCT features in a standardized manner to enhance to a sufficient confidence level in diagnosing ACR, avoiding TBBx especially in more critical and fragile LTX patients.

In the present study we analysed 169 transbronchial lung biopsy combined with BAL sampling and CT scans. Our diagnostic TBBx yield data are broadly consistent with literature reports, at approximately 81.5% [8]. 34% of cases were diagnosed with ACR observing an incidence close with literature data [17].

Our findings show elevated BAL lymphocyte levels in patients with acute rejection, particularly CD8 + cytotoxic T-lymphocytes. This lymphocytic population is consistent with rejection mechanisms, alveolar lymphocytes coordinate adaptive immune responses, modulating inflammation to prevent excessive tissue damage. CD8 + T cell accumulation characterizes acute rejection, as these cells target epithelial cells of the graft recognized as "non-self" [18].

Lymphocyte levels correlated significantly with acute rejection severity, and a cutoff of 25% was identified as a

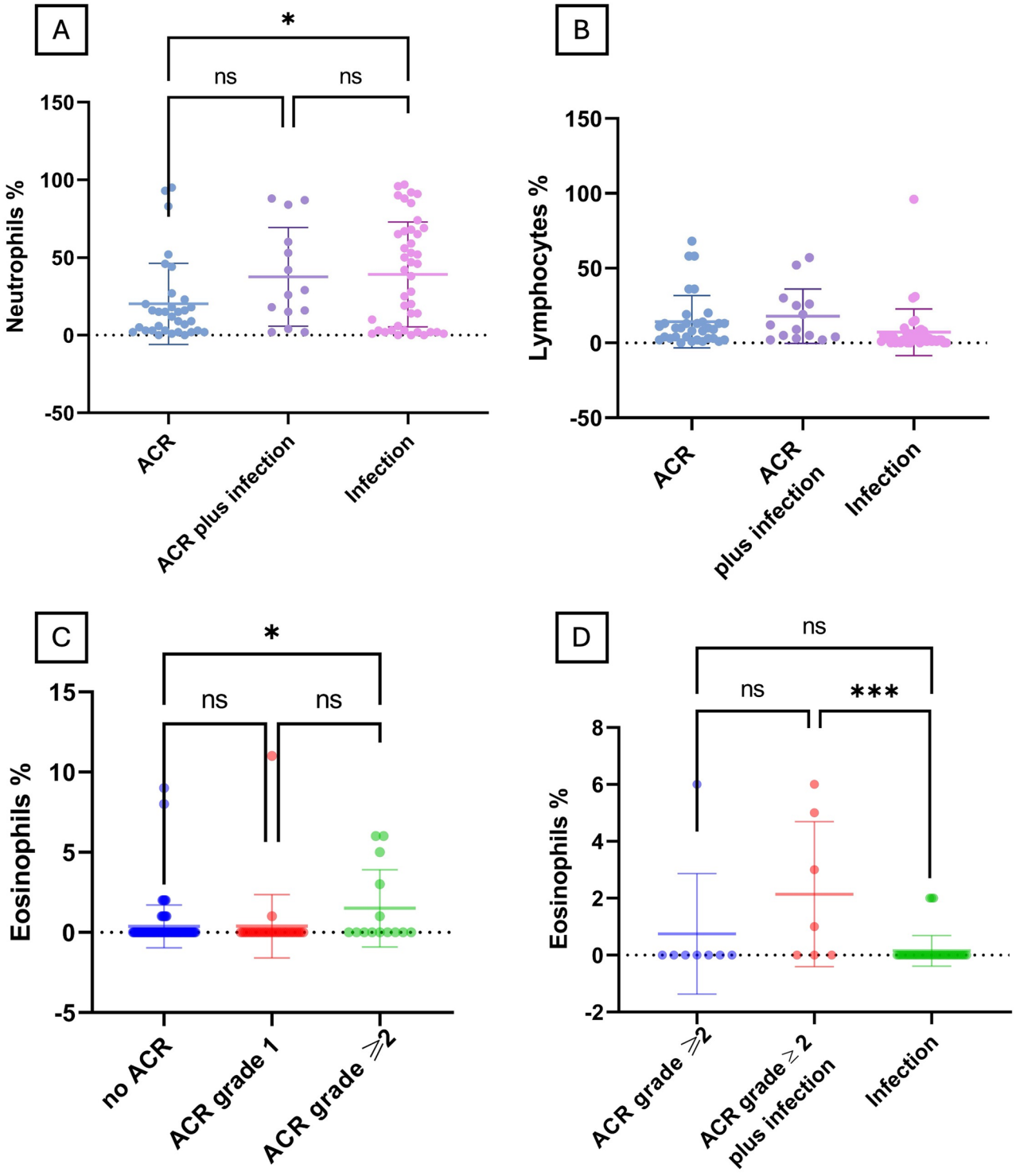

Fig. 3 Panel (**A**) Percentage of neutrophils in BAL from patients with ACR, ACR and *plus* infection and infection only. Panel (**B**) Percentage of lymphocytes in BAL from patients with ACR, ACR and *plus* infection and infection only. Panel (**C**) Percentage of eosinophils in BAL from patients without ACR, with mild (A1 grade) and moderate-severe (≥ A2 grades) acute rejection. Panel (**D**) Percentage of eosinophils in BAL from patients with moderate-severe (≥ A2 grades) acute rejection alone, with concurrent infection and infection only. * $p \leq 0.05$, *** $p \leq 0.001$

Table 6 CT abnormalities observed in ACR, as well as in ACR plus infection and infection only are reported as the percentage of cases in which each finding was present

	ACR	ACR plus infection	infection	*p*
Number	33	14	42	
Ground glass	24 (72%)	12 (85.7%)	29 (69%)	0.47
Air trapping	16 (48.5%)	8 (57%)	16 (38%)	0.40
Parenchymal consolidations	18 (54.5%)	9 (64.3%)	23 (54.8%)	0.80
Micronodules	7 (21.2%)	4 (28.6%)	9 (21.4%)	0.83
Pleural effusion	16 (48.5%)	8 (57%)	19 (45%)	0.74
Septal thickening	11 (33.3%)	6 (43%)	14 (33.3%)	0.79
Lymphadenopathy	2 (6%)	0	2 (4.8%)	0.65

non-invasive biomarker of ACR, with a specificity of 92% for its diagnosis. Therefore, this parameter appears more suitable for ruling in ACR rather than ruling it out.

Next, we tried to enhance diagnostic power for acute rejection by combining these data with radiological findings. Although ground-glass opacities were more prevalent in patients with ACR, pleural effusion was the only CT abnormality that increased diagnostic power when combined with the percentage of lymphocytes in BAL. The contemporary presence of BAL lymphocytosis over 25% and pleural effusion at HRCT let specificity raise up to 95%, making this approach clinically significant that can be considered reliable in more severe and fragile and in single LTX recipients in which execution of invasive procedure like transbronchial biopsy could raise some concerns. No other CT features could increase diagnostic yield.

BAL analysis also showed other interesting findings. Eosinophil levels were also elevated in ACR, particularly in more severe cases and in those with concurrent infection compared to infection alone, confirming the central role of eosinophils in both pathogen defence and the mechanisms underlying alloreactivity. These observations are consistent with previous studies that reported higher eosinophil levels in both BAL and peripheral blood of patients with acute and chronic rejection, correlating with poorer prognosis [19, 20].

These cells play roles in host defence and immune responses to respiratory pathogens. During infections, eosinophils recognize antigens, migrate to infection sites, and release granular proteins and cytokines to activate defense mechanisms. They also regulate immune responses by attracting other immune cells. Their role in rejection likely involves recruiting and activating alloreactive immune cells, but need further clarification yet [19].

Given the frequent occurrence of infectious conditions in lung transplant patients, distinguishing between infection and acute rejection is challenging. We assessed the contribution of BAL cytology in patients with concurrent infection. Neutrophil levels were significantly elevated in patients with infectious conditions and in those with concurrent ACR compared to those with acute rejection without infection. In cases of acute rejection, it is not uncommon to observe neutrophilia in the BAL, reflecting a non-specific inflammation. A high percentage of neutrophils in the BAL is associated with a deterioration in pulmonary function, measured by a decline in FEV1 and is reported in CLAD patients, in particular in those with BOS phenotype, where it is considered a marker of positive response to Azithromycin [21, 22].

The data we observed are quite interesting from a clinical perspective. As mentioned, the main differential diagnosis for rejection is infection, and identifying specific cytological patterns in the BAL can help guide the diagnosis. The predominance of lymphocytes (especially if above 25%) and the absence of neutrophils clearly point toward rejection, whereas in the presence of elevated neutrophils, excluding ACR becomes more challenging, particularly in patients with concurrent infection.

There are many ongoing studies on non-invasive biomarker for ACR detecting and dd-cfDNA is a very promising one. It allows early detection of rejection episodes, and provides a broader assessment of graft health without traditional biopsy's procedural risks. Conversely, it still has limitations such as no standardized thresholds cut offs and confounding factors (infections, tissue injury) [15, 16]. Other non-invasive endoscopic methods, such as confocal laser endomicroscopy probe (pCLE), are under investigation for real-time airway and parenchyma evaluation without the need for biopsy [23]. However, these techniques lack global standardization and are expensive, unlike BAL and HRCT, which are routinely available in lung transplant centers.

The main limitations of our study include the relatively small sample size, its single-center design, and its retrospective nature, and HRCT image interpretation by a single radiologist, even though expert in thoracic pathology. Additionally, 18% of transbronchial lung biopsies were inadequate for pathological analysis, representing a significant diagnostic limitation. A further limitation is the low sensitivity of BAL lymphocytosis, which reduces its value as an effective standalone screening tool for ACR and underscores the need to interpret this parameter

only in combination with clinical and radiologic findings as we propose in this study. Moreover, immunologic cell analysis of BAL is not easily performed in all laboratories and requires dedicated personnel.

In conclusion, BAL lymphocyte profiling effectively discriminated acute rejection from infection, with opposite cellular patterns, and diagnostic accuracy was further improved by combining BAL lymphocytosis with pleural effusion on CT. These findings are particularly relevant in critically ill patients, in whom transbronchial biopsy carries higher risk, supporting the use of less invasive diagnostic approaches. Multicenter studies on larger cohorts are needed to confirm these results and define clinical recommendations.

Abbreviations

LTX	Lung transplantation
ACR	Acute cellular rejection
BOS	bronchiolitis obliterans syndrome
CLAD	Chronic lung allograft dysfunction
TBBx	Transbronchial lung biopsies
BAL	Bronchoalveolar lavage
HRCT	High-resolution computed tomography
dd-cfDNA	Donor-derived cell-free DNA
PGD	Primary graft dysfunction

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Authors' contributions
EP and DB design of the work; the acquisition, analysis, interpretation of data; have drafted the work or substantively revised it. MG, FP, ES, MF LB, CG, LL, CC, EB, AF, CB, CP the acquisition of data. MAM the acquisition and interpretation of data.

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Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
All patients provided informed consent to participate in this study, which was approved by the Local Ethics Committee (Protocol Respir1, Prot n 15732, Comitato Etico Regionale per la Sperimentazione Clinica della Regione Toscana, Sezione: AREA VASTA SUD EST). The study was conducted in compliance with the Helsinki Declaration. Lung harvesting was performed in accordance with Italian regulations issued by the National Transplant Center (Centro Nazionale Trapianti). No organs were procured from prisoners.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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